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Kluwer Academic Publishers
Boston/Dordrecht/London

Distributors for North America:
Kluwer Academic Publishers
101 Philip Drive
Assinippi Park
Norwell, Massachusetts 02061, USA

Distributors for the UK and Ireland:
Kluwer Academic Publishers
Falcon House, Queen Square
Lancaster LA1 1RN, UNITED KINGDOM

Distributors for all other countries:
Kluwer Academic Publishers Group
Distribution Centre
Post Office Box 322
3300 AH Dordrecht, THE NETHERLANDS

The figure on the cover is from Samson, A.C.R., "Virus Structure."
The figure appears on page 24 of this book.

Library of Congress Cataloging-in-Publication Data

Newcastle disease.

(Developments in veterinary virology)
Includes bibliographies and index.
1. Newcastle disease. 2. Newcastle disease virus.
I. Alexander, Dennis J. II. Series. [DNLM: 1. Newcastle Disease. 2. Newcastle Disease Virus.
SF 995.6.N4 N536]
SF995.6.N4N48 1988 636.5'0896925 88-16721

ISBN-13: 978-1-4612-8987-6 e-ISBN-13: 978-1-4613-1759-3
DOI: 10.1007/978-1-4613-1759-3

THIS BOOK IS DEDICATED TO THE MEMORY OF PROFESSOR R.P. HANSON

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PREFACE

Most of the chapters of this book were written during 1987 which was the Diamond Jubilee year of the publication of the first reports of Newcastle disease in 1927. During the intervening years the nature of the Poultry Industry throughout the World has changed, or is in the process of changing, dramatically from one based on small village or farm flocks, frequently kept as a sideline, to an industry based on large flocks, sometimes consisting of hundreds of thousands of birds, run by multinational companies. To all these flocks, both large and small, Newcastle disease poses a considerable threat to their well-being and profitability and it is not unreasonable to state that hardly a single commercial flock of poultry is raised in the world without Newcastle disease having some effect due to actual disease, prophylactic vaccination or restrictions placed on rearing, movement, processing, sale or export of birds and products.

In addition, recent years have produced developments in virology and associated biological technology which would have been unbelievable when Newcastle disease virus was first isolated. The economic importance of Newcastle disease virus and its use as a laboratory model has meant that major advances have been quickly applied to the field situation whenever possible and, as a result, a much fuller understanding, not only of the biochemistry and basic virology of the virus but also the ecology, epizootiology, antigenicity, immunology and other important aspects in the control of the disease has been achieved.

The objective of this volume has been to bring together the current views and opinions of recognised experts to give comprehensive coverage of Newcastle disease, the causative virus and its control. It is to be hoped that it will appeal equally to those interested in Newcastle disease virus as a laboratory model and those concerned with the diagnosis and control of the field disease, and that, for those specialising in a single area, the presence of all these aspects in one volume will prove a catalyst for greater interest in and understanding of the wider parameters of the problem.

NEWCASTLE DISEASE

1

HISTORICAL ASPECTS

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INTRODUCTION.

The almost simultaneous occurrence of Newcastle disease (ND) as an apparently new disease in several different geographical locations, the rapid spread of the disease and the great variation in seriousness has meant that it is extremely difficult to place the important events in the history of ND in chronological order. There is no doubt that the importance of the disease is due to the development of the poultry industry during the 20th Century to a highly efficient industry run on an international basis which is dependent on trade between countries. If poultry meat and egg production had stayed at a backyard or village level the significance of the disease and its ability to spread throughout the world might have been greatly diminished.

In most countries, the appearance of the disease was the stimulus for the initiation of work to control or understand it and each new approach has its own history. Some aspects, such as control by quarantine and trade restriction have been developed as a result of both scientific and political considerations and the historical reasons for such policies and the effects on trade and disease control could fill a whole book by themselves. Nevertheless, the histories of four parameters of ND have an important bearing on the understanding and level of control that we have reached today, they

are:- the emergence of the disease, the understanding that isolates of the virus showed different virulence for poultry, the development of vaccines and the panzootic nature of disease outbreaks. It is the intention to cover these aspects of the history of ND in this introductory chapter.

EMERGENCE OF A "NEW" DISEASE.

Newcastle disease was the name given to a highly pathogenic disease seen in chickens in England in 1926 by Doyle (1). Doyle reported the first outbreak to have occurred, in the Spring of 1926, on a farm near Newcastle-upon-Tyne and hence the name. Doyle (2) considered the name "obviously unsuitable" and to be used only until "a more applicable one is coined". Nevertheless he considered it to be better than names which attempted to describe the disease signs as these led to confusion with other diseases. This is probably the reason for the name remaining after more than 60 years.

The disease had also emerged in March 1926 on the island of Java, Indonesia (3). Possible links between these initial outbreaks have been postulated by many authors. Generally it has been considered that the presence of the virus in England resulted from transportation to the port of Newcastle-upon-Tyne from S.E. Asia by ship, either in frozen meat or as a result of the practice of keeping live chickens on board for eggs and meat.

The disease also appears to have been present in Korea in 1926 (4) although Levine (5) quotes Ochi and Hashimoto (6) as evidence that the disease was present in that country as early as 1924. An outbreak also occurred in Ranikhet in India in July, 1927 (7), but in this case direct links with shipping could be ruled out as the town is in the foothills of the Himalayan mountains 600 miles from the sea.

What cannot be excluded is that outbreaks may have occurred earlier elsewhere but gone unnoticed due to lack of available expertise in recognising an apparently new disease. Wherever the first outbreak occurred it is obvious from the literature that a highly virulent disease of poultry appeared within a very short time in England, Java, Philippines, India, Ceylon, Korea and Japan (2)

and that the disease was sufficiently different from other highly virulent diseases to be recorded as distinct and recognised as the same disease. That the disease was caused by the same organism was established by experiments, carried out in several countries, showing cross immunity in surviving poultry which were summarized by Doyle (2). Not all workers were convinced of the novel nature of Newcastle disease and Manninger (8), after some experimentation with the virus, concluded that it was a mild form of fowl plague, a claim that Doyle meticulously refuted (2). Nevertheless, at a time when two very similar diseases of fowl were prevalent in various countries of the World and before the current sophisticated diagnostic techniques were available, differential diagnosis must have been a considerable problem and misdiagnosis undoubtedly occurred.

Wherever it began and however it was spread, in 1926 a new disease emerged or was recognised and within a few years had spread throughout the World. Many authors have put forward theories on the origins of ND virus (NDV) but basically these have fallen into three categories as stated by Hanson (9). The first possibility is that a major mutation of a precursor virus of low virulence took place resulting in virulent Newcastle disease. The second possibility is that the disease was present in the poultry of S.E. Asia for a long time but while it affected only poultry raised at a village level it was afforded little significance and it was only with the development of large scale poultry operations that the disease and the enormous economic losses attributable to it were noticed. The third possibility is that the virus was present as an enzootic of some entirely different species and it was only by the chance bringing together of this species and poultry that the disease emerged. This last possibility has some attractions in view of the association of the panzootic virus seen in the early 1970s with psittacine species (10).

ISOLATES OF DIFFERENT VIRULENCE

The initial recognition of ND in different parts of the World was as a highly pathogenic disease with levels of mortality up to

100% (2). Although this disease spread to most parts of the World in subsequent years it did not appear to reach the United States of America. However, in California a relatively mild respiratory disease, sometimes with nervous signs, of apparent viral aetiology had been first observed during the mid-1930s (11, 12). This disease, termed "pneumoencephalitis" (13) differed markedly from the disease reported by Doyle (1), not least in its low mortality which rarely rose above 15%. However, it was shown to be attributable to a virus indistinguishable from NDV in immunological tests (14). How long this virus had been circulating in the USA is not clear, but there was good evidence that the virus was also on the east coast prior to 1944 (14) and Beaudette and Hudson (15) reported retroactive identification of viruses isolated as early as 1938 as NDV. Despite the milder nature of the disease it still represented a major threat to the poultry industry throughout the USA in the 1940s.

Once it was established that NDV did not always cause a highly pathogenic disease and with the development of the techniques required to easily isolate and identify the virus (16,17) numerous reports began to appear of isolations of NDV with low pathogenicity for poultry.

Early isolates of viruses of low virulence for chickens were made in USA (18,19), England (20), Northern Ireland (21) and Australia (22) and each of these was later used as a live vaccine.

The isolations made in Northern Ireland and Australia were of some interest as these countries were considered free of ND at the time and had no vaccination taking place. The N. Ireland incident began in 1964 when birds in a healthy poultry flock were shown to have antibodies to NDV (23). The isolated virus, designated strain Ulster 2C (21) proved to be of extremely low virulence even for day-old chicks, replication occurring mainly in the gut. Despite the mild nature of the disease a slaughter and quarantine policy was invoked. In Australia, outbreaks of virulent disease had occurred in 1930 and 1932 but from then until 1966 the country was considered free of any form of NDV. In that year a mild infection was detected on a farm in Queensland (22). It was shown that this virus was widespread in Australia and as a consequence no quarantine and slaughter policy was invoked. Mild viruses similar to the first

isolate, V4, are still prevalent in Australia today and have been used as vaccine (24).

There have been many instances of the isolation of NDV from feral birds. Luthgen (25) lists 117 species of bird covering 17 of the 24 orders of the class Aves that have been shown to be infected with NDV. A large number of the cases may have been a result of contact with infected poultry but NDV of low virulence appears to be frequently enzootic in waterfowl from many parts of the World (26). Many of the isolates from waterfowl have very similar properties to Ulster 2C and V4 which suggests a source from which these viruses may be introduced to poultry.

VACCINES

Since the initial outbreaks of ND, studies on the prevention and control of the disease by vaccination have been carried out. Three strategies of vaccination have been used: immunization with inactivated virus, infection with viruses manipulated to produce mild disease and infection with naturally occurring mild viruses. The early reports of such vaccines and their efficacy are listed and discussed in detail by Lancaster (40).

The first studies usually involved inoculation of inactivated material but generally it was found difficult to produce good protection and attenuation of the virulent virus was attempted. Iyer and Dobson (27) passaged the 1933 Herts isolate through chick embryos to produce a virus of lower virulence, strain H, which could be used as a vaccine. Iyer repeated this attenuation with the Ranikhet isolate on his return to India (28) and as a result the Mukteswar mesogenic vaccine strain was developed (29). This strain is still widely used throughout Asia. Another similar vaccine was produced by Komarov by serial intracerebral passage of a field isolate in ducklings (38). The problem with these vaccines is that, while attenuated to some extent, they are still capable of causing disease and high mortality in fully susceptible birds.

In the USA inactivated vaccines were also used at first to combat the milder form of ND that was widespread. However, the known presence of viruses showing quite marked differences in virulence

led to the search for a live vaccine. Beaudette et al (30), after screening 105 field isolates, selected the Roakin strain for use as a live vaccine. This virus was widely used in the USA but was quite virulent in its own right and could not be given to birds less than 4 weeks of age. This proved an unacceptably long period as maternal immunity did not confer protection for the whole of the period. The problem was overcome by the selection and use of two milder naturally occurring isolates, B1 (18) and La Sota (31), which could be given much earlier in the life of the chicken.

It is now well known that B1, possibly the most widely and frequently used vaccine in veterinary medicine, was obtained by Hitchner as an infectious bronchitis virus culture from Beaudette who, in turn, developed the La Sota vaccine as an alternative to B1. The history of the discovery of these vaccines, the personalities involved and the acrimony that developed between the main characters has been covered in two very readable papers by Hitchner (32) and Goldhaft (31).

At about the time the mild vaccines were being developed in the USA, Asplin (33), in England, was developing another naturally occurring virus, strain F, as a live vaccine. This is very similar to B1 in virulence and immunogenicity and has been employed in many countries as a vaccine.

Although inactivated vaccines were the first used for ND in the USA (34), they were largely replaced by the more easily applied live vaccines. Inactivated vaccines, usually adsorbed to aluminium hydroxide, were used widely in Europe up to the 1970 panzootic but during these outbreaks they did not perform well and failed to contain the spread of the disease. As a result most countries allowed the use of live vaccines, usually B1 and La Sota. However, in more recent times considerable developments have been made in the preparation and manufacture of inactivated vaccines. In particular the use of oil emulsions (35) has produced vaccines much superior to aluminium hydroxide vaccines (36) and it has been shown that these may be used efficaciously even at day-old in the face of maternal immunity (37).

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PANZOOTICS

At least three panzootics of ND can be recognised from the literature. However, just as it is difficult to say precisely how and when ND emerged it has been difficult to determine the period each panzootic has covered.

The first began with the emergence of ND in 1926 and spread to most countries of the World. The passage of the disease in different countries varied considerably. For example in England the disease had disappeared by 1928 (2) whereas in India it spread rapidly all over the sub-continent (39). On examining the first dates that ND was recorded in different countries listed by Levine (5) and Lancaster (40) it is noticeable that two distinct groups appear: Asia and East Europe, where disease spread during 1926-1942, and the rest of Europe, Africa and the Americas [including the USA (41)], where spread of Doyle's form or Asiatic ND appears to have occurred in the late 1940s and early 1950s. Bearing in mind that no antigenic or biological markers existed at that time for the panzootic virus, this may represent a second panzootic on the tail of the first or, perhaps more likely, a second wave of disease. Certainly Doyle (2) felt that there was a mainstream of disease moving through S.E. Asia and that occasionally chance introductions to other countries occurred, such as those in England in 1926 (1), Australia in 1930 (42) and Kenya in 1935 (43), so that the later outbreaks occurred when the mainstream of disease reached those geographical areas. In Great Britain this was to give quite a different disease situation to the earlier introductions. In 1947 the first outbreak since 1933 occurred and was the forerunner of frequent outbreaks each year into the 1960s. In 1959-1960, for example, 2,724 outbreaks were recorded 391 of which were considered to be primary introductions (44).

The second panzootic of ND appears to have emerged in the late 1960s in the middle east, although it is difficult to see why it should have begun in that location if the transportation of exotic birds was responsible for the spread as discussed below. Allan et al (45) considered strain AG 68 from Iraq to represent an early isolate of the panzootic virus, but Russell and Alexander (46) point out that this strain appears to be more closely related to earlier

viscerotropic velogenic isolates. The second panzootic spread considerably faster than the first, reaching all continents and most countries by 1973. It has been suggested that this rapid spread was due to the association of the virus with psittacine species and that the enormous trade in these birds involving airborne shipment was largely responsible for the rapid dissemination of the disease (10). The circumstantial evidence relating many of the outbreaks in this period with importations of psittacines is good. For example, Walker et al (41) were able to associate most of the outbreaks that occurred in the USA during 1970-72 to the importation of infected exotic birds from South America, Central America or S.E. Asia. What has not been explained is why virulent NDV should emerge in feral psittacines in both Asia and S. America at about the same time.

The third panzootic occurred much more recently and related to a mainly neurotropic disease of racing pigeons caused by an NDV strain distinguishable from other strains by monoclonal antibodies (47). This panzootic will be described in more detail in a subsequent chapter. There is some evidence that the disease may have emerged in the middle east in the late 1970s (48) and spread across Europe and further mainly by contact between pigeons at races and trade in such birds. To date the disease has been confirmed by virus characterisation in at least 20 countries including many European countries, Canada, USA, Hong Kong and Sudan (47,49) and the disease signs have been reported in many other countries. The potential to infect poultry was demonstrated in Great Britain where a non-vaccination policy was in effect and the variant virus was responsible for 20 outbreaks in the fully susceptible chickens during 1984 as a result of contamination of food by infected pigeons (50). This suggests that the spread from pigeons to poultry in other countries was prevented by vaccination or because the circumstances which resulted in the spread were unique to Great Britain.

History indicates that from time to time NDV strains emerge from unknown sources for uncertain reasons and that they have the capacity to spread throughout the World in susceptible birds. To what extent the current prophylactic use of vaccines, practised in most countries, will prevent the emergence and spread of future panzootic viruses is unclear. But there is sufficient evidence from

the continued isolation of virulent viruses from exotic birds in quarantine and the reported outbreaks of disease in poultry from some parts of the World that it would be unwise for most countries to relax existing control policies.

CONCLUSION

Although the history of ND covers only 60 years and began at a time when viruses were recognised as disease causing entities it could serve as a model for the degree of confusion and misunderstanding that can be achieved in such a short time by the use, misuse and random application of control measures, which usually involved live vaccines, plus the acceptance of dubious epizootiological observations as fact. To the epidemiologist the history of ND still offers a considerable challenge since modern techniques enable better characterisation and grouping of isolates and could be applied to the numerous isolates that were made early in the history of ND and maintained or stored in various NDV repositories.

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NEWCASTLE DISEASE VIRUS - AN AVIAN PARAMYXOVIRUS

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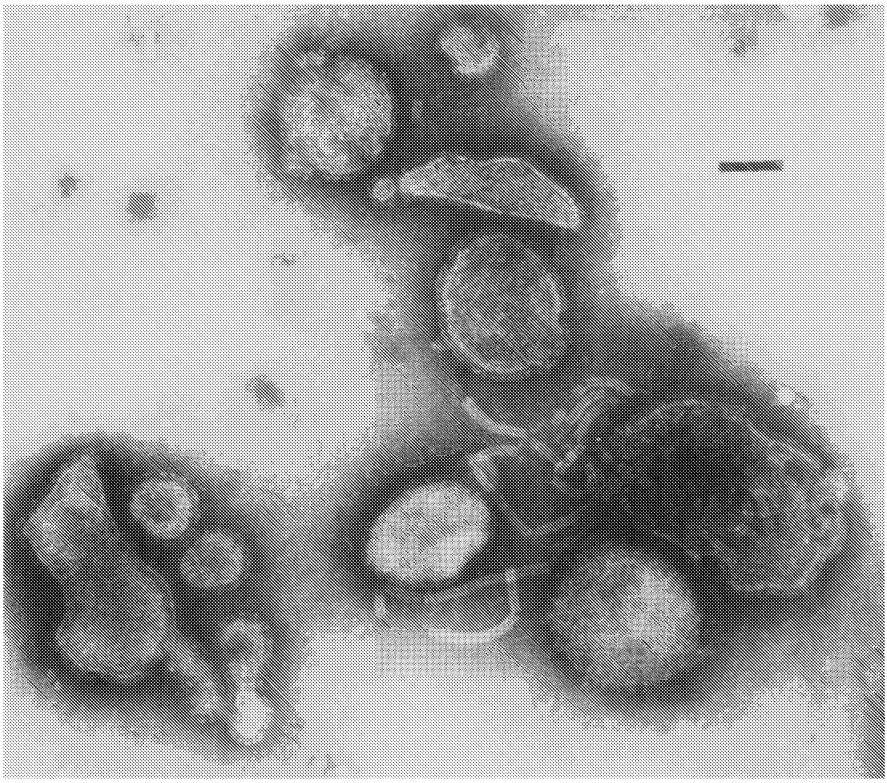
CLASSIFICATION

The virus family PARAMYXOVIRIDAE consists of enveloped RNA viruses which show helical capsid symmetry, posess a non-segmented, single stranded genome of negative polarity, undergo capsid assembly in the cytoplasm and are budded from the cell surface in an envelope of modified cell membrane (1).

Virus particles of members of the group are very pleomorphic when viewed by negative contrast electron microscopy. They generally appear as rounded particles of 100-500nm diameter but often filamentous forms of about 100nm across and variable length are seen. The surface of the virus particle is covered with projections. Inevitably in electron microscope preparations of Newcastle disease virus (NDV) the "herring bone" nucleocapsid may be seen either free or emerging from disrupted virus particles, this may be less evident for other members of the family.

Viruses grouped as the Paramyxoviridae have been further divided into three genera:

The genus Morbillivirus consists of measles, rinderpest and canine distemper viruses. The Pneumovirus genus is formed from the mammalian respiratory syncytial viruses and mouse pneumonia virus. A virus showing many of the properties of the Pneumovirus genus has been isolated from turkeys showing rhinotracheitis (2) and chickens with swollen head syndrome (3). The third genus, Paramyxovirus, includes the mammalian parainfluenza viruses, mumps virus and NDV



Bar = 100nm

FIGURE 1: NEGATIVE CONTRAST ELECTRON MICROGRAPH OF NEWCASTLE DISEASE VIRUS
The "herring bone" nucleocapsid can be seen free and emerging from the pleomorphic particles.

This micrograph was supplied by M.S. Collins, Central Veterinary Laboratory, Weybridge, Surrey, U.K.

which is designated the prototype of the genus. Numerous other viruses, fulfilling all criteria of the Paramyxovirus genus but serologically distinct from NDV have been isolated from avian species and have been grouped, unofficially, with NDV at the sub-genus level as 'avian paramyxoviruses' (4).

AVIAN PARAMYXOVIRUSES

In 1956, thirty years after the initial isolation of NDV (5), a paramyxovirus, serologically distinct from NDV, was obtained from chickens in Yucaipa, California by Bankowski et al (6). A widespread distribution of turkeys with antibodies to this virus was demonstrated in the USA (7) and occasionally serious disease episodes in these birds were associated with the presence of the virus (8).

A third paramyxovirus, serologically distinguishable from the other two types, was obtained from turkeys in Canada in 1967 and USA in 1968 (9). Surveys again indicated widespread presence of the virus in turkeys in the USA (9).

During the 1970s the number of isolations of paramyxoviruses, distinct from NDV, from avian species throughout the world showed an enormous increase. Two factors were responsible for this. Firstly, many countries imposed quarantine and testing of imported birds as a result of the 1970-1974 panzootic and routine virus isolation attempts produced many paramyxoviruses. Secondly, interest in the presence of influenza in feral birds resulted in surveillance programmes being undertaken throughout the world aimed at the isolation of such viruses. In addition to numerous influenza viruses, paramyxoviruses were also frequently isolated. While the former source generally resulted in viruses serologically related to the three recognised serotypes at that time, the latter source frequently produced viruses of apparently new serotypes (10). Faced with so many isolates it became clear that some form of nomenclature and a systematic classification scheme was needed if a proper understanding of the ecology and epizootiology of these

viruses was to be achieved.

Tumova et al (11) suggested that groups of viruses formed on the basis of serological tests should be termed PMV-1 for NDV, PMV-2 for Yucaipa-like viruses, PMV-3 for the North American turkey isolates etc. This form of nomenclature has been adopted by most avian virologists and at present nine serotypes are recognised, PMV-1 to PMV-9, and several other viruses are being considered as candidates for further serotypes (4).

In an effort to bring about some conformity in naming individual isolates it was also suggested that the rules recommended for naming influenza viruses (12) should be adopted for avian paramyxoviruses. Names should therefore include:- i) serotype, ii) species or type of bird from which the isolation was made, iii) country, state or other geographical location, iv) reference number or name, if any, v) year of isolation. For example PMV-1/pigeon/England/617/83, PMV-2/chicken/Yucaipa/California/56, PMV-8/pintail/Wakuya, Japan/20/78.

Use of this method for naming isolates and strains has been widely adopted for avian paramyxoviruses other than PMV-1 (NDV). For the latter it has been less readily taken up for historical, well characterised strains although in reports concerning new isolates of PMV-1 viruses it has been found to be most convenient to adopt the recommended system.

Prototype strains of the avian paramyxovirus serotypes have also been designated and these are listed in Table 1. Some of the serotypes have shown association with particular hosts and these are also indicated.

To date there has been no attempt to define a serotype specifically. Viruses have been mainly grouped serologically on their similarities in haemagglutination inhibition (HI) tests (10). However, neuraminidase inhibition (NI) tests (13-16), serum neutralization (SN) tests (9, P.H. Russell personal communication) and immunodouble diffusion (IDD) tests (16-19) have also been employed and produced similar serogroups.

Table 1 - Avian paramyxovirus serotypes

Prototype virus	Common hosts	Other hosts
PMV-1 = Newcastle disease virus	numerous avian species	
PMV-2/chicken/California/ Yucaipa/56	turkeys passerines	chickens psittacines, rails
PMV-3/turkey/Wisconsin/68*	turkeys only	-
PMV-3/parakeet/Netherlands/ 449/75*	psittacines	passerines
PMV-4/duck/Hong Kong/D3/75	ducks	geese, rails
PMV-5/budgerigar/Japan/ Kunitachi/75	budgerigars only	-
PMV-6/duck/Hong Kong/199/77	ducks and geese	turkeys
PMV-7/dove/Tennessee/4/75	pigeons and doves	-
PMV-8/goose/Delaware/1053/75	ducks and geese	-
PMV-9/duck/New York/22/78	domestic ducks	

* monoclonal antibodies may distinguish between these two groups see text

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RELATIONSHIPS BETWEEN NDV (PMV-1) AND OTHER PARAMYXOVIRUSES

Variation within the PMV-1 serogroup

To fully assess any antigenic relationship between viruses considered to represent different serotypes it must be understood that variation within a serogroup will inevitably exist if examined in sufficient detail. Studies using only one or a few strains and isolates aimed at detecting subtle similarities or differences between different groups of viruses may therefore be extremely limited in the inference that may be drawn from results obtained.

For the PMV-1 serotype (NDV) the working hypothesis, for control policies practised for over 50 years, has been that no significant antigenic variation occurs between strains and isolates and that vaccines derived from a single strain will protect against all virulent field viruses (20). This assumed antigenic homogeneity is in marked contrast to the considerable variation in the biological properties of the different strains, particularly the severity of disease resulting from infection. Beard and Hanson (21) placed ND viruses into six pathotypes, on the basis of disease produced in chickens, varying from extremely virulent to those producing asymptomatic infections. Attempts to relate other properties of the viruses, particularly antigenic properties to these pathotypes have been largely unsuccessful. However, markers such as plaque type, thermostability and haemagglutination of and elution from red blood cells from various hosts have been employed to differentiate ND viruses and have shown some general relationship with pathotype (22, 23).

Antigenic diversity unrelated to biological variation has been demonstrated by modified 'classical' serological methods (24-26), one dimensional polypeptide mapping (27), oligonucleotide finger printing (28) and lectin binding (29).

Recently several groups have produced mouse monoclonal antibodies to NDV and the application of these to the detection of variation in PMV-1 viruses has been reviewed in detail by Russell

in a chapter of this book (30). Monoclonal antibodies produced by Russell and Alexander (31) have proven particularly useful in differentiating and grouping NDV isolates in that the groups formed appear to share biological and epizootiological properties (31, 32). While those produced by other groups allow distinction between commonly used live vaccine strains and field viruses (33, 34).

Possibly the viruses showing the widest variation in antigenicity from the more 'classical' PMV-1 strains have been those responsible for the panzootic in pigeons during the 1980s (35, 36). This virus shows sufficient variance to produce detectable differences in standard haemagglutination inhibition tests and there has been some question of the efficacy of classical vaccines against this virus, especially when used to protect pigeons (36).

Relationships between PMV-1 and other avian paramyxovirus serotypes

The hypothesis that phylogenic relationships may exist between avian paramyxoviruses forming different serogroups which are assessable by measurement of antigenicity does not seem unreasonable and several studies have been undertaken to establish similarities between viruses placed in different serotypes. However, most of these studies have used a very limited number of viruses from each serogroup and, bearing in mind the variations seen within the serotypes discussed above, these may be inadequate to correctly evaluate any relationships.

Cross reactivity in serological tests between PMV-1 and PMV-3 viruses, especially PMV-3 viruses isolated from psittacines and other exotic birds, have been recorded in many studies. Smit and Rondhuis (37) reported low cross reactions between NDV and PMV-3/parakeet/Neths/449/75 in HI and SN tests. Alexander and Chettle (38) confirmed these results and found a similar relationship in NI tests. A further study (39) showed that several PMV-3 viruses were capable of conferring some levels of protection in chickens against challenge with the virulent Herts '33 NDV strain (PMV-1/chicken/

England/Herts/33). After challenge surviving birds showed an increase in PMV-3 HI titres as well as PMV-1 titres. A similar effect was seen in ND-vaccinated turkeys naturally infected with PMV-3 viruses when a sharp rise in both PMV-1 and PMV-3 titres was recorded (40).

Some PMV-3 viruses, particularly those isolated from exotic birds, show marked inhibition by conventional NDV chicken antisera in HI tests. This represents a potential for confusion in diagnosis which can usually be avoided by adequate use of control sera.

Several groups that have prepared monoclonal antibodies to NDV strains have also tested PMV-3 isolates. Most have shown no cross reactions, but Abenes et al (41) reported a monoclonal antibody directed against the F polypeptide of PMV-1 viruses also reacted with PMV-2, PMV-3 and PMV-4 representatives but not PMV-7 or PMV-9. The same authors reported (42) other monoclonal antibodies, to the HN polypeptide, which reacted to all PMV-1 isolates tested, representatives of six avian PMV serotypes and influenza virus A/PR/8/34. Such cross-reactive antibodies may be directed against the carbohydrate of the glycopolypeptide.

Anderson et al (43) produced monoclonal antibodies against a turkey PMV-3 isolate which were able to distinguish between turkey and exotic bird PMV-3 isolates in HI tests. None of these antibodies produced HI titres with NDV strain F or representatives of the PMV-1 pigeon variant. However, a monoclonal antibody, prepared against a pigeon PMV-1 variant virus, which inhibited all 'pigeon PMV-1' isolates in HI tests but not other NDV strains and isolates (32) also showed high HI titres with PMV-3 exotic bird isolates but not PMV-3 viruses from turkeys. It would appear, therefore, that at least one epitope on the HN molecule is shared by 'pigeon PMV-1' and 'exotic bird' PMV-3 viruses, while the results with polyclonal sera suggest the latter also have at least one common epitope with other PMV-1 viruses. Since turkey PMV-3 isolates and classical PMV-1 viruses also show some relationship using polyclonal sera at least one epitope is shared between those groups.

PMV-1 isolates have also been reported to have serological

relationships with viruses representing other avian paramyxovirus serotypes, although these have been generally much less marked than with PMV-3. Hoshi et al (44) prepared monoclonal antibodies against a PMV-1 variant virus one of which gave positive HI titres to the PMV-2 viruses tested. Many other studies have reported minor relationships in conventional HI or NI tests using polyclonal antisera, i.e. between PMV-1 and PMV-4 (13), PMV-1 and both PMV-8 and PMV-9 (19), PMV-1 and a virus provisionally typed as PMV-7 (45).

Detailed analyses of the cross-relatedness of representatives of the PMV serotypes detected in HI and NI tests have been undertaken by Lipkind et al (46-48). They conclude, on the basis of the minor cross relationships detected, that supergroups of the avian paramyxoviruses could be formed consisting of: i) PMV-1, PMV-3, PMV-4, PMV-7, PMV-8 and PMV-9, ii) PMV-2 and PMV-6. While this has not been borne out by other studies such groups had been tentatively suggested on the basis of similarities in polyacrylamide gel analysis of the structural polypeptide (49).

Relationships between NDV (PMV-1) and mammalian paramyxoviruses

Studies aimed at assessing similarities between NDV and mammalian paramyxoviruses have also tended to suffer from the use of too few viruses of each serotype to fully represent the two groups in view of the variations reported between viruses placed in the same serogroup.

Early reports based on in vivo studies suggested a possible relationship between NDV and mumps virus (50) which was not confirmed by in vitro studies (51). There was also some evidence of a serological relationship between the neuraminidases of NDV and Sendai virus (52).

More recent serological studies have produced no greater insight into the possible relationships between these viruses. Orvell et al (53) compared the Montana strain of NDV with mammalian paramyxoviruses in a study using monoclonal antibodies raised against Sendai virus (parainfluenza type 1). Although these authors

were able to suggest phylogenic relationships between Sendai, parainfluenza virus type 3 and mumps, NDV showed no comparable relationships. Ito et al using immunoprecipitation with specific antisera included several strains of NDV and each mammalian paramyxovirus group in their study to assess any relationship (54). They concluded that NDV belonged to a separate antigenic group but reported some minor cross-relationships between some of the NDV and parainfluenza strains which emphasised the importance of strain variation in such studies.

Molecular cloning with subsequent nucleotide and amino acid sequencing of various paramyxoviruses has indicated some similarities between different serotypes. Chambers et al (55) reported an overall homology of the F protein sequence of 33% between NDV and SV5 and 25% between NDV and Sendai. A 24% homology of the F protein has been reported between NDV and measles virus with up to 64% in some areas (56). For the HN polypeptide 32% overall homology has been reported between NDV and SV5 and 23% with Sendai virus (57) with much higher levels in some areas. In contrast 96.5% homology has been reported for the HN sequences obtained for two different NDV strains in separate laboratories (58). Chambers et al (59) reported only a 17% level of amino acid identity between the matrix protein of NDV and those of Sendai and measles viruses.

The exact biological significance of findings of homology in amino acid sequences at these levels is not clear, but serves as confirmation of the closeness of viruses in the paramyxovirus family.

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3

VIRUS STRUCTURE

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INTRODUCTION

The Paramyxovirus genus of which NDV is the type species, also includes mumps, human parainfluenza, Sendai and simian virus 5. Much of our understanding about the structure and function of components of paramyxovirus germane to NDV is gleaned from a variety of paramyxoviruses of which the above play a major role. In addition the rhabdovirus vesicular stomatitis virus (VSV) has provided a useful model, especially with respect to transcription and genome replication, for paramyxovirus studies.

Paramyxovirions are large pleomorphic membrane enveloped virions of roughly spherical shape ranging in size from 150 to 400nm (1) which contain a long helical nucleocapsid structure (1,000nm long, 17-18nm diameter, 5nm per turn). The envelope is covered with spike glyco-proteins (HN and F) 8-12nm long (1,2). Many excellent reviews dealing with paramyxovirus structure and function are available and the reader is recommended to refer to these for background and further details (3-6).

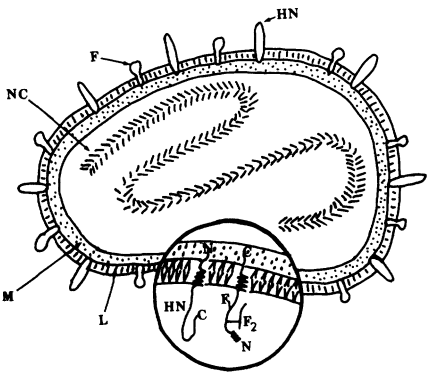
The genome of NDV is a single strand of RNA of negative sense, i.e. complementary to the messenger RNA which codes for virus proteins, and has a molecular weight of 5.2 to 5.7 x 10^6 daltons which is approximately 15 Kilobases (Kb) of RNA (7). This RNA genome codes for the following six gene products listed in order from the 3' end of the minus strand: nucleocapsid protein (NP), nucleocapsid associated protein (NAP or P), matrix or membrane (M), fusion protein (F_o uncleaved, $F_{1,2}$ cleaved), haemagglutinin-neuraminidase (HN) and large polymerase protein (L). The above order is that deduced from

DNA cloning studies (8,9) and is consistant with earlier ultra-violet light genome inactivation studies (10).

GENOME STRUCTURE

The large single stranded RNA genome of NDV (approximately 15Kb) has a sedimentation coefficient of 50S and directs both the transcription of three size classes of mRNA viz 35S, 22S and 18S (11), and the synthesis of complementary genome size plus strand RNA necessary for virion minus strand genome RNA production. The 35S size class directs the synthesis of the large polymerase protein L of size 220 kilodaltons (Kd) (12) and contains a unique messenger RNA. The 18S size class anneals to the remainder of the genome and contains all the remaining unique mRNAs. The 22S size class does not contain additional unique sequences and is thought to comprise bi- and other polycistronic genome transcripts (13,14).

Figure 1 Schematic diagram of NDV virion



M = matrix protein F = fusion protein C = carboxy terminus

HN = haemagglutinin-neuraminidase protein N = amino terminus

NC = nucleocapsid (containing RNA genome, nucleocapsid, nucleocapsid-associated and polymerase proteins)

MMM = hydrophobic region in polypeptide

The 3' polyadenylated 18S class has been fractionated into five

different mRNAs using acid agarose-urea gels and the mRNAs identified by *in vitro* protein synthesis. In order of decreasing mobilities in these gels, the following correlations were made: $mRNA_1$, M protein; $mRNA_2$, NAP or P protein (this mRNA also directs the synthesis of 33Kd and 36Kd polypeptides - see later section); $mRNA_3$, NP; $mRNA_4$, F; $mRNA_5$, HN (15).

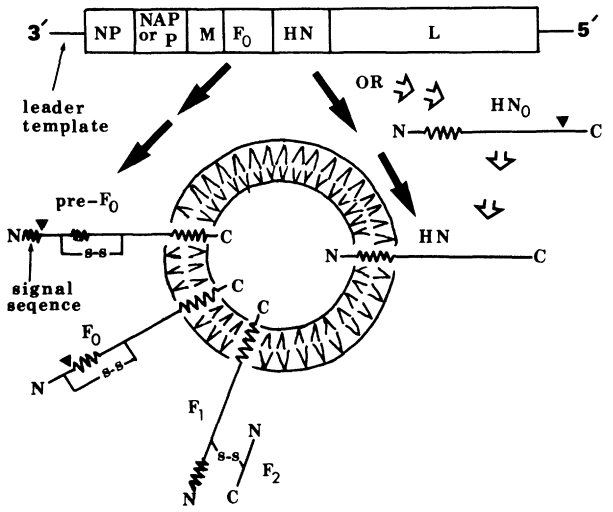
In Figure 2 the arrangement of these mRNA coding regions and leader RNA template is indicated together with the post-translational cleavage site within F_o (and HN_o where appropriate) and the orientation of these glycoproteins within the lipid bilayer.

In addition to mRNA classes the minus genome RNA contains a sequence starting at the 3' end which is complementary to leader RNA. In NDV this leader RNA was found by RNA sequencing studies to be 47 (*in vivo*) or 53 (*in vitro*) nucleotides long (16). In a more recent study based on DNA sequencing the first 2,617 nucleotides derived from the 3' end of NDV genome RNA, a leader sequence of 53 nucleotides was also identified (17). This study showed that the 5' ends of mRNAs synthesized in infected cells were m^7GpppA suggesting that transcription of viral mRNAs starts with an A and not a G as suggested from the earlier RNA sequence studies (16). The different NDV strains used in these studies may account for this. Following the 53 base leader sequence there are a further 68 bases before the first AUG start codon triplet belonging to the NP (the OP1 open reading frame), is found. Both OP1 and OP2 (NAP or P protein) are preceded by a common sequence (N1) 3' UGCCCAUCUUCC (minus RNA strand) which may be a concensus sequence which forms part of the transcription initiation site. Furthermore a sequence (N2) located after the OP1 but before the initiation site N1 for OP2 was found (3' AAUCUUUUUU), this closely resembles the transcription termination signals found for the HN gene (3' AUUCUUUUUU) and F gene (3' AAUCUUUUUU) of NDV (16,17,18).

By analogy with the rhabdovirus vesicular stomatitis virus (VSV) the regulatory domains which are concerned with entry of polymerase, +ve and -ve RNA initiation, and the formation of nucleocapsid structures, are all likely to be located at the termini

of the genomic RNA (16). Now that sequencing of the NDV genome nears completion in several laboratories, the details of both the 3' and the 5' end sequences will soon shed further light on these most important processes.

Figure 2 NDV genome structure



(for protein abbreviations see Table 1)

N = amino terminus C = carboxy terminus

MM = hydrophobic region in polypeptide

* HN is formed by post-translational cleavage of HN_0 in certain strains (33)

VIRUS PROTEINS

Background and overview

The early analysis of NDV proteins made use of the then new powerful analytical tool, polyacrylamide gel electrophoresis in conjunction with the denaturing anionic detergent sodium dodecyl sulphate (SDS-PAGE) (19). In this technique a complex mixture of proteins can be resolved into its component polypetides by boiling

with SDS in the presence of a reducing agent. The denatured polypeptides (with any disulphide bridges now broken) bind SDS and are converted to polyanions. These migrate towards the anode, are separated on the gel as a function of their size (polypeptide molecular weight) and can be detected by staining or by radioactive tracer methods. Although biological activities of the proteins are destroyed by this process, excellent separation of polypeptides is achieved and a reliable size can be allocated to each species.

An early study by Haslam et al (19) showed that NDV virions contained three major proteins of approximate molecular weight 80Kd, 54Kd and 38Kd, and broadly similar results were found by Alexander and Reeve (20). More comprehensive analyses were achieved by Mountcastle et al (21) who compared nucleocapsid subunits of simian virus 5 (SV5), NDV and Sendai, and then compared proteins and glycoproteins from the same set of viruses (22). The latter study was particularly significant in establishing that these paramyxoviruses contain at least five or six proteins, two of which were glycoproteins. Moreover in NDV the smaller glycoprotein had the same apparent molecular weight as that of the nucleocapsid protein (56Kd) thus these two proteins could not be separated by SDS-PAGE.

Following this, an unexpected observation was made that both the haemagglutinin and neuraminidase activities of paramyxoviruses, initially with SV5 and later with NDV (23,24), were associated with the larger glycoprotein (74Kd) in contrast to the situation known to obtain for orthomyxoviruses where these activities reside on different glycoproteins (25), Scheid and Choppin suggested that the smaller NDV glycoprotein might be involved in haemolysis or cell fusion (24).

Another observation which was to have important consequences for understanding the role of the smaller NDV glycoprotein (and for paramyxoviridae in general) was the discovery that one of the NDV proteins of size 53-56 Kd was formed from a larger precursor protein of 67Kd by a post-translational cleavage event (26,27,28). This was the first example of post-translational cleavage in this family of viruses and is now known to be essential for the activation of the

fusion protein (for a review see reference 5). The 67Kd precursor glycoprotein is now called F_o and the 53-56Kd product is called F_1. Details of the structure of the F protein and the evidence for its vital role in the infection process is dealt with in a later section.

By analogy to other paramyxoviruses it was anticipated that NDV should possess a phosphorylated protein associated with the RNA polymerase activity of the virion i.e. a P protein. The isolation and analysis of a transcriptive complex from NDV virions by Colonno and Stone (29) showed that in addition to genome RNA, this complex contained the nucleocapsid protein NP, a minor protein of 53Kd, the L protein (150Kd) together with traces of HN and M proteins. These workers suggested that the 53Kd protein might be the analogue of the non-structural protein (NS) of vesicular stomatitis virus. The identity of the paramxyovirus P analogue was independently reported by two groups at the same time (30,10). The former group made use of isoelectric focussing: SDS-PAGE to separate virion and virus induced proteins and showed that a new 56Kd virus-coded protein, (dubbed NAP for nucleocapsid associated protein), was the paramyxovirus P analogue. The reason this protein had eluded detection earlier was that it comigrated with F_1 and NP proteins on SDS-PAGE. The latter group working with different NDV strains showed that the P proteins could be separated from other NDV proteins using SDS-PAGE and that ultra-violet light transcription mapping placed the P gene between NP and (F_o, M) genes. The NAP or P protein was shown to be phosphorylated, non-glycosylated, associated with nucleocapsid and exist as disulphide-linked oligomers (30,31).

Table 1 summarises the set of NDV proteins gleaned from PAGE analysis of virions and virus infected cells. Protein sizes given refer to polypeptides separated on SDS-PAGE under reducing conditions, they are approximate because of strain to strain differences and different SDS-PAGE protocols.

Haemagglutinin-neuraminidase protein

Scheid and Choppin (24) had shown that the larger NDV glycoprotein contained both haemagglutinin and neuraminidase activities in contrast to the distribution of these activities on two separate

Table 1 Catalogue of NDV coded proteins*

Name of Protein	Abbrev.	Approx. size Kd	Function
nucleocapsid	NP	53-56	major structural component of nucleocapsid:complexed with genome RNA
nucleocapsid-associated	NAP (or P)	53-56	associated with nucleocapsid, phosphorylated, role in transcription/replication
matrix or membrane	M	38-40	virus assembly organiser, moderates transcription
uncleaved fusion	F_o	67	precursor to $F_{1,2}$
larger cleaved fusion	F_1	55	fusion of virus and host membranes, necessary for infection and haemolysis
smaller cleaved fusion	F_2	12	
haemagglutinin-neuraminidase	HN†	72-75	dual function: receptor binding protein responsible for haemagglutination and cleavage of sialic (neuraminic) acid residues from glycoproteins/lipids
large	L	180-220	RNA directed RNA polymerase

* In addition breakdown products of NP and HN are found in infected cells together with NAP(P) related polypeptides (10,21,32).

† In some NDV strains HN is derived from a larger precursor HN_o of size 82K (33).

glycoproteins in orthomyxoviruses. By analogy with orthomyxoviruses the haemagglutination activity (HA) is a consequence of the adsorption of virus to cells *via* the virus glycoprotein and cell surface receptors. These receptors contain sialic (neuraminic) acid (34,35) and one of the presumed roles of the neuraminidase (NA) is to aid elution of budding virions from the host cell by destroying local receptors. Sialic acid residues are not found on glycoproteins from virions which contain neuraminidase and this is thought to be significant in preventing virions from adsorbing to one another forming clumps and thereby frustrating dissemination.

Many studies have been conducted with ortho- and paramyxoviruses in an attempt to determine the role of neuraminidase in these virusess. Smith and Hightower (36,37) studied various revertants of a temperature sensitive mutant of NDV which had defective glycoproteins. One of these revertants (L1) had less than 3% of the neuraminidase activity of the wild type but was normal for haemagglutinin. This revertant was virulent *in ovo* and grew normally in cultured cells but had seven times more virion associated N-acetylneuraminic acid than the progenitor strain AV. However the revertant did exhibit a lower rate constant of attachment to HeLa cells and showed impaired elution from red blood cells. Clearly a separation of HA and NA sites is indicated within HN and evidence for this has been provided in a variety of studies. Iorio and Bratt (38) isolated monoclonal antibodies against the NDV HN protein and showed that, although antibodies to two antigenic sites were capable of inhibiting haemagglutination, only antibodies to one to these sites could inhibit neuraminidase activity (NA) using the small substrate N-acetyl neuraminlactose. These workers also showed that antibodies for all four antigenic sites was needed for the complete neutralization of NDV (39). Portner (40) obtained evidence for the separation of these two activities working with Sendai virus temperature sensitive mutants and antigenic variants selected using monoclonal antibodies. For both Sendai and NDV he showed that an analogue of N-acetyl neuraminic acid (2-deoxy-2,3-dehydro-N-acetyl neuraminic acid) inhibited NA by $\geq$ 95% at 10^{-4}M but did not inhibit HA at 10^{-2}M.

Inhibition of NA also prevented the elution of virus from agglutinated red blood cells. The recent analysis of NDV (strain Hitchner B_1) HN sequence by Jorgensen et al (41) suggested that the sialic acid binding analogue to that of the influenza NA protein is the sequence: asn arg lys ser cys ser, between amino acid positions 234 and 239 in the NDV HN. This sequence is well conserved among other paramyxoviruses analysed (parainfuenza 3, Sendai, SV5) and exactly the same amino acids are predicted at the same position in the HN of the other NDV strain sequenced so far, Beaudette C (42). This latter study identified a conserved region between NDV, SV5 and Sendai: gly ala glu gly arg leu/ile at amino acid positions 399 to 404 in NDV which shows similarity to the influenza HA sialic acid receptor binding site (43). This sequence is also found in the B1 strain (41).

The role of NA in myxovirus induced cell fusion was addressed by Huang et al (44) by constructing liposomes containing various myxovirus glycoproteins. Cleaved ($HA_{1,2}$) uncleaved (HA) and NA from influenza virus was mixed in various combinations with HN or F from NDV to make liposomes which were then tested for their fusability. Fusion with F occured in the presence of either HA + NA or HN but not in the presence of either $HA_{1,2}$ or HA alone. These studies indicated that some NA is needed for fusion to take place. The authors suggested that the role of NA (be it influenza NA or NDV HN derived) is to remove neuraminic acid from the primary receptor following adsorption, allowing the membranes to come closer together and permiting the F_1 or HA_2 hydrophobic sequences to make contact as a prelude to membrane fusion (see section on F). Neuraminidase is also considered responsible for removing neuraminic acid from glycoproteins and glycolipids synthesized in NDV infected cells. The mode of inhibition of NA by halide ions led Merz et al to suggest that most of this removal occured within, rather than without, the cell membrane (45). The neuraminidase (sialidase) from NDV has been shown to exhibit strict specificity for hydrolysis of the Neu Acα2 $\rightarrow$ 3 Gal linkage contained in glycoprotein oligosaccharides both N-linked to asparagine and O-linked to threonine or serine (46).

In some NDV strains (avirulent strains such as Queensland V4 and Ulster 2C), the HN protein is synthesised as an inactive precursor HN_o (47,48) of 82Kd. Proteolytic activation involves the loss of a glycopeptide of approximately 8Kd which is not found in the mature spike protein (32). The amino- and carboxy-terminal amino acids of HN_o and HN from NDV Ulster were analysed, both N-termini were blocked but the C-termini were different. However the small cleavage fragment had a free N-terminus and Schuy et al concluded that HN is inserted into the membrane envelope with its C-terminus exposed and that activation occured by removal of the peptide from the C-terminus of HN_o (49). The suggestion that HN is embedded in the membrane at its N- terminus (analogous to influenza NA (50,51)), has been amply vindicated by recent sequence studies of a number of paramyxovirus HN genes (52,53,54), including NDV (8,42,41).

The recent NDV HN gene sequence studies (8,42,41) have revealed the following general features. There is a single open reading frame coding for 577 amino acids (both Beaudette C (42) and Hitchner B_1 (41) strains), with predicted unglycosylated molecular weights of 63,149 and 63,250 daltons respectively. Five potential glycosylation sites (asn X thr/ser) are found at the same sites in both studies but a sixth site in Beaudette C (asn pro thr, residues 500-502) was not found in the B_1 strain. Both strains contain a highly hydrophobic sequence near to the N terminus (residues 27-50) which is highly conserved between the two strains. There is no evidence for a cleavable signal sequence or a carboxy terminal anchor sequence. This accords with an N-terminal location for the membrane anchor. In both sequences 12 cysteine residues are found in the same sites and 10 of these are conserved among other paramyxovirus HN genes. This high degree of conservation underlines the importance of cysteine residues in HN protein structure in both intra- and possibly inter-HN molecule disulphide linkages (55,56).

Fusion protein

Although in some NDV strains cleavage of a precursor to HN is necessary for adsorption of virus to host cells, all strains of NDV require the activation of the fusion protein by a specific protease

mediated post-translational cleavage as a pre-requisite for virus-cell fusion and hence infection. (For an excellent review of this topic see (5)).

The now classic studies of Homma and Ohuchi (57) and Scheid and Choppin (58) demonstrated that when Sendai virus was grown in certain cell lines such as HeLa, MDBK or L cells, progeny virus was not infective for susceptible host cells. Whereas when Sendai virus was grown in ovo or in chick embryo cells it was infective. Moreover uninfective virus could be rendered infective by treatment with the protease trypsin. Trypsin converted a 65Kd glycoprotein (F_o) to a 53Kd glycoprotein (F) in MDBK cell grown virus and chick embryo grown virus contained F but not F_o. An analogous post-translational cleavage event was also found in NDV infected cells (26,27,28).

Scheid and Choppin (59) isolated a new class of Sendai virus mutants which exhibited an altered specificity with respect to the activating protease. Some of the mutants which were activated by chymotrypsin (pa-c mutants) or by elastase (pa-e mutants) could no longer undergo multiple cycle replication in ovo unless the appropriate protease was added to the allantoic fluid. The nucleotide sequence of one of the pa-c mutants shows a change of arginine to isoleucine at the Sendai virus cleavage site and accounts for its failure to be activated by trypsin (60).

The importance of the F cleavage event with respect to host range and tissue tropism was indicated by the aforementioned work with Sendai mutants (59). This expectation was realized in a series of studies on NDV by Klenk and Nagai (47,48) They examined HN and F glycoproteins synthesised in a variety of host cell systems infected with virulent (Italien, Herts, Field Pheasant, Texas, Warwick) and avirulent (La Sota, B1, F, Queensland, Ulster) strains of NDV. In all strains F (56Kd) was derived by proteolytic cleavage from a precursor glycoprotein F_o (68Kd). Cleavage of F_o was shown to be necessary for cell fusing and haemolytic activity. This cleavage was shown to be a function of both the virus strain and the host cell system. With virulent NDV strains cleavage of F_o occurred in all

host systems analysed whereas with avirulent strains cleavage only occurred in embryonated hens eggs or in cultures of chorioallantoic membrane cells. Scheid and Choppin later showed that cleavage of F_o in SV5, Sendai and NDV produced two glycoproteins, the larger F_1 (48 to 54Kd) and the smaller F_2 (10 to 16Kd) which were held together by disulphide bonds (between cysteine residues). The cleaved but disulphide bonded F protein is called $F_{1,2}$. No free N- terminus could be detected on the F_o or F_2 polypeptide of Sendai virus but an N-terminal phenylalanine was found on F_1. From this the following order of polypeptides within F_o was deduced: NH_2-F_2-F_1-COOH (61). The fact that F_1 and F_2 were linked by disulphide bonds was exploited by Samson et al (62) in a salt-shock experiment to determine the order of F_1 and F_2 in vivo for NDV. The same order was found as deduced for Sendai virus.

At least the first six N- terminal amino acid residues of F_1 polypeptides from Sendai, SV5 and NDV were found by Scheid et al (63) to be hydrophobic. Later analysis of these viruses by Richardson et al (64) extended the run of hydrophobic amino acids to twenty which showed a very high degree of sequence conservation. The latter group showed that synthetic peptide analogues of the N terminal F sequences were very effective at inhibiting plaque formation by these viruses and that this inhibition was highly sequence specific. It was realized that the N- terminus of F_1 of paramyxoviruses ressembled the hydrophobic N- terminus of the HA_2 subunit of influenza virus haemagglutinin, that these proteins are each produced by post-translational cleavage events, and are necessary for infection by paramyxoviruses and influenza virus respectively (65,66).

It may be asked why the paramyxovirus and orthomyxovirus proteins which are involved in virus-host membrane fusion, are always produced by a post-translational cleavage event? The answer may be that if these proteins were made de novo with free highly hydrophobic N termini, these might become trapped in the lipid bilayer of the infected host and serve to anchor the fusion protein (at its N- as well as its C- terminus) and leave no free hydrophobic region to

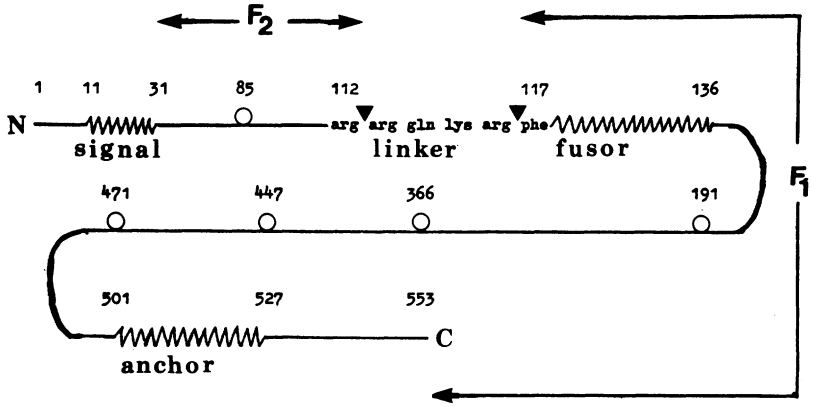
interact with membranes of the next host cell to be infected. The presence of a 'masking' polypeptide (the uncleaved F_2 or HA_1 moieties) may somehow prevent the hydrophobic sequence from being trapped in the infected cell membrane, it being cleaved off later to reveal the N terminal hydrophobic sequence (62).

Recently the complete sequence of two F genes has been worked out for NDV strains Beaudette C (18) and AV (67). The overall features of these sequences closely resemble other sequenced paramyxovirus F genes; SV5 (68), Sendai (69), respiratory syncytial virus (70) and parainfluenza 3 (71). Both NDV analyses predict that the unprocessed F protein (pre-F_o- unglycosylated) comprises 553 amino acids with a molecular weight of 59Kd. Three highly hydrophobic sequences of amino acids are predicted for this protein (see Figure 3 and (18,67) for details); the signal sequence near the N-terminus (residues 11-31), the F_1 N- terminal sequence (dubbed 'fusor' in Fig. 3) (residues 117-136), and the presumed membrane anchor sequence near the C-terminus (residues 501-527) see figure 3 and references (18) and (67) for details. Both sequences predict 13 cysteine residues within pre-F_o of which 11 are in the same positions. There is only one cysteine predicted in F_2 (residue 76) which must therefore be involved in the disulphide linkage to F_1. Both sequences also predict that there are 5 potential glycosylation sites within F (one in F_2, four in F_1) a sixth potential site was found in Beaudette C but on the C terminal side of the anchor sequence. A group of highly basic amino acids immediately precedes the F_1 N- terminal hydrophobic sequence and is the candidate linker peptide between F_2 and F_1 (18,64.67,72) (see Figure 3). It may be anticipated that as more NDV F gene sequences become available that the virulence of a given NDV strain (due to cleavability of F) will correlate with the structure of this linker region.

Very recently Patterson and Lamb (73) reported some very elegant gene construction/expression experiments involving the 'fusor' region of the F_1 protein from simian virus 5 and an anchor-less influenza virus HA protein. They showed among other things that the 'fusor' region (which they call FRED for Fusion Related External

Domain) can act as an anchor when placed at the C-terminus of the truncated HA protein.

Figure 3 NDV fusion gene product



N = N-terminus, C = C-terminus, O = potential glycosylation site

MWM = hydrophobic region in polypeptide

numbers refer to amino acid residue position.

They conclude that the FRED sequence is on the threshold of hydrophobicity required to function as an anchor and can do so only if at the end of a molecule (C-terminus in these studies). However, when present in an internal position (as in uncleaved F_o), it is not sufficiently hydrophobic to arrest the passage of the F protein through the lipid bilayer. Cleavage of the F protein in vivo places the FRED sequence at the end of a molecule (N-terminus of F_1) which can now interact with lipid bilayers (i.e. the next host cell). This

exciting finding strongly supports the modus operandi for fusion proteins presented earlier (and 62). If another construct or deletion variant which removed the whole of the F_2 plus linker were made the model would predict that F_1 would be linked as a hairpin by both N- and C-termini (62).

Matrix (or Membrane) protein

The matrix protein is thought to play a key organisational or marshalling role in paramyxovirus assembly by locating nucleocapsid structures beneath those regions of the plasma membrane in which the F and HN glycoproteins are anchored (74-80). In addition M protein has been implicated in moderating the activity of virion RNA polymerase activity (81) possibly in interactions with cellular actin (82,83) and in protein kinase activity (84). An excellent account of how M protein is involved in the assembly of virus prior to budding is given by Dubois-Dalq et al (1).

Complete sequences for a number of paramyxovirus M genes are now available; Sendai (85,86) measles and canine distemper viruses (87) and NDV (88,89). The matrix protein for NDV predicted by the gene sequence is a hydrophobic and highly basic protein of 364 amino acids in length and mass 39,605 daltons (Beaudette C (88)) or 39,742 daltons (AV (89)), which is very close to SDS PAGE estimates for this protein. Both NDV studies found homologies with other sequenced paramyxovirus M proteins, and in the former (88) some homology was also found with respiratory syncytial virus M protein (90) and in the latter with the rhabdovirus VSV (91,92). There is no extensive hydrophobic sequence within M (which one would anticipate for a transmembrane protein), rather the distribution of hybrophobicity is consistant with the peripheral location of M protein with respect to the lipid bilayers indicated by M protein elution studies (74,75). Most of the positively charged basic amino acids lie in the C-terminal portion of the molecule. The net positive change on the M protein may enable it to bind to negatively charged portions of the nucleocapsid and may be also the basis of the interaction with negatively charged actin (see 88). The region of VSV M protein which shows some homology to NDV M protein (N-terminal region) has been

shown to be involved in binding VSV M to the lipid bilayer (93) and McGinnes and Morrison (89) point out that it will be of interest to see if this region of NDV M is also the site which binds to membrane. It can be anticipated that in the next few years site-specific mutagenesis and the expression of M gene deleted clones will go a long way in delineating the multifarious assembly/organization features of this key paramyxovirus protein.

Nucleocapsid, nucleocapsid-associated and large proteins

The three protein species, which, in association with the RNA genome, make up the helical nucleocapsid structure, will be discussed together. Like VSV, transcription in NDV requires RNA complexed with nucleocapsid protein (NP) and both nucleocapsid-associated protein (NAP or P) and large (polymerase) protein L (29,94). Involvement of some of these proteins in RNA metabolism has also been infered from NDV mutant studies (95,96) and from proteolytic cleavage studies (97).

Paramyxoviruses synthesize non-structural (i.e. non-virion) proteins and the P (or NAP in NDV) gene has been shown to code for at least some of these proteins. In Sendai virus for example, the C (non-structural) and P proteins are both coded by the P gene but using different overlapping reading frames. A single species of mRNA is made from this region and can code for either protein (98). A similar situation obtains for measles virus (99). In respiratory syncytial virus, separate genes exist for the non-structural proteins (100). In contrast in NDV, SV5 and mumps virus the non-structural proteins are thought to be coded in the same reading frame by the same mRNA that codes for the NAP or P proteins and to be related to the NAP or P proteins since they share common peptides (30,15,32, 101,102). In NDV the NAP (P) related proteins are known as 36K and 33K. The role(s) of NDV 36K and 33K protein is still unknown. Virion nucleocapsids do not contain these proteins but are capable of mRNA transcription. Synthesis of full length positive and negative strands of RNA could possibly require these additional non-structural proteins. In VSV (the model negative single-strand virus for RNA transcription/replication studies), the non-structural protein mRNA

has recently been shown to possess an internal initiation site for a second non-structural protein (103). At present only part of the NAP (P) gene of NDV has been sequenced and this corresponds to the first 243 amino acids (17). These workers also described an open reading frame (ORF) which overlapped with that of the NAP (P) gene. The size of the peptide if it is coded by this open reading frame (104 amino acids) does not correspond to any known NDV protein. It may be noted that other ORFs for NDV have been described in addition to the established six genes (NP, NAP, M, F_o, HN and L). For example Millar et al (42) described an ORF of 41 amino acids near the proposed start of the HN gene. Earlier Hiebert et al (103) described an ORF of 44 amino acids between the F and HN genes of SV5. This hydrophobic protein called SH (small-hydrophobic) has been identified in SV5 infected cells. The NDV ORF is not hydrophobic but basic and has not yet been detected in NDV infected cells. As more paramyxovirus sequences become available more ORFs may become evident. The predicted amino acid sequences of ORFs will allow specific methods to be devised (e.g. based on antibodies raised against synthetic oligopeptides) to detect proteins in infected cells.

The NP gene of NDV was sequenced by Ishida et al (17) and shown to comprise an ORF of 1,467 nucleotides (489 amino acids) extending from nucleotides 122 to 1,588 from the 3' end of the negative RNA genome strand. The molecular weight predicted for this polypeptide is 53,161 daltons which is in excellent agreement with SDS PAGE estimates for this protein. Moderate amino acid sequence homology was found between the NDV, Sendai and measles virus NP in the middle of the sequence (105,106).

The largest NDV protein (L approx. 220Kd) together with the NAP (P) protein constitute the RNA-directed RNA polymerase found in virions. All negative stranded RNA viruses are obliged to carry within their virions an RNA polymerase, for without it no positive (coding) sense mRNA could be made within the infected cell. Our perception of the role of nucleocapsid protein, nucleocapsid-associated and large protein draws heavily upon the model for negative strand RNA viruses viz the rhabdovirus VSV. The interested

reader should refer to the recent mini review by Banerjee (107) and references cited within for details. The following represents a summary from the above. The transcription complex makes leader and mRNA in relative amounts reflecting the gene order (i.e. leader > NP > NAP(P) > M > F_o > HN > L in NDV) there being a distinct pause and attenuation at each intergenic junction. The polymerase, mediated by newly synthesized nucleocapsid protein, switches from transcription to replication to give full length plus (and later minus) strands. The L component of polymerase acts catalytically whereas the phosphoprotein (NAP or P in NDV) acts stoichiometrically. L appears to be required for synthesis of small uncapped RNA and requires protein NS (VSV) for their extension. The phosphorylation of NS (by L ?) probably plays a key role in transcription. The VSV NS protein contains three domains; a negatively charged N-terminal region which may react with the nucleocapsid protein, a second which binds to L protein and a third C-terminal basic region which appears to be tightly associated with the RNA-nucleocapsid protein complex (see 107 for details). How far these VSV features are reflected in the paramyxovirus NDV will have to await sequence analysis of NAP(P) and L protein and their comparison with homologous proteins from other paramyxoviruses.

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NEWCASTLE DISEASE VIRUS REPLICATION

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INTRODUCTION

Newcastle disease virus (NDV) has long been known as one of the most diverse and deadly avian pathogens. The replication cycle of NDV has also been studied effectively as a prototype for the Paramyxoviridae family of negative strand RNA viruses (1). In this role, NDV has several distinct advantages. It is easy to work with in cell culture because it is relatively stable, and replicates in many cell types. The NDV replication cycle is the most rapid of all paramyxoviruses. It replaces host protein synthesis with viral protein synthesis within 6 hr (2), producing maximal yields of viruses within 12 hr post-infection.

The many NDV strains which cause widely varying disease patterns have provided a starting point for the study of viral functions and pathogenicity. However, strains of NDV contain multiple genetic differences, making specific assignment of function or pathogenicity to specific proteins difficult in some cases. In an attempt to avoid this problem, mutants from a single strain of NDV (3,4) have been isolated and studied.

As with most RNA viruses, NDV displays a relatively high mutation rate (3). Since the NDV genome is a single strand of RNA which appears incapable of recombination (5-7), there is no method to evaluate the function of one mutant NDV gene on a background of "normal" NDV genes. However, the recent cDNA cloning of most of the NDV genes provides the tools to sequence mutant genes and their revertants to determine the exact location of the mutation, and to determine if one and only one gene has changed in a mutant.

Expression of individual NDV genes or pairs of genes in eukaryotic cells by recombinant techniques should eventually allow definite assignment of functions to viral proteins and allow determination of viral protein interactions with the host cell.

In this discussion of NDV replication, I will limit my comments to information available for NDV. If there is a gap in the NDV information which probably can be extrapolated from that known about other negative strand RNA viruses, it will be discussed. This approach is taken for the sake of brevity, and is not meant to indicate that our first insights into the replication of paramyxoviruses necessarily came from studies with NDV.

INITIATION OF INFECTION

When a bird is infected with NDV, the virus can replicate in, and damage many different organs. The particular disease pattern depends on the infecting isolate of NDV, as discussed elsewhere in this volume. In cell culture, NDV is also able to infect a wide variety of cell types, such as primary chick embryo lung (8), secondary chick embryo (9-11), baby hamster kidney (11,12), Madin Darby bovine kidney (12), mouse L cells (13), Chinese hamster ovary (14), and human cervical carcinoma (HeLa) (15-17). Since the first step in virus infection is attachment to a target cell via a cell receptor, it would appear that the receptor for NDV is a common molecule found on most cells.

Attachment

NDV particles contain two virus-encoded glycoproteins. Both are embedded in the virus particle's lipid membrane and both are required for the initiation of infection. The hemagglutinin/neuraminidase (HN), which is the larger of the two viral glycoproteins, provides the attachment function, both to erythrocytes and to target cells (18). As with all viruses, NDV requires the presence of salt for attachment to target cells (9), indicating that electrostatic forces are important. Attachment to cells in culture is very rapid. It is nearly complete within 10 min (9). Neuraminic acid is a required part of the target cell receptor for NDV (18). Whether or not neuraminic acid, alone,

functions as the target cell receptor is not known. However, the wide range of cells and organs that can be infected with NDV and the fact that all cells have neuraminic acids on their surface, make neuraminic acid the likely cell receptor for NDV.

Penetration

The second NDV glycoprotein, the fusion (F) glycoprotein, provides the penetration function for the virus particle. This glycoprotein is produced as a precursor (F_0). To function, F_0 must be cleaved to the disulfide linked $F_{1,2}$. Nagai et al. (12) clearly demonstrated the importance of F in this process by infecting cultured cells with virulent or avirulent strains of NDV. The virulent strains produced particles which contained the cleaved $F_{1,2}$ glycoprotein, were infectious, and could lyse erythrocytes by fusing with them. The avirulent strains which produced particles containing the precursor F_0 glycoprotein, were not infectious, and were unable to lyse erythrocytes. If the inactive particles produced in the avirulent infection were treated with trypsin, they were activated, by cleaving F_0 to $F_{1,2}$. These particles became infectious and could lyse erythrocytes.

The importance of cleaved $F_{1,2}$ for penetration is clear, but its mechanism of interaction with the host cell membrane is not. Some electron microscopic studies have found evidence for penetration of the cell by endocytosis (16,19). The envelope of the virus particle would probably then fuse with the endocytotic vesicle surrounding it, releasing the viral nucleocapsid into the cytoplasm (20). However, there is also electron microscopic evidence for direct fusion of the viral envelope with the cell membrane (21), releasing the nucleocapsid into the cytoplasm. While either, or both of these methods of penetration may occur, it is interesting to note that NDV (22-24), as well as other paramyxoviruses, are capable of fusing with erythrocytes or cultured cells at the neutral pH present at the cell surface. Other viruses such as influenza, VSV, or togaviruses, require the low pH of the endosomes to fuse and release their nucleocapsids into the cytoplasm (20).

Once in the cytoplasm, the NDV nucleocapsid complex with its associated transcriptase enzyme initiates infection. The entire replication cycle appears to take place in the cytoplasm of an infected cell and requires no nuclear function. This idea is derived mainly from two lines of evidence. 1) NDV replicates in cells treated with actinomycin D (25). Actinomycin D prevents the host cell from synthesizing RNA from DNA, but does not affect the ability of the virus to make RNA from its RNA genome. 2) Polyvalent antisera detect NDV antigens only in the cytoplasm of infected cells (8,15). However, monoclonal antibodies against one of the viral proteins display a different pattern, as discussed below.

VIRUS RNA SYNTHESIS

Huang et al. (26) demonstrated that the NDV virus particle itself contains an RNA synthetic (transcriptase) activity which is activated when the viral envelope is removed with a detergent. In vivo, the viral envelope is removed by penetration into a host cell, as described above. RNA synthesis by the invading viral nucleocapsid is defined as primary transcription, since it occurs before viral replication. In order to detect primary transcription, cells are infected with a high multiplicity of virus in the presence of actinomycin D, which prevents the much larger amount of host cell transcription. (Cycloheximide is also added to prevent virus replication, as discussed below.) Secondary transcription is viral RNA synthesis detected later in infection, after genome replication. Primary and secondary transcription are probably identical processes, differing only in the source of their template: parental nucleocapsids versus progeny nucleocapsids.

The viral nucleocapsid is composed of a single strand of genomic RNA covered with the 55 kilodalton (kDa) nucleoprotein (NP). The genomic RNA is 50S in size, as determined by its sedimentation rate. Its molecular weight is 5.5-7.5 x 10^6 daltons (18). The RNA-NP complex assumes a very regular helical configuration, but the helix itself is flexible (18). Two other viral proteins are associated with the nucleocapsid complex: the 52

kDa phosphoprotein (P) and the 180 kDa large protein (L) (27-29).

Transcription

The viral proteins involved in transcription have been examined in several ways. Chinchar and Portner (30) found that the transcriptase activity of nucleocapsid complexes was sensitive to limited proteolysis. Loss of transcriptase activity correlated with cleavage of the P protein, suggesting that the P protein is a component of the transcriptase. Madansky and Bratt (31) found that several of the noncytopathic NDV mutants which they had isolated (4) were deficient in viral RNA synthesis. These mutants accumulated less L protein in infected cells, implicating L in transcriptase activity. Peeples et al. (32) found that members of two temperature-sensitive RNA^- complementation groups (3), A and E, were deficient in both primary and secondary transcription. The mutations in group A probably reside in the L gene, and the mutation in group E probably resides in the P gene. This determination was made by examining the ultraviolet (UV) radiation sensitivity of the ability of a group A mutant to complement a group E mutant, and vice versa (33). Interestingly, the defect in RNA synthesis in some of the noncytopathic mutants could be complemented with the group E mutant but not with a group A mutant (31), providing further evidence for the localization of these mutations to the L gene.

These interpretations were confirmed by Hamaguchi et al. (34) who removed the NDV particle envelope with detergent and then removed the P and L proteins from the NP-RNA complex with high salt. The resulting nucleocapsid had no transcriptase activity. Only when both P and L were added back to the NP-RNA complex, was the transcriptase activity restored. All of this evidence indicates that the viral RNA synthetic complex is composed of the NP-covered genomic RNA and the associated P and L proteins.

Peeples and Bratt (33) also found that there are two mechanisms by which a group A mutant can complement a group E mutant: by a UV radiation-sensitive target, presumably genomic RNA, and by a UV radiation-resistant target, probably a protein. It was suggested that the P protein from the group A mutant was capable of

dissociating from nucleocapsids and reassociating with the group E mutant nucleocapsid, complementing its P protein defect. These data may indicate that the transcribing nucleocapsid is a dynamic structure, with at least one of the polymerase complex proteins able to dissociate, reassociate, and function.

Most of the RNAs produced by the nucleocapsid transcriptase complex in infected cells are complementary to genomic RNA isolated from virions (25). By convention, genomic RNA has been designated the negative strand (-) since it cannot be translated. RNAs complementary to the genome are designated positive (+) RNA because they contain the sequences which can be translated into viral proteins. The virus RNAs in an infected cell compose several size classes which were orginally described by their sedimentation in sucrose gradients: 18S, 22S, 35S, and 50S (25). The intracellular 50S (or 57S, the assigned value depended on the laboratory) RNA contains both (+) and (-) RNAs (25) and is the same size as the NDV genomic RNA (35). The roles of these genome and genome complement RNAs in replication and virus production will be discussed below. The 18S RNAs are (+) sense, polyadenylated mRNAs (36). As a group, the 18S RNAs are complementary to 50% to 60% of the genome (25,37,38). These mRNAs can be separated into 5 species by electrophoresis (39-42). Collins et al. (42) have determined the coding assignments for each of the 18S mRNAs by electrophoretically separating them and translating them in vitro. The 22S RNAs include several large species of viral "polytranscripts" which contain information from two or more NDV genes (43-46). These transcripts compose approximately 25% of the RNA produced by the NDV transcriptase and are found associated with ribosomes, suggesting that they are functional mRNAs (45). The 35S RNA anneals to the remaining 40% of the NDV genome (37), and must code for the L protein (47).

The order of the NDV genes on the genome was first examined using UV radiation to randomly inactivate the template RNA. UV-induced lesions block RNA transcription (48) presumably by forming uracil dimers in the genomic RNA. The size of the gene plus its distance from its promoter will determine its "target size", or

susceptibility to inactivation. Collins et al. (49) treated virus particles with UV radiation and measured the translation products from the mRNA produced in vivo and in vitro. They determined that the NDV genes were all inactivated with target sizes larger than expected from the size of their RNA transcripts, except for the NP gene. The results fit a model in which there is one virus promoter at the 3' end of the genome and the genes are transcribed sequentially, as shown in Fig. 1. The only placement left ambiguous in this study, was the order of M and F_0.

Since the transcriptase transcribes the NDV genes sequentially, the 22S polytranscripts should represent RNA from adjacent genes. Wilde et al. (46) used cDNA clones generated from NDV mRNA as probes to determine which mRNA sequences were included in each polytranscript. One polytranscript included sequences from both NP and P, another included P and M, another included M and F,

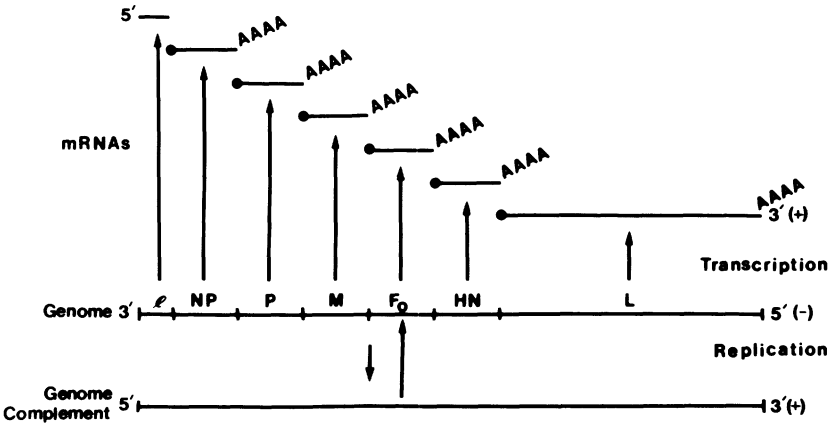

Fig. 1. Transcription and replication of the NDV genome, which is negative strand (-). The "ℓ" represents the leader gene and "NP,P,M,F_0,HN and L" represent the genes for the 6 NDV proteins. Transcription (upward arrows) results in 6 mRNAs, which are capped, methylated, and polyadenylated, and one leader transcript which is not. The approximate abundance of each transcript is signified by the length of each arrow. Replication results in a full length genome complement (+) RNA which in turn is the template for more genome (-) RNA. Again the length of the arrow represents the relative abundance of each RNA.

and another included F and HN. These polytranscripts are only compatible with a gene order of NP,P,M,F_0,HN, confirming the gene map of Collins *et al.* (49) and removing the uncertainty of the order of M and F_0. Chambers *et al.* (50) have also confirmed the NDV gene order by determining the sequence of a series of overlapping cDNA clones generated from genomic RNA. They assigned cDNA clones to particular NDV mRNAs by Northern blot analysis. The NP gene was the only gene for which they did not find an overlapping cDNA clone to orient it with the adjacent gene.

All of the NDV mRNAs are polyadenylated at their 3' ends (36). They are also capped at their 5' ends, at least when synthesized *in vitro* (27). The cap structure is methylated (51) at the blocked terminal G residue. However, unlike many eukaryotic mRNAs, NDV mRNAs are not methylated at the penultimate residue (52,53). The enzymes required to modify these mRNA transcripts are probably a part of the viral transcriptase complex since these functions are performed *in vitro* by purified virus particles whose envelopes have been disrupted with detergent. Genetic evidence that the virus codes for some of these functions comes from VSV mutants which express aberrant polyadenylation (54) or methylation (55). The viral origin of these functions would make sense because viral transcription takes place in the cytoplasm, removed from the cellular mRNA processing functions of the nucleus.

The method by which NDV polyadenylates its mRNA is distinct from the method by which eukaryotic cells polyadenylate their mRNA. At the end of each NDV gene is the sequence UCUUUUUU (53,56,57). This sequence is thought to serve as a template for the addition of multiple adenosine residues by "chattering", transcribing several A's on the UUUUUU template, and then slipping back to repeat the process (58). The poly(A) on NDV mRNA is approximately 120 nucleotides long (36), which would correspond to transcribing the UUUUUU template at least 20 times. Eukaryotic mRNA's are also polyadenylated at their 3' ends, but this process involves transcription of an mRNA beyond the polyadenylation site, cleavage at a specific polyadenylation site, followed by the addition of adenosine residues (59).

A small "leader" RNA is transcribed from the first 47 or 53 nucleotides of the genome (60). This leader RNA is not capped or polyadenylated and is not functional as mRNA. Its role in the viral replication or host cell interactions is not completely clear. It probably contains the encapsidation sequence and acts to switch from transcription to replication, as described below. It may also interact with the host cell. The leader RNA of VSV has been shown to inhibit host cell transcription in vitro (61).

The nucleotides between the genes are probably involved in terminating mRNA transcription from the preceeding gene, before initiating transcription of the subsequent gene. In the NDV genome, there is no common sequence or number of nucleotides in these "intergenic" regions. There are 0 to 31 nucleotides between each pair of NDV genes (53,56,57,60,62). This finding is surprising since VSV (63), Sendai (64), parainfluenza 3 (65) and measles (66) viruses all have a constant number (2 or 3) of nucleotides between genes, with a generally conserved sequence for each virus. However, two other paramyxoviruses, respiratory syncytial virus (67) and SV5 (68) display heterogeneity in their intergenic regions, like NDV.

It is interesting that the amount of viral mRNAs produced during infection and their protein translation products roughly correspond to the placement of the gene in the viral genome (39): more NP is produced than P, more P than M, more M than F_0, etc. The intergenic region may behave as an attenuator sequence between each gene. When the transcriptase reaches this sequence, it may have a certain likelihood of releasing the template, or of initiating mRNA transcription from the following gene, as suggested for VSV (69). While likely, this scenario is difficult to prove. It is possible (58) that the polymerase reads through the intergenic region, and that the mRNA transcript is subsequently cleaved and the bases complementary to the intergenic sequences removed. In fact, the polytranscripts described above might be intermediates in mRNA production. However, Wilde and Morrison (45) have shown that there is no poly(A) between the two mRNA sequences in the polytranscript, indicating that the first mRNA in the

polytranscript would not only have to be cleaved from the polytranscript, but it would subsequently have to be polyadenylated.

Genome Replication

Primary transcription results in mRNA corresponding to each of the viral genes. This mRNA is subsequently translated into the viral proteins. However, in order to amplify this process and to produce progeny, the genome must replicate itself. It must first produce a full-length (+) RNA rather than the leader RNA and 6 small mRNA molecules. The mechanism by which this switch from mRNA to full-length (+) RNA takes place has not been studied with NDV. In vitro, VSV transcription can be switched to replication by the addition of the nucleocapsid protein (N). It is thought that the N protein (which is analogous to the NDV NP), acts as a transcription anti-terminator, preventing the polymerase from stopping (or cleaving) at the end of the leader sequence and from polyadenylating and stopping (or cleaving) at each subsequent gene junction. The transcript from the leader sequence probably contains the nucleocapsid packaging sequence needed to bind N protein and initiate the formation of the helical nucleocapsid structure (70). However, it is not clear how encapsidation of the nascent RNA could prevent the transcriptase from halting at the next gene junction on the template. The VSV NS protein (analogous to the NDV P) may be a regulatory element in this process (71). Replication halts when Sendai virus infected cells are treated with cycloheximide (72), probably due to lack of a supply of soluble NP. Under these conditions, mRNA transcription continues (72), since this process is not dependent on the presence of soluble NP.

The second half of replication involves making full-length genomic (-) RNA from the full-length (+) RNA, again resulting in amplification. Presumably, this process occurs in a similar manner. It is interesting that in infected cells there are twice as many copies of the genome as of the genome complement (25). The control of this process is not understood. Both (-) and (+) 50S RNAs of Sendai virus are encapsidated in the NP protein (72).

These nucleocapsids can be used as template for new RNA (replication or secondary transcription), or the nucleocapsids containing (-) RNA can be packaged into progeny virus particles in the budding process.

The NDV group A and E temperature-sensitive mutants are also defective in replication at the nonpermissive temperature (32). These results suggest that the NDV L and P proteins, which are represented by these ts mutants (33) are involved in genome replication, as well as primary and secondary transcription, as discussed above. Peeples and Bratt (33) also found that UV radiation damage blocks replication, just as it blocks transcription (49).

TRANSLATION AND PROCESSING OF NDV PROTEINS

With the exception of primary transcription, all of RNA synthesis in the infected cell requires new copies of the viral proteins. Viral mRNAs produced by either primary or secondary transcription are translated into the six viral proteins: the three nucleocapsid-associated proteins NP, P, and L; the two glycoproteins HN and F_0; and the matrix (M) protein (42). The location of three of the viral proteins in an infected cell is shown by immunoperoxidase staining in Figure 2.

A monoclonal antibody directed against the HN glycoprotein stains intact cells (Fig. 2A), indicating that it is present on the cell surface. The diffuse, speckled staining with HN antibody is also seen with a monoclonal antibody to the F glycoprotein, but this staining is less intense (data not presented). A monoclonal antibody against the P protein (or against the NP protein, not shown) stains a concentrated perinuclear region of the cell (Fig. 2E and F) after the plasma membrane is disrupted with a nonionic detergent.

Monoclonal antibodies against the M protein weakly stain the cytoplasm in a diffuse manner (Fig. 2H and I). Surprisingly, antibodies against the M protein intensely stain the nucleus of infected cells (Fig. 2I). The nuclear staining is only detected under conditions where the nuclear membrane has been disrupted

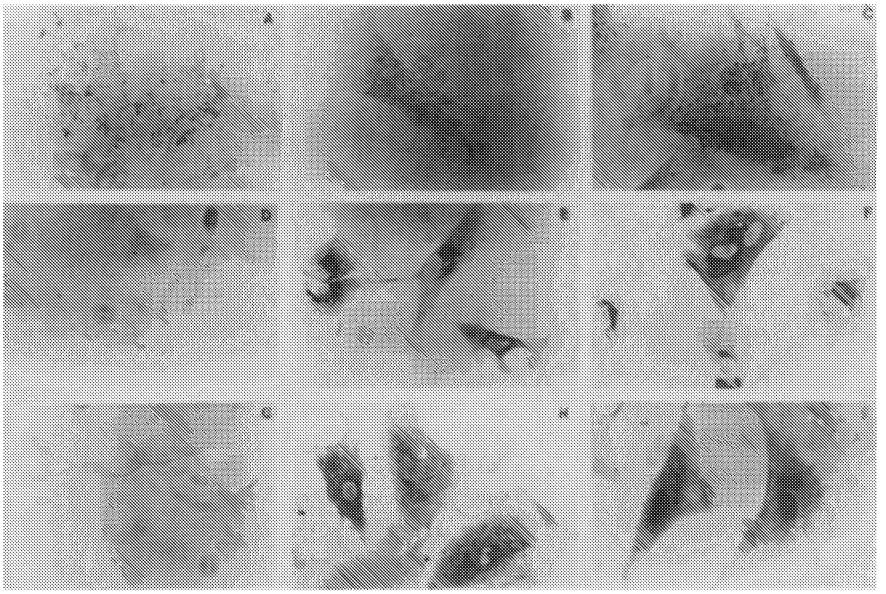

Fig. 2. Immunoperoxidase staining of NDV-infected cells as a function of Triton X-100 concentration. Cells were fixed with 3% paraformaldehyde and treated with no Triton (A,D, and G), 0.02% Triton (B,E, and H), or 0.05% Triton (C,F, and I). Cells were subsequently stained with mouse monoclonal antibodies against HN (A,B, and C), against P (D,E, and F), or against M (G,H, and I); followed by goat anti-mouse Ig-biotin; followed by streptavidin-peroxidase; followed by diaminobenzidine and H_2O_2. The details of this method and its characterization have recently been submitted for publication (M. Peeples).

(Fig. 2I; 0.05% Triton X-100). The nuclear M protein was not detected under conditions where the cytoplasmic membrane has been disrupted but the nuclear membrane has remained intact (Fig. 2H; 0.02% Triton X-100), indicating that the M protein is not on the cytoplasmic surface of the nucleus, but is actually within the nucleus. The apparent nuclear location of the NDV M protein has been briefly reported previously (73,74). The reason that the M protein was not detected in the nucleus with polyvalent anti-NDV sera (8,15) might lie in the finding that, at least in mice, the NDV M protein is poorly antigenic (Faaberg and Peeples, submitted for publication). It may be that some polyvalent anti-NDV sera do not contain high titer antibodies against the M protein. The

location of the M protein within the nucleus of infected cells raises the possibility of a nuclear role in some aspect of NDV replication.

In addition to their intracellular location, the translation, postranslational processing, and transport of the NDV proteins are important clues for understanding NDV; its interaction with the host cell and assembly into infectious virus particles. The control of the abundance of each viral protein is probably exercised at the level of transcription, as shown schematically in Fig. 1. There is no evidence for temporal control of viral protein expression (2,75).

The NP protein

NP, a 55 kDa protein, is the most abundant NDV protein in infected cells and in virus particles. Within 5 min of synthesis, much of the NP protein is found associated with the cytoskeleton of the infected cell (76), probably associated with growing nucleocapsids. NP is present in 3 different electrophoretic forms under nonreducing conditions, but as a single species under reducing conditions (77), possibly indicating several populations of NP molecules with alternate uses or numbers of intrachain disulfide bonds. Some of the NP molecules are also phosphorylated (77). It is possible that the various disulfide-bonded conformations or the phosphorylation of some NP molecules may be important in their roles in the assembly of the helical nucleocapsid, in the transcription process, or in the folding of the nucleocapsid helix in virus particles.

The P protein

The NDV P protein was an enigma for a long time. Sendai virus, like other paramyxoviruses, nad a prominent 78 kDa phosphoprotein associated with its nucleocapsid (78), while NDV did not. Actually, the NDV P protein detected by electrophoresis migrating close to NP was thought to be the F_1 glycoprotein. Several groups (49,77,79,80) then recognized that the NDV 53 kDa protein was similar to the Sendai virus P protein, though much smaller. In infected cells, P is found in 5 forms, separable by

2-dimensional gel electrophoresis: 3 phosphorylated forms and 2 nonphosphorylated forms (81). One of the phosphorylated forms is lacking in virus particles, while one of the nonphosphorylated forms is in greater abundance in virions (81). Under nonreducing conditions, some of the P protein migrates as a 53 kDa monomer while much of it migrates as a 180 kDa disulfide-linked trimer (77). Four forms of the P protein are found in the virus particle, where each is found both in monomers and in trimers (81). The P protein is required for virus transcription (32,34), as discussed above. However, the specific functions of these multiple electrophoretic, isoelectric, phosphorylated, and disulfide-linked forms of the P protein are unknown. They may play different roles in control of L protein binding to the nucleocapsid, as a cofactor in transcription, or in the other enzymatic activities of the nucleocapsid such as polyadenylation, capping, methylation, or cleavage (if it occurs) of mRNAs. Like NP, most of the P protein is found associated with the cytoskeleton soon after synthesis (76).

The P genes of the paramyxoviruses Sendai, parainfluenza 3, and measles are interesting for another reason. In these viruses, the P gene encodes one mRNA which is translated into several proteins: P and one or two nonstructural proteins, C, and C' (82-84). C and C' are produced from translation start sites downstream from the P start site, in a different reading frame. Therefore, C and C' share much of their amino acid sequence, but both have a sequence different from P. C and C' appear to be nonstructural proteins since they have not been found in virus particles (85). In NDV-infected cells, a nonstructural 36 kDa phosphoprotein has also been found (2,75,79). Collins et al. (42) have found that the P mRNA translated the P protein and two small proteins (33 kDa and 36 kDa) in vitro. Both of the small proteins shared two of their three tryptic peptides with P (42). Recent in vitro translations of the NDV P mRNA transcribed from a cDNA copy of the gene also indicate that the gene may encode two smaller proteins in addition to P. All three of these proteins are immunoprecipitated with a monoclonal antibody against the P protein indicating that they

share some amino acid sequence (Morrison et al., manuscript in preparation). Unlike the C and C' proteins of Sendai virus, the NDV small proteins must be translated from the same reading frame as the P sequence, since there are no other significant open reading frames in the P gene (Morrison et al., manuscript in preparation). The function of the "extra" small proteins from the P gene is not known. Presumably, they have some significance, since they are found in infections with several paramyxoviruses.

The L protein

Little is known about the structure of the L protein. Under reducing conditions, it migrates as a 150 kDa to 220 kDa protein (28,29,75). Under nonreducing conditions it does not appear to migrate into the gel, either indicating that it is disulfide linked to itself or another protein, or that it forms aggregates if it is not reduced. Hamaguchi et al. (34) have directly demonstrated that the L protein is a required part of the viral transcriptase, as described above. It probably provides the polymerase function of the transcriptase complex. An L protein mutant of VSV has also been shown to add longer than normal poly(A) tails to its mRNA (54), indicating that the VSV L protein is involved in polyadenylation. Whether the L protein is responsible for capping and methylating viral mRNA has not been proven.

The HN glycoprotein

HN is a 74 kDa glycoprotein which contains the virus attachment and neuraminidase functions. HN expresses its erythrocyte attaching function when it is in the plasma membrane (hemadsorption) of an infected cell, as well as when it is in the virus particle (hemagglutinin). Likewise the neuraminidase function of HN is expressed on the plasma membrane and in the virus particle. The weight of the polypeptide chain, without its attached carbohydrates, as determined from cDNA clones is 63-65 kDa (56,62,86) in good agreement with the 67 kDa, derived from in vitro translation (14) and tunicamycin-treated infected cells (80). Unlike the HN glycoprotein of most NDV strains, the HN of the Ulster and Queensland strains are synthesized as a precursor, HN_0,

which is activated by cleavage (12). Chambers et al. (50) have sequenced a cloned cDNA copy of the HN gene and the putative start of the L gene from the Beaudette C strain of NDV. They found that the 3' non-coding region of the HN gene is longer than that of Sendai and SV5 viruses. They suggest that a mutation in the stop translation codon could allow synthesis of a protein which is 55 amino acids longer, similar to the HN_0 of other strains.

Schuy et al. (87) have used the HN_0 cleavage to determine the orientation of HN in the membrane. Both HN_0 and HN_1 have blocked amino termini, but they have different carboxy termini, indicating that the cleavage takes place near the carboxy terminus. This result suggests that HN is anchored in the membrane near its amino terminus. HN does not contain a cleavable signal sequence since nonglycosylated HN translated in vitro is the same size as nonglycosylated HN from tunicamycin-treated cells (80). In fact, within the HN sequence, there is only one stretch of hydrophobic amino acids long enough to act as a translocation signal sequence or as a membrane anchor (56,62,86). This sequence follows a short group of charged amino acids at the amino terminus. It appears then, that HN is anchored in the membrane near its amino terminus, like the influenza neuraminidase glycoprotein and a small group of other glycoproteins. Microsomal membranes isolated from infected cells contain HN which is slightly reduced in size by trypsin treatment, indicating that a small portion of the HN terminus is exposed on the cytoplasmic surface of cellular membranes (88,89).

HN is processed and transported through the infected cell by a series of discrete, host cell controlled steps. A description of these processing steps follows, and is summarized in Fig. 3. HN is synthesized in the rough endoplasmic reticulum (90,91). Wilson et al. (88) have demonstrated in vitro that HN is cotranslationally inserted across microsomal membranes and glycosylated. This process requires signal recognition particles, as has been shown for other glycoproteins. During translation, the cellular glycosylating machinery adds N-linked, high mannose chains (91) to 2 to 4 of the 5 or 6 potential sites on HN (56,62,86). In the Golgi, some of the carbohydrates on each molecule are processed to

complex carbohydrate chains, which are insensitive to endoglycosidase H. The other carbohydrate chain(s) remain in the high mannose form, sensitive to endoglycosidase H even in the mature, virion HN (91). Tunicamycin which prevents glycosylation, does not prevent HN from reaching the cell surface indicating that carbohydrate chains are not required for transport to the plasma membrane (91,92). However, the unglycosylated HN on the plasma membrane has no hemadsorbing or neuraminidase activities (92). Unglycosylated HN is incorporated into virus particles but these virus particles are not infectious (92). These results indicate that the carbohydrate chains are involved in the function of HN, or that they maintain a conformation required for function. Parallel experiments with VSV indicate that blocking carbohydrate addition to its glycoprotein had no effect on the infectivity of virus particles (92).

Before transport from the rough endoplasmic reticulum (T. Morrison, personal communication), the HN molecules of some strains form disulfide-linked dimers while the HN of other strains remain as monomers (29,77,93). The positions of cysteine (cys) residues in the Australia Victoria strain HN (86) which does dimerize, and the B1-Hitchner strain HN (62), which does not dimerize, are identical except that the Australia Victoria HN has two additional cysteines. Recently, Sheehan et al. (94) have sequenced the area of cys_{123} in 11 NDV strains. Only the strains which contain cys at this location form HN dimers. The area of the second additional cysteine in the Australia Victoria HN was not conserved in two other strains whose HN molecules form dimers. The role that this dimerization plays in HN is not known but it must not be absolutely required for transport or function. However, it is also possible that the HN of some strains maintain HN dimers by forces other than disulfide bonds.

HN reaches the plasma membrane very slowly (91). Morrison and Ward (93) have found that 60 min is required for half of the NDV HN to reach the trans Golgi, while the VSV G glycoprotein requires only 13 min. However, HN and G transit time from the trans Golgi to the plasma membrane are similar, approximately 15 min. These

results indicate that HN is delayed in reaching the trans Golgi and not in migrating from the trans Golgi to the plasma membrane. The HN protein also appears to associate with the cytoskeleton of infected cells witnin 30 min of synthesis (76) which may slow its progress through the cell. Whether association with the cytoskelton represents a step in virus assembly or an obligatory step in intracellular transport or processing is unknown.

Marcus (17) has followed the point of appearance and the distribution of HN on the surface of infected cells by describing binding of individual erythrocytes at various times after infection. Erythrocytes were first found to adsorb to isolated areas on the periphery of cells. Witn time, they encircled the entire cell periphery and then the central cell surface, absorbing last over the nucleus. The centripital movement of HN was directly demonstrated by finding that the same erythrocytes bound to the periphery of individual cells relocated to the center portion of these cells after incubation (17).

The F glycoprotein

The F glycoprotein mediates fusion of the virus envelope with the cell membrane, accomplishing penetration and uncoating of the viral nucleocapsid in one step. If cultured cells are infected with high multiplicities of NDV, they may fuse into polykaryocytes. This fusion, which does not require that the virus be infectious (UV-inactivated virus works) or that the cells be metabolically active (insensitive to cycloheximide), is termed "fusion from without" (FFWO). Interestingly, there is wide variation in the ability of different strains to cause FFWO (22).

The F glycoprotein undergoes a number of post-translational modifications which are described in detail below and summarized in Fig. 3. F is synthesized as a nonfunctional precursor, F_0, with a molecular weight of 66 kDa. The molecular weight of the peptide portion of F_0 is 59 kDa, as determined by sequencing cDNA clones (96-98). F_0 is cleaved (2,75) during transit through the cell, yielding the disulfide-linked $F_{1,2}$ (90,91): F_1 is 55 kDa and F_2 is 12.5 kDa (99). Scheid and Choppin (100) suggested that F_2 contains

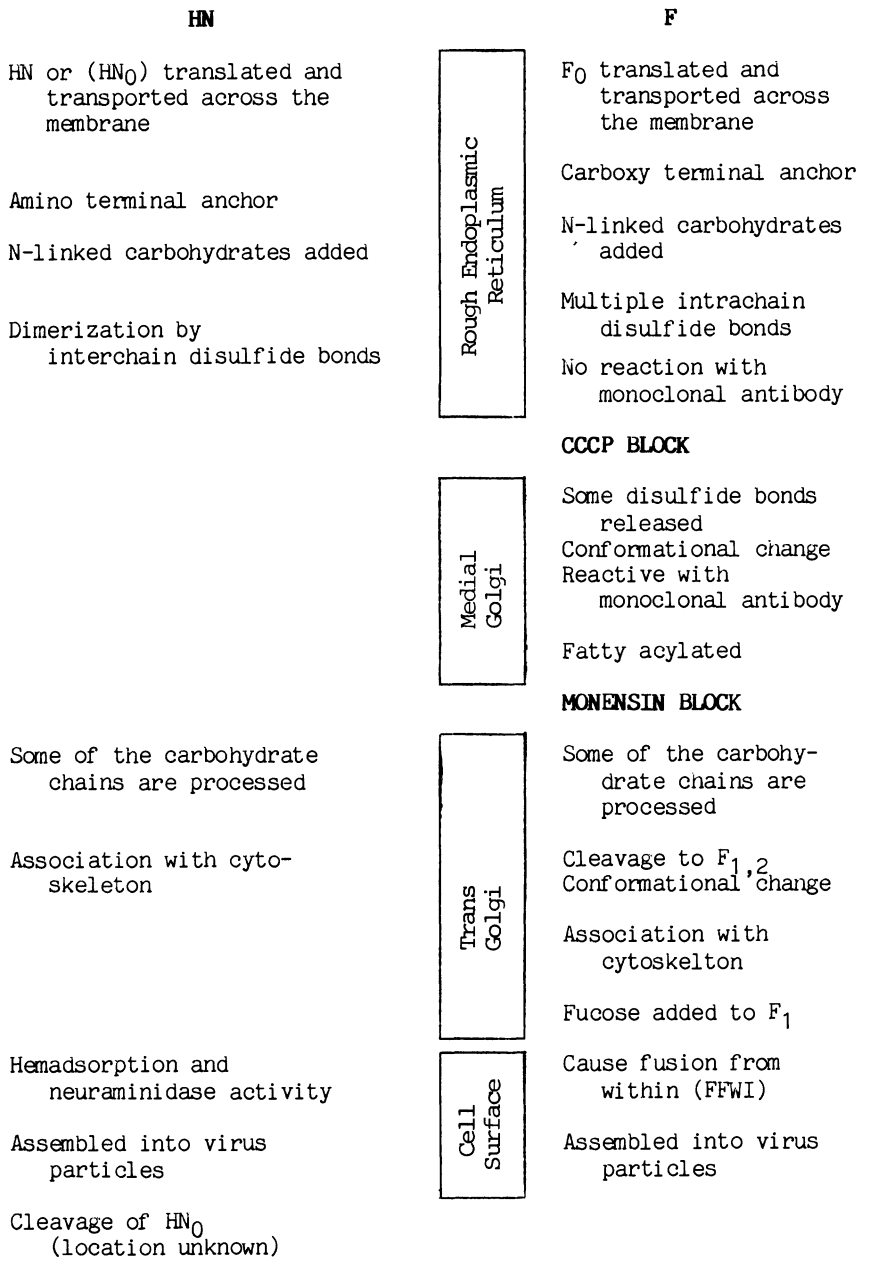

Fig. 3. Synthesis and processing of the HN and F glycoproteins of NDV.

the amino terminus of F_0 because the termini of both F_0 and F_2 were blocked to protein sequencing, but the amino terminus of F_1 was not. Therefore F_1 contains the carboxy terminus of F_0 and the new amino terminus generated by the cleavage of F_0. Localization of the cleavage to the amino terminal region was confirmed by synchronizing translation in an infected cell with high salt, followed by radioactively labeling proteins for various lengths of time after release from the salt block (99). From cDNA sequencing (96-98), it was recognized that F_0 has 3 hydrophobic regions: one near the amino terminus which probably represents the translocation signal sequence; one near the carboxy terminus which probably represents the membrane anchor; and one which is known from direct protein sequencing (101) to be the amino terminus of the F_1 fragment. F_0, then, belongs to the major class of glycoproteins which are anchored in membranes via their carboxy termini and differs from HN which has the opposite orientation in the membrane. Microsomal membranes isolated from infected cells contain F_0 which is slightly reduced in size by trypsin treatment, indicating that, like HN, a small portion of the F_0 terminus is exposed on the cytoplasmic surface of the cellular membranes (88,89). While there is no direct evidence that the amino terminus of F_0 includes a signal peptide which is cleaved, the gene sequence encodes an amino terminus with the characteristics of such a peptide (96-98).

F_0 is probably transported across the rough endoplasmic reticulum as it is being translated. The cellular glycosylating enzymes add N-linked carbohydrate side chains to some of the 5 potential sites (97) on F_0 during or soon after translation (91). As F_0 is transported through the cellular membranes, it undergoes a novel processing event. McGinnes et al. (102) have shown that in a pulse/chase experiment, F_0 is found first in a highly disulfide-linked form: by electrophoresis, it migrates more rapidly under nonreduced conditions (57 kDa) than under reduced conditions (66 kDa). After a nonradioactive chase, the nonreduced 57 kDa form shifts to a migration rate of 64 kDa. This change is blocked if infected cells are chased in the presence of carbonyl cyanide m-

chlorophenylhydrazone (CCCP) which prevents glycoprotein migration out of the rough endoplasmic reticulum. It is not blocked by monensin (102) which prevents migration beyond the medial Golgi. Therefore, F_0, which contains 13 cysteine residues (96-98), appears to be synthesized in a compact, highly intrachain disulfide-linked, rapidly migrating form. After leaving the rough endoplasmic reticulum, but before entering the trans Golgi, some of these disulfide bonds are released.

Morrison et al. (103) have further demonstrated that F_0 undergoes a conformational change at the same time: 2 monoclonal antibodies directed toward F will not precipitate F_0 after a 5 min pulse with ^{35}S-methionine, but will precipitate F_0 from a chase with non-radioactive medium. Furthermore, when the pulse F_0 was reduced, it was precipitated by these antibodies, confirming that the loss of disulfide bonds in F_0 leads to a more "mature", antibody-reactive conformation (103). This aspect of F_0 may be unique in eukaryotic cells, but seems more likely to represent a new post-translational processing event in the maturation of some glycoproteins.

F_0 is cleaved intracellularly (90,91) to the disulfide linked $F_{1,2}$ form, the active form of the glycoprotein (12,104). The cleavage occurs after the medial Golgi because it is blocked by monensin, but before it leaves the trans Golgi, because F_0 is not labeled with fucose, while F_1 is (105). Fucose addition occurs in the trans Golgi or soon thereafter. Neither cleavage nor transport require the carbohydrate chains since F_0 produced in the presence of tunicamycin is cleaved (80,91) and transported to the plasma membrane (91). The conformational change due to disulfide bond release must occur before cleavage since monensin does not block that conformational change, but does block cleavage. In addition, the two monoclonal antibodies against F react with both F_0 and $F_{1,2}$, indicating that they recognize an epitope present on F_0 and not simply an epitope which is generated after cleavage (103).

Cleavage occurs in an arginine-rich region of the F_0. In virulent strains, 4 of the 5 residues in this cleavage region are basic amino acids (98,106). The same region of the avirulent

strains of NDV contains 2 basic amino acids (98,106). Presumably, proteases which are able to cleave at the nighly basic virulent sequence are found in many cells and tissues, while the protease(s) which are able to cleave the less basic avirulent sequences are found in fewer locations. The amino acid sequence of the highly hydrophobic amino terminus of the F_1 polypeptide has been directly determined (101). This sequence is probably directly involved in the membrane fusion activity because peptide analogs of the F_1 amino terminus of several other paramyxoviruses are able to block its fusing activities (101).

Cleavage appears to induce a second conformational change in the fusion glycoprotein, as detected by a change in its circular dichroism spectrum (107). Cleavage also alters the isoelectric point of the fusion protein. It migrates as a more acidic protein, which might reflect either a rearrangement of charges resulting from the conformational change, or the loss of basic amino acids (107). Garten et al. (108) have shown that the influenza HA glycoprotein is cleaved by a trypsin-like protease followed by removal of several basic amino acids by a carboxypeptidase B type enzymatic activity.

F_0 is also modified by the addition of fatty acid, as detected by incubating infected cells with ^{3}H-palmitate (109). Fatty acylation probably occurs in the cis Golgi (110). However, F_0 was not as efficiently labeled with ^{3}H-palmitate as the VSV G protein. It is interesting that the NDV HN was not labeled with ^{3}H-palmitate (109).

F also appears to associate with the cytoskeleton (76) and to display a longer transit time to the cell surface than the VSV G protein (93). Once the cleaved $F_{1,2}$ glycoprotein reaches the plasma membrane, it is able to induce fusion between an infected cell and neighboring cells, resulting in polykaryocytes. Fusion induced after a cycle of infection is described as "fusion from within " (FFWI) to distinguish it from FFWO, discussed above. The level of FFWI varies greatly among NDV strains and is most efficient at high pH. Interestingly, the strains which are highly active in FFWI are poor inducers of FFWO (22).

The M protein

The paramyxovirus M protein is a basic protein (79), thought to be a controlling element in RNA synthesis and in virus assembly. When the Sendai virus M protein is added to the transcriptase complex, it inhibits 80% of the *in vitro* RNA synthesis (111). Paramyxoviruses which produce defective M protein are unable to produce virus particles (112,113). The M of NDV is synthesized as a 40 kDa (28,57,114) nonglycosylated, nonphosphorylated (77) protein with a single mobility when it is reduced before electrophoresis. Under nonreducing conditions M migrates in several forms (115), probably indicating a heterogeneity in the extent of intrachain disulfide bond formation in individual M molecules. The M protein amino acid sequence, deduced from a cDNA cloned sequence, contains 5 cysteine residues, 4 of which are concentrated within a 49 amino acid stretch (114).

Some of the M protein associates with the plasma membrane soon after synthesis (90,91). This M protein is thought to interact with the cytoplasmic surface of the plasma membrane via hydrophobic interactions with the lipid bilayer and/or the HN or F glycoprotein tails. Sequence analyses of the M gene cDNA confirms that M is highly basic and generally hydrophobic, though no stretches are long enough to constitute a membrane spanning domain (57,114). The NDV M protein will associate with liposomes, regardless of net charge, as shown in Fig. 4 possibly indicating a hydrophobic interaction with membranes. The finding that M will interact with these liposomes even in the presence of 0.5 M NaCl (Fig. 4) which should shield most electrostatic charges, strengthens the argument for a hydrophobic interaction with these membranes. Freeze-fracture studies of Sendai virus-infected cell membranes suggest that the M protein may actually span the inner leaflet of the lipid bilayer (116,117). The M protein appears to form a very uniform crystalline-like array on the cytoplasmic side of the plasma membrane (116,117).

Whether or not *in vitro* interactions with liposomes represents the most important M protein interaction with the plasma membrane is not clear. Yoshida *et al.* (89) have reported that monensin

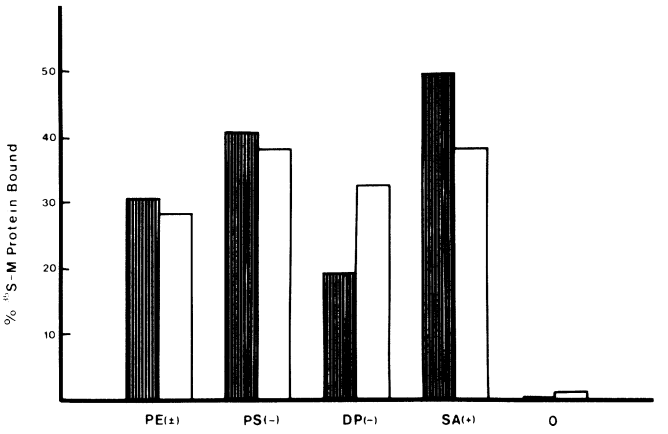

Fig. 4. Interaction between the NDV M protein and liposomes. ^{35}S-methionine-labeled strain Australia Victoria virus was produced in chicken embryo cells, partially purified on a step sucrose gradient, and dissociated with Triton X-100 and 1 M KCl. The M protein was isolated by affinity chromatography with a monoclonal antibody. Liposomes were prepared from a mixture of phosphatidylcholine, cholesterol, and a third lipid, in a 40:30:4 molar ratio. The third lipid was phosphatidylethanolamine (PE), phosphatidylserine (PS), dicetylphosphate (DP), or stearylamine (SA). PE does not give the liposomes a net charge (±), while PS and DP give the liposomes a net (-) charge, and SA gives the liposomes a net (+) charge. Affinity purified ^{35}S-M protein was incubated with each liposome preparation or without liposomes (0) in PBS (0.15 M NaCl) for 2 hr. Liposomes were washed by pelleting. ^{35}S-M protein pelleted with all 4 liposome preparations (striped bars), but not without liposomes. The same experiment was performed in the presence of 0.5 M NaCl which should prevent electrostatic interactions (open bars), with similar results (Faaberg, K. and M. Peeples, manuscript in preparation).

treatment of infected cells, which inhibits transport of the NDV glycoproteins to the plasma membrane, also inhibits the association of M with the plasma membrane. This result implies that one or both of the viral glycoproteins must be located in the plasma membrane for M to bind. The F glycoprotein (88,89,96,97) and the HN glycoprotein (56,62,86,88,89) have cytoplasmic tails (30 and 26 amino acids long, respectively) which could interact with the M protein.

The intranuclear location of the majority of the M protein in NDV-infected cells (73,74, Fig. 2) is a puzzling finding, as discussed above. Interestingly, two highly basic amino acid sequences, Lys-Lys-Gly-Lys-Lys and Arg-Lys-Ile-Arg-Arg, separated by 7 amino acids are found in the M protein sequence (114). Perhaps one of these sequences contains the signal for transport into the nucleus in a manner similar to the SV40 large T antigen. Large T contains a nuclear transport signal composed of 7 amino acids, of which the central 5 amino acids are basic (Pro-Lys-Lys-Lys-Arg-Lys-Val) (118-120).

The role that M might play in the nucleus is also a subject for speculation. The M protein appears to be particularly concentrated in the nucleolus where it might modify ribosomes, leading to the observed (2) host cell protein synthesis shut-off and/or the enhancement of viral protein synthesis. Collins and Hightower (121) have demonstrated that NDV infection of chick embryo cells stimulates the heat shock mRNAs and proteins. It is also possible that nuclear M protein might be involved in stimulating this response.

Using similar antibody localization techniques, the M protein of measles virus has been found exclusively in the cytoplasm of infected cells, but some of the NP has been found in the nucleus (122). It is interesting that a small amount of the Sendai virus M protein is found in the infected cell nucleolus (123).

ASSEMBLY

NDV assembles its components at the plasma membrane of infected cells and produces infectious virus by budding (124,125) as do many enveloped viurses (126). Several lines of evidence point to the central role that the M protein plays in this process. Sendai (113) and measles (112) virus mutants which produce defective M protein, do not form infectious virus efficiently. VSV ts mutants in the M protein are likewise unable to form virus particles under nonpermissive conditions (127). In vitro, the Sendai virus nucleocapsid will not form a complex with the viral glycoproteins unless the M protein is added (123). It

has also been found that inhibition of protein synthesis, even late in infection, quickly stops the production of infectious NDV (128,129). Since the M protein is incorporated into virus particles soon after it is synthesized (90,91), it has been suggested as the limiting factor in virus budding (18).

NDV strain Australia Victoria ts mutant D1 contains a mutation in its M protein, as determined by the altered electrophoretic migration rate of its M protein and by the analysis of ts^+ revertants (115). The mutation has been localized to one of the M protein termini (130). This mutant forms virus particles at nonpermissive temperature which lack the F glycoprotein and are less infectious than particles produced at the permissive temperature (115). The M protein of mutant D1 either fails to interact with the F glycoprotein or interacts improperly. Both interpretations imply that there is a normal, important M-to-F interaction in wild type NDV. Further studies with D1-infected cells indicate that the F protein is synthesized but never gains reactivity with the monoclonal antibody (74) described above, implying that F_0 never leaves the rough endoplasmic reticulum and does not undergo the disulfide bond-release conformational change (102,103) also described above. The M protein might arrest the processing of the F glycoprotein by failing to interact with F at the proper time, or by interacting with F before the proper time.

All three of the group D mutants are able to form virus particles without the F glycoprotein, though the process is somewhat less efficient (115). When these mutants are grown in embryonated eggs at permissive temperature, they contain less $F_{1,2}$ than wild-type virus and are less infectious (115). There is a rough correlation between the amount of $F_{1,2}$ in a virus particle preparation and its infectivity, possibly indicating a stoichiometeric requirement for the $F_{1,2}$ protein in infectivity.

In this regard, it is interesting that the NDV group B,C, and BC mutants, all of which represent lesions in the HN protein (131,132), incorporate greatly reduced amounts of HN into their virus particles (131). However, their virus particles are just as infectious as wild-type NDV. Since all of these mutants

incorporate a small amount of HN into virus particles under all conditions, it may be that some HN is required to form virus particles (unlike the suggestion made above, that $F_{1,2}$ may not be required for physical particle formation). These results also imply that the amount of HN contained in a virus particle is unimportant for infectivity, as long as the virion contains some HN.

The actual mechanism behind the budding process is unknown. It may involve the M protein. The Sendai virus M protein appears to form a crystalline array on the inner surface of the virus membrane (116,117). The Sendai virus M protein which has been removed from virus particle nucleocapsids with detergent and high salt also forms crystalline arrays in vitro when the salt is removed (133). Russell and Almeida (134) have described the regular crystalline appearance of the virus particle surface glycoproteins of the avirulent LaSota strain of NDV. They suggest that this appearance may represent a stage in virus morphogenesis in which the glycoprotein spikes are associated with the M protein. If the M protein does interact with the nucleocapsid and the viral glycoproteins, the protein-protein interactions between molecules of M protein might gather the viral components in preparation for budding. M protein interactions with the nucleocapsid might also drive the evagination of the membrane, forming a bud.

Actin is found as an integral component of many enveloped viruses (135). It is possible that actin is in some way involved in driving the budding process. Electron micrographs depicting actin filaments growing into budding measles virus have been presented (136). Actin has been found in NDV-infected cells in close association with viral antigens (137). Actin bundles appear to be disrupted during NDV infection (138). A specific interaction between actin and the NDV M protein has been demonstrated by affinity chromatography, by the formation of actin-M protein complexes, and by a change in the circular dichroism pattern of the M protein in this complex (139). Most of the NDV proteins in an infected cell are associated with the cytoskeleton, as determined by detergent extraction methods (76). Disruption of actin

microfilaments by cytochalasin D has been shown to speed the release of NDV proteins and virus particles from infected cells (76). These results imply that actin actually slows the release of virus particles. The role of actin in virus assembly is, therefore, not yet clearly defined.

It is interesting that NDV is able to preferentially package genomic (-) RNA into virus particles. In the infected cell, one-third of the genome-length RNA is (+) (25). Both (+) and (-) genome-length RNAs are found in the cytoplasmic nucleocapsid structures (72), yet little, if any, (+) sense RNA is found in virus particles (25,60,140). The mechanism of this preferential packaging is unknown.

CONCLUSIONS

The replication of NDV is a relatively complex process. The virus uses its own proteins to transcribe and process its mRNA and to replicate its genome. It uses the host cell to translate its mRNA into proteins, insert two of them into membranes, glycosylate them and transport them to the plasma membrane. The virus is capable of assembling its structural components at the plasma membrane but it is still not clear how the virus components rendezvous on the plasma membrane or whether or not the virus and cell collaborate to form the virus bud.

Studies of cells infected with NDV mutants, biochemical separations and analyses, and in vitro reconstitution experiments, have shed light on the viral proteins required for basic viral processes. How the viral proteins perform their functions is still a puzzle in most cases. The recent advances in NDV gene cloning and sequencing have provided the pieces to this puzzle. Expression of the NDV genes, alone or in concert, from these cDNAs may eventually assemble these pieces. Three of the NDV proteins are particularly interesting: HN as a representative of the small class of glycoproteins attached to membranes by their amino termini; F as the first glycoprotein reported to be reduced as it migrates through the cell membrane system; and M whose unexpected nuclear location opens a new area for exploration of the interaction of NDV

with the host cell.

This review has attempted to summarize our knowledge of NDV replication. Several excellent reviews of paramyxoviruses (18,141,142) and their assembly (143,144) will be useful for further exploration.

ACKNOWLEDGEMENTS

The author would like to thank Michael Bratt and Trudy Morrison and their laboratories for sharing unpublished and soon to be published work and for critically reading this manuscript, Patrick Dillon, Kay Faaberg, Kailash Gupta, Diana Huang, and Esther Schechter for critically reading this manuscript, and Barbara Newton, Alice Robinson, and Kaarin Soma for help in preparing this manuscript. This work was supported by the United States Public Health Service, National Institues of Allergy and Infectious Diseases, grant AI 21924.

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5

MOLECULAR CLONING AND NUCLEOTIDE SEQUENCING OF NEWCASTLE DISEASE VIRUS

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INTRODUCTION

Newcastle disease virus (NDV) is an enveloped RNA virus with a nonsegmented, single stranded genome of negative polarity. The NDV genome comprises six genes which encode the six known viral structural proteins. Three of these are associated with the lipid envelope of the virion: the haemagglutin-neuraminidase (HN) and fusion (F) glycoproteins are anchored in the membrane and appear as protruding spikes on the virion surface, the matrix protein (M) is non-glycosylated and is peripherally attached to the inner surface of the envelope. The remaining three proteins are associated with genomic RNA to form the viral nucleocapsid, these are the nucleocapsid protein (NP), the phosphoprotein (P) and the large protein (L).

The Paramyxoviridae virus family, of which NDV is a member, is thought to be most closely related to the Rhabdoviridae which is the only other family of enveloped, negative stranded RNA viruses which have nonsegmented genomes. The most intensively studied member of either group, and the first to which cDNA clones were obtained, is Vesicular stomatitis virus (VSV), a rhabdovirus. Several paramyxoviruses are now being studied using recombinant DNA techniques. Of these, Sendai virus was the first to be cloned and its complete nucleotide sequence has now been determined.

The first detailed account of the molecular cloning of NDV (in 1986) was of the moderately virulent strain, Beaudette C (1). Prior to 1986, the only nucleotide sequence data of NDV was of a region of approximately 250 bases obtained by direct chemical sequencing of the

RNA genome from its 3' end (2). It was not until cDNA clones were obtained from NDV genomic and messenger RNA that the complete nucleotide sequence and deduced amino acid sequence of viral genes could be determined. The nucleotide sequence of various genes from several strains of NDV are now known (3-10), see Table 1.

Table 1.

NDV STRAIN	GENE	REFERENCE
Beaudette C	F	(3)
	HN	(4)
	M	(5)
	L	(6)
Australia-Victoria	F	(7)
	M	(8)
Hitchner B1	HN	(9)
D26	NP	(10)
Ulster	F	*
	HN	*

* (Unpublished work, this Laboratory)

The methods used to obtain cDNA to NDV and other RNA viruses require either the viral genomic RNA or its mRNAs as a template for the enzyme reverse transcriptase. A suitable primer for reverse transcriptase is annealed to the single stranded RNA template. In the case of genomic RNA, short oligonucleotide primers are often used which hybridize at random sites. For mRNA a poly (T) primer is usually hybridized to the 3' poly (A) tail. The resulting RNA:DNA hybrid can either be cloned directly into a suitable plasmid vector, or it can be first converted to double stranded DNA. This is done by removal of the original RNA strand by the use RNase H, or some other means, and then synthesis of a second strand by DNA polymerase. Details of the techniques used to obtain cDNA clones and methods used to map their position within the genome have been described fully elsewhere (1,9-11).

The construction of cloned NDV genes enables the expression of individual viral mRNAs and proteins to be examined *in vivo* and *in*

vitro. Cloned genes of several paramyxoviruses and rhabdoviruses have been expressed in mammalian cells by insertion into a eukaryotic expression vector under the control of a simian virus 40 promotor (12-15).

Both live and inactivated virus vaccines are available for the control of Newcastle disease. However, the cloning of NDV provides the means of developing an alternative, recombinant vaccine. One posssible approach is to insert cloned NDV genes into a poxvirus expression vector. This has been done successfully in the case of the glycoprotein gene of the rhabdovirus, rabies virus (16) and has been shown to protect vaccinated animals against severe challenge infection (17,18). Vaccinia and related poxviruses have the advantage for this type of vaccine since they have a large capacity for foreign DNA (19). This approach may enable the development of a multivalent vaccine by the expression of cloned genes from several viral pathogens in a single vector.

The cloning and sequencing of NDV has provided a greater understanding of the viral genome organisation and structure, as well as detailed information about the individual mRNAs and their products. Sequencing data also provides information about evolutionary relationships between NDV and members of the Paramyxoviridae, and other virus families.

GENOME ORGANISATION

The single stranded RNA genome of NDV and other paramyxoviruses is generally thought to contain a single promoter located towards its 3' end (20,21). This is similar to the situation in the rhabdoviruses, (22,23) but is in contrast to that in the segmented genomes of orthomyxoviruses (24,25). The six genes of NDV are thought to be transcribed in a sequential manner from the 3' end of the genome by the viral RNA-dependent RNA polymerase.

Before the molecular cloning of NDV, a gene order had been proposed on the basis of UV transcriptional mapping experiments (26,27). This, however, left some ambiguity, particularly in the order of the F and M genes (27). Recent data derived from the cDNA cloning of the NDV genome has unambiguosly established the gene order

to be 3'-NP-P-M-F-HN-L-5' (1,11), which is identical to that of other paramyxoviruses studied in detail (see reference (28) for review).

It was shown as early as 1967 that NDV transcripts could be resolved into three size classes with sedimentation coefficients of 18S, 22S and 35S (29). Subsequent work has suggested that the 35S component corresponds to a single species, the L mRNA, and that the 18S class represents the five mRNAs coding for the NP, P, M, F and HN proteins (30,31). These six monocistronic mRNAs account for the total coding capacity of the genome. This information and the results of hybridization-competition experiments (32,33), which showed that the 22S transcripts contained sequences present in the smaller 18S transcripts, suggested that the 22S class may represent polycistronic messages.

Northern blot analysis, using cDNA clones, has now confirmed the presence of NDV polycistronic transcripts (1,11). This technique involves the hybridization of a radioactively labelled cDNA to viral mRNA, isolated from NDV infected cells, which has been separated on a denaturing agarose gel and transferred to a nitrocellulose membrane. The combination of genes found in polycistronic form (NP/P, P/M, M/F etc.) confirms the proposed gene order of NDV (1,11). These polycistronic transcripts have been shown to account for 15-25% of transcribed RNA and are found in association with polyribosomes in infected cells (34) suggesting that they may represent genuine functional mRNAs. These transcripts are polyadenylated at their 3' ends but do not appear to contain internal poly (A) sequences (34). Sequencing studies show that polycistronic transcripts of Sendai virus do not contain intervening poly (A) sequences, but are accurate copies of the genomic sequence (35). The observation of poly (A) at intercistronic boundaries in VSV polytranscripts (36,37) may be an artifact due to transcription *in vitro*, since *in vivo* polytranscripts do not appear to contain such sequences (38). It is still uncertain whether polycistronic transcripts are synthesized as a result of aberrant transcription due to failure of transcription termination, or represent intermediates in the synthesis of monocistronic mRNA.

To date, no NDV strain has been completely sequenced but it is possible to determine the size of the NDV genome on the basis of data

from two strains. The NP and partial P sequence of strain D26 (10) and the M, F, HN, L and partial P sequence of strain Beaudette C (3-6, and unpublished data, this laboratory) define the length of the NDV genome as 15156 nucleotides (see Fig. 1). In calculating this genomic length it has been assumed that the clone used to sequence the extreme 5' end terminates six nucleotides before the true end of the genome, as previously suggested (6). A length of 15156 for the NDV genome is similar to that of Sendai virus (15383 nucleotides), which is the only other paramyxovirus completely sequenced to date (39, and references cited therein). The size of the NDV genome, determined from sequencing data, is also in good agreement with the estimated size of approximately 5 x 10^6 daltons (15 Kb) suggested by Kolakofsky et al. in 1974, on the basis of sedimentation analysis in sucrose gradients and on electron microscopic length measurements (40).

Data from the two NDV strains mentioned above reveals that 98.8% of the viral genome is transcribed into the six monocistronic polyadenylated mRNAs and that 90.7% of the genome corresponds to regions which are translated into proteins. These figures are similar to those reported for Sendai virus: 99.17% and 93.63%, respectively (39), and demonstrate the efficient organization of the paramyxovirus genome.

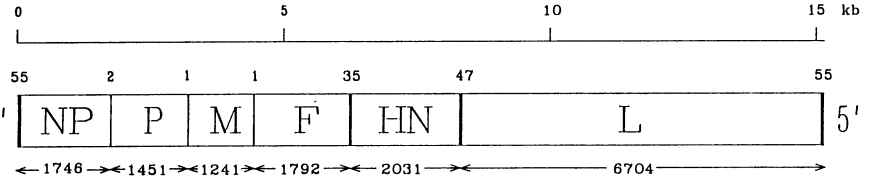

Fig. 1. NDV genome organization. The genome is represented in its 3' to 5' direction and shows the position of the six genes (NP, P, M, F, HN and L). The length, in nucleotides, of each gene is shown below and the lengths of intergenic and leader regions are shown above the diagram. A scale in kilobases (kb) is also shown.

In the related paramyxovirus, Sendai virus, in addition to the six known structural proteins (NP, P, M, F, HN and L) there is a nonstructural protein (C) present in virus infected cells but not in

mature virions (41). Nucleotide sequencing studies (42,43) have shown that the C protein is encoded by the same mRNA as P and constitutes a second overlapping reading frame. The only published sequence of the NDV P mRNA, a partial sequence (10), also reveals a second overlapping reading frame. Whether this is analogous to the C protein of Sendai virus is not yet certain. The two nonstructural proteins of NDV of molecular weight 33000 (33K) and 36K, which have been identified (30,44), have been suggested to be N-terminal fragments of the P protein, rather than proteins derived from a different reading frame.

Sequencing studies of another paramyxovirus, simian virus 5 (SV5), revealed the presence of a previously unrecognized gene between the F and HN genes coding for a small hydrophobic (SH) protein of 44 amino acids (45). Sequencing of NDV in this region (3,4) reveals no analogous gene, although there are small open reading frames of 41 and 50 amino acids at the starts of the HN and L genes, respectively (3,6). In both cases, they overlap the start of, and are in a different phase to, the major open reading frames. They are preceded by a sequence resembling a typical NDV mRNA start and are followed by a region resembling a polyadenylation site. Such transcripts or their encoded peptides have not been detected conclusively, but there is some evidence suggesting that transcription may occur at these positions (3,6 and unpublished data, this laboratory).

As has been shown for other paramyxoviruses (46,47) and the rhabdovirus, VSV (46,48), the six NDV mRNAs are preceded by a conserved start signal and terminate with a conserved polyadenylation signal. Sequencing data from the six NDV genes in this region (see Fig. 2) allows the proposal of a consensus sequence for the NDV mRNA start site (3'-UGCCCAUC$\begin{smallmatrix}U\\C\end{smallmatrix}$U-5') and polyadenylation site (3'-A$\begin{smallmatrix}A\\U\end{smallmatrix}$UCUUUUUU-5'). Both sequences are shown in the genomic (negative) RNA sense. Between the polyadenylation and mRNA start site of VSV there is a conserved dinucleotide intergenic region (3'-GA) and in Sendai virus a trinucleotide intergenic region (3'-GAA, or 3'-GGG). In NDV, however, the intergenic regions are of less uniform length, between 1 and 47 nucleotides (Fig. 2).

	POLY A	INTERGENIC REGION	mRNA START
NP			UGCCCAUCUU
NP/P	AAUCUUUUUU		CA UGCCCAUCUU
P/M	AUUCUUUUUU		A UGCCCAUCUU
M/F	AAUCUUUUUU		G UGCCCAUCUU
F/HN	AAUCUUUUUU	GAUGGCCAACAUCTUCUGGUUUCCUGCUAUA	UGCCCAUCUU
HN/L	AUUCUUUUUU	ACAUUCAUUGUUACUCUAUGUUCCGUUUUGUCGAGUACCAUUUAUCA	UGCCCAUCCU
L	AAUCUUUUUUU		

Fig. 2. NDV mRNA polyadenylation, intergenic and start sequences. The sequences are shown in the genomic (negative) RNA sense. Data taken from references (4,6,10).

The exact 5' terminus has been determined for several NDV mRNAs synthesized *in vivo*. This has been done both by the method of nuclease-mapping (6) and by primer extension analysis (7-9) and suggests that these transcripts begin with an A residue, as appears to be common among negative-strand RNA viruses. Analysis of the 5' cap structure of NDV mRNAs confirms this. By the techniques of paper electrophoresis and thin-layer chromatography the NDV mRNA cap structure was identified as m^7GpppAm (10). Analysis of the NP mRNA by the wandering spot method (2) contradicts this, since the first nucleotide was found to be G rather than A. However, this discrepancy may be the result of the synthesis of mRNA by *in vitro* transcription and may not represent the situation *in vivo*.

Both paramyxoviruses and rhabdoviruses synthesise a short leader RNA which is complementary to the 3' end of genome. Sequencing of this region of the NDV genome (10) identified a sequence resembling an mRNA start site 56 nucleotides from the 3' terminus (Fig. 2) which was assumed to be the start of the NP mRNA. It was proposed that the leader sequence corresponded to the first 53 nucleotides of the genome and was followed by a dinucleotide (3'-CA) as found between the NP and P genes (10). Earlier work, in which NDV leader RNA was isolated from both *in vivo* and *in vitro* transcription reactions, identified species of both 53 and 47 nucleotides. The larger species

was the most abundant *in vitro*, whereas the shorter species was most abundant *in vivo* (2). This may be a result of degradation of the larger species *in vivo*. Sequencing of the 5' end of the NDV genome (6) suggests the presence of a negative strand leader RNA, as well as the known positive strand leader. This is similar to the situation in VSV (49).

MEMBRANE ASSOCIATED VIRAL PROTEINS

Fusion glycoprotein (F)

The fusion glycoprotein is responsible for fusion of the virus with and penetration through the host cell membrane (50,51). The protein is synthesized as an inactive precursor (F_0) which requires proteolytic cleavage to generate the disulphide-linked fragments F_1 and F_2 for viral infectivity (50-54). The order of the two fragments in the intact F_0 was determined as NH_2-F_2-F_1-COOH by the technique of salt shock mapping (55) and has now been confirmed by sequencing (3,7).

The fusion protein is thought to be the major determinant of the wide ranging virulence of NDV strains. Cleavability of the precursor glycoprotein (F_0) in a range of cell types correlates well with the pathogenicity of viral strains in infected birds (51).

In pathogenic strains of avian influenza virus the haemagglutinin precursor (HA_0) is readily cleaved in a wide range of cell types and has a highly basic peptide at the cleavage site of HA_0, whereas apathogenic strains which are not readily cleaved have a significantly less basic peptide in this region (56,57). Sequencing data has shown that there is a similarly convincing correlation between basic amino acids at the cleavage site of NDV F_0 and pathogenicity of strains (Fig. 3). In the moderately virulent strain, Beaudette C, a highly basic sequence preceeds the cleavage site (4) while in the extremely avirulent strain, Ulster, this region is less basic (unpublished data, this laboratory).

The correlation between cleavage site sequence and known cleavability of the F_0 precursor can be extended to other paramyxoviruses. For example, the F_0 precursor of SV5 has a basic sequence in this region (58) and, like that of NDV strain Beaudette

C, is readily cleaved in a wide range of cell lines (59), whereas the Sendai virus protein which has a much less basic sequence (39,60), is cleaved in only a restricted range of cell lines (61).

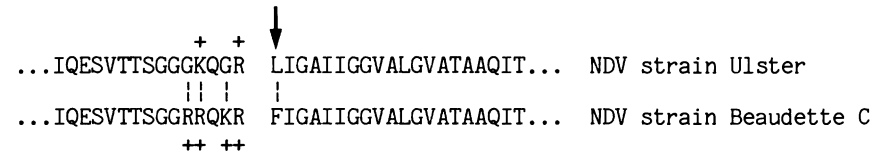

Fig. 3. Amino acid sequence at the F_0 cleavage site. A partial sequence of the virulent Beaudette C strain and avirulent Ulster strain is shown. Basic amino acids are indicated by '+', differences between strains by dashed lines and the N-terminus of F_1 by an arrow.

The F_0 precursor has a blocked N-terminus (as does HN) and this has prevented direct N-terminal amino acid sequencing of these proteins. However, after proteolytic cleavage of F_0, the newly exposed N-terminus of the F_1 fragment can be sequenced. The N-terminal 20 amino acids of F_1 were sequenced directly by the Edman degradation method several years before the full sequence was determined (62). This showed the region to be highly hydrophobic and well conserved among paramyxoviruses. Analysis of the complete amino acid sequence of the fusion protein of NDV, deduced from nucleotide sequencing (3,7), and that of other paramyxoviruses has identified three strongly hydrophobic regions (Fig. 4). These correspond to the cleaved N-terminal signal sequence, a C-terminal membrane attachment site and an internal region which is at the N-terminus of the F_1 cleavage fragment. This internal hydrophobic peptide is thought to be responsible for the fusion activity of the virus, and presumably interacts with the host cell so as to facilitate virus-cell fusion.

Recent work with SV5 has helped in the understanding of the role of the hydrophobic domain at the N-terminus of F_1 (63). The construction of hybrid proteins containing this hydrophobic region in both internal and external positions suggests that it is close to the threshold of hydrophobicity required to interact with lipid membranes. When in an internal position, as in F_0, it is not sufficiently hydrophobic to halt translocation, which explains how the protein is able to cross the rough endoplasmic reticulum. However, when in an external position, as occurs after cleavage

activation of F_o, this region is sufficiently hydrophobic to interact with membranes. Similar work with the F protein of Sendai virus indicated that the hydrophobic region at the N-terminus of F_1 is unable to act as a protein membrane anchor when situated in an internal position (64). When inserted into a prokaryotic membrane protein, the C-terminal region of the Sendai virus F protein halted transfer of the hybrid protein through the cytoplasmic membrane of Escherichia coli, whereas the N-terminus of F_1 did not.

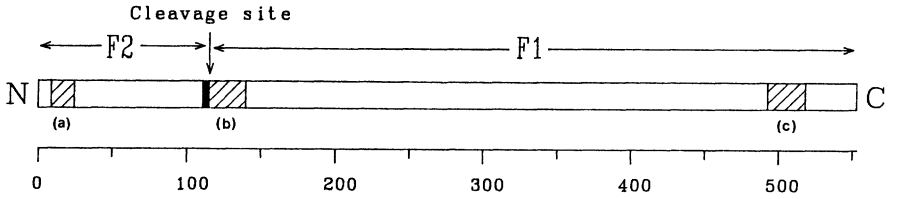

Fig. 4. Fusion glycoprotein structure. The three major hydrophobic regions are cross hatched. These correspond to (a) the signal sequence, (b) the N-terminal region of F_1 and (c) the membrane attachment site. The site at which F_o is cleaved to generate the F_1,F_2 fragments is indicated and is located in the highly basic region of the protein (heavily shaded). A scale of amino acid number is also shown.

Haemagglutinin-neuraminidase glycoprotein (HN)

The HN glycoprotein contains both the haemagglutinating and neuraminidase activities of NDV which are responsible for the initial attachment of the virus particle to its cellular receptor and receptor-destroying activity, respectively (65,66). In the orthomyxoviruses, in contrast to the paramyxoviruses, these activities are on separate glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA).

The influenza virus NA has been known for some time to be unusual with respect to its orientation in the viral membrane, being anchored at a site close to its N- rather than C-terminus (67,68). Before sequencing data was available there was evidence to suggest that this was also the case with the NDV HN glycoprotein. A 9K fragment removed from the HN_o of intact virions was shown by N- and C-terminal amino acid analysis to be derived from the C-terminus of the protein, while the N-termini remained blocked (69). Analysis of

the complete amino acid sequence of the NDV HN (4,9) supports this interpretation by showing that the major hydrophobic region of the protein is close to the N-terminus. Furthermore, the relatively hydrophilic nature of the extreme N-terminal amino acids suggests the absence of a signal sequence which is cleaved after translation, since this region does not have the features identified by von Heijne as being necessary for cleavage (70,71). There does not appear to be a cleaved N-terminal hydrophobic signal in the influenza virus NA (67,68) and other N-terminally attached viral glycoproteins (72-77).

Monoclonal antibodies have been used to show that the haemagglutinin and neuraminidase sites of HN are antigenically separate (78). The location of the two active sites have not been identified conclusively by sequencing data. However, three recent papers present evidence which argues that the NA site is located towards the centre of the paramyxoviral HN glycoprotein, whereas the HA site is towards the C-terminus. Blumberg *et al*. compared the Sendai virus HN sequence to that of influenza virus HA and NA sequences and found homology which suggested this order for the active sites (72). In the reported sequencing of the Beaudette C strain of NDV (4) a sequence, Gly-Ala-Glu-Gly-Arg-Leu, in the C-terminal region of the protein was suggested to be similar to the influenza HA sialic acid binding site, while in the reported sequence from the B1 strain (9) a sequence, Asp-Arg-Lys-Ser-Cys-Ser, was identified in the centre of the HN sequence, which was suggested to be homologous to the influenza NA sialic acid site.

The ability to express cloned viral proteins *in vivo* has enabled the location of a neutralising epitope on the HN glycoprotein of NDV. Progressively smaller fragments of HN cDNA were cloned into a bacterial expression vector, fragments of HN were expressed either alone or fused to the alpha-peptide of beta-galactosidase. Examination of these peptides by Western-blot analysis using a monoclonal antibody which bound to the reduced form of HN localised the binding site of the antibody to a hydrophilic, eight amino acid peptide (unpublished data, this laboratory).

In the extremely avirulent strains of NDV, Ulster and Queensland, the structure of the HN as well as the F glycoprotein is

ILKDDGVREARAGRLSQLREGWKDDIVSPIFCDAK**NQT**EYRRELESYAASWP* Strain Ulster

.....S.* Strain B1

...N.....S.* Strain Beaudette C

Fig. 5. Amino acid sequence at the C termini of the HN_o glycoprotein precursor of NDV strain Ulster and the HN glycoproteins of NDV strains Beaudette C (4) and B1 (9) are shown boxed. Amino acids identical to the Ulster sequence are indicated by dots and termination codons by asterisks. A potential asparagine-linked glycosylation site is shown in bold type.

thought to be responsible for the lack of virulence (51). In these two strains the HN glycoprotein appears to be synthesized in a precursor form (HN_o) which requires cleavage of a C-terminal glycopeptide to produce active HN (79). The presence of an HN_o precursor has not been observed in other paramyxoviruses. The long non-coding region at the end of the HN gene of the moderately virulent Beaudette C strain, which has the capacity to code for up to 55 further amino acids, provided a possible explanation for the origin of HN_o (1,4). Nucleotide sequencing of the HN gene of strain Ulster reveals that the major open reading frame of HN_o extends 39 amino acids beyond the C-terminus of the HN glycoprotein of the more virulent strains Beaudette C and B1. Within the C-terminal 'extension' of HN_o a potential asparagine-linked glycosylation site is present (Fig. 5).

Matrix protein (M)

The M protein of NDV and other paramyxoviruses forms a shell on the inner surface of the viral lipid envelope (80) and is thought to play an important role in paramyxovirus assembly (80,81). Nucleotide sequence analysis of the M gene of NDV (5,8) has gone some way towards explaining the functions of this protein.

The amino acid sequence reveals the generally hydrophobic and highly basic nature of the M protein. It is assumed that the major interaction of the M protein with the viral membrane is hydrophobic, as suggested for the M protein of influenza virus (82). There do not appear to be any areas of the sequence which are of sufficient length

and hydrophobicity to represent a transmembrane region, which is consistent with the known peripheral attachment of the M protein with the viral membrane (83).

The basic nature of the M protein may help to explain its role in the assembly of virions in infected cells. The M protein is thought to bind to the viral nucleocapsid (81,84), the major structural proteins of which are acidic. The regions of the nucleocapsid proteins which interact with the viral genome are presumably basic, suggesting that the surface available to bind with the basic M protein would display a pronounced negative charge. The M protein of NDV has been shown to bind actin *in vitro* (85). The basic nature of the M protein may account for this association with actin, which has an overall negative charge and is frequently found in enveloped viruses (86).

Bellini *et al*. compared the sequences of the M proteins of Sendai, measles and canine distemper viruses and showed the positions of glycine, proline and paired basic amino acid residues were well conserved (87). These features are also conserved to some extent between the M proteins of these viruses and NDV (5,8). It is possible that paired basic amino acids play a role in the association of the M protein with the viral nucleocapsid. However, the overall amino acid sequence of the M protein appears to be less well conserved among paramyxoviruses than are the NP, F, HN or L sequences, suggesting that the functions of the protein may be mediated by its overall characteristics (basic and largely hydrophobic) rather than by any particular conserved sequences (5).

NUCLEOCAPSID ASSOCIATED VIRAL PROTEINS

The NP, P and L proteins, in association with the genomic RNA, form the viral nucleocapsid. They occur in the approximate relative abundances of 3000, 300 and 30 molecules per virion. These proteins have been studied in less detail than the viral glycoproteins and less can therefore be inferred from sequence analysis data. Rather more is known about the analogous proteins of VSV (see reference (88) for review). VSV has been studied more thoroughly than any paramyxovirus, partly because it replicates more efficiently in

tissue culture cells and is more transcriptionally active in vitro.

Nucleocapsid protein (NP) and phosphoprotein (P)

The helical nature of the nucleocapsid is due to the binding of NP, the most abundant viral protein, to both genomic RNA and positive strand (anti-genome) RNA, which serves as the template for replication of genomic RNA. In VSV, investigation of the self-assembly of viral RNA with the N protein, which is analogous to the NP protein of paramyxoviruses, showed that both genomic and leader RNAs were encapsidated by protein. This work also identified a 14 nucleotide sequence at the 5' end of the leader RNA, and hence the complementary region of genomic RNA, as being important in initiating nucleocapsid assembly (89). There is evidence that the nucleocapsid protein (N) of VSV (its most abundant viral protein) is responsible for the control of genome replication (90). By analogy, the paramyxovirus NP protein may have a similar reglulatory role in the virus infected cell.

Several paramyxovirus P genes have been sequenced and a general feature which has emerged is the greater divergence in amino acid sequence of the P protein between viruses compared to that of other viral structural proteins. However, the various P proteins do show similar hydropathy profiles (91), which is similar to the situation with the NS protein in different serotypes of VSV (92). The recently published partial NDV P sequence suggests that, like other paramyxoviruses, this is the least conserved of the viral proteins (10) and implies that P might have a role which is largely virus specific. The presence of nonstructural protein(s) encoded on the P mRNA, which appears to be a feature of paramyxoviruses was discussed above in the section dealing with genome organisation.

Large protein (L)

The gene which encodes the L protein is approximately 6.7 Kb long (6) and therefore constitutes almost half of the viral genome. The protein was known to have an extremely high molecular weight (>200 K) from analysis by SDS-polyacrylamide gel electrophoresis (27). The amino acid sequence of the protein derived from cDNA sequence gives a molecular weight of 249 K (6).

The precise functions of the L protein are not yet known, but

are assumed to include the RNA-dependent RNA polymerase activity responsible for both viral transcription and replication. The L protein of NDV together with the P protein (or NS in the case of VSV) have been shown to constitute an active viral transcriptive complex when added to viral nucleocapsids which have been stripped of these proteins (93,94). The L protein is the least abundant of the viral proteins, suggesting that it has an enzymatic rather than a structural role. The L protein of VSV and, by analogy, paramyxoviruses is thought to be responsible not only for synthesis of viral mRNA but also for capping, methylation and polyadenlyation of the newly transcribed mRNAs (88,95,96). In vitro studies with the L protein of VSV have demonstrated that it is able to phosphorylate NS (97), suggesting that the L protein also has a protein kinase activity.

The L genes from Sendai virus (39,98) and VSV (99) have been sequenced in addition to that of NDV (6). Comparison of these sequences at the amino acid level has shown a high degree of similarity (6). This might be expected for NDV and Sendai virus, on the basis of comparisons with other gene sequences, but this is the only NDV protein to show convincing sequence similarity to a VSV, or any rhabdoviral protein. This provides evidence for an evolutionary relationship between the Paramyxoviridae and Rhabdoviridae virus families.

Comparison of the three available L sequences suggests that most of the RNA synthetic and modification activities are located in the N-terminal two-thirds of the L protein (6). This assumes that regions likely to have important enzymatic or conformational functions are those which are most highly conserved. The most variable parts of the three sequences are their C-terminal regions (6) which might, therefore, be responsible for virus-specific functions such as interaction with other viral proteins.

The cloning of individual NDV genes will provide further opportunities to study the structure and function of the viral proteins. The cloned L gene of VSV has been expressed in mammalian cells to give a protein which is indistinguishable in size and functions from the L protein synthesised in virus infected cells

(13). Similar work with the L gene of NDV may lead to a better understanding of this complex multifunctional protein.

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6

MOLECULAR BASIS OF INFECTIVITY AND PATHOGENICITY OF NEWCASTLE DISEASE VIRUS

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INTRODUCTION

Newcastle disease virus (NDV) can be isolated from a wide variety of different avian species. Differences in virus pathogenicity have been observed which depend on the host infected as well as on the virus strain involved. Chickens seem to be the most susceptible species. Ducks and geese, on the other hand, are reported to be refractory even to the most pathogenic viruses for chickens (27). There is evidence that adaptation of a particular NDV strain to a novel host may affect pathogenicity. Thus, Alexander and Parsons (1) reported that NDV isolates from pigeons required several passages in chickens, before their potential pathogenicity became manifest in this species. Although the reason for this host specificity of NDV is not known, such observations are of epidemiological significance. They explain how potentially pathogenic virus strains can be generated and maintained in a particular species without harm. This pathogenic potential could then become fully manifest when a different species of highly susceptible birds, such as chicken, is exposed to these viruses.

With respect to the causative virus of Newcastle disease it became evident that it comprises a wide range of virus strains which differ markedly in pathogenicity for the chicken and other susceptible hosts both in natural and experimental infected birds (64). One should be aware of observations, however, that disease patterns, which overlap classification, have been frequently described in field outbreaks.

Pathogenicity can be measured on a quantitative basis in the chick embryo or in young chickens, though these tests are not without problems. Numerous comparative studies have been carried out on different NDV strains in search for in vitro properties linked to pathogenicity. Of the many parameters analysed, only the capacity to form plaques at all or plaques of different size (14, 25, 36, 51) and to induce cell fusion (42) in chick embryo cells were found to be related to pathogenicity. Through the application of biochemical methods it became possible to gain an insight into the molecular basis underlying these properties. It has been shown that the structure of the envelope glycoproteins of NDV plays a critical role in infectivity of the virus, its spread in the infected organism and thus, for pathogenicity. It is the subject of this article to explain these interdependencies.

THE PROTEINS OF NDV

The genome of NDV is a single strand of RNA of negative polarity. It has a molecular weight of 5.2×10^6 to 5.6×10^6 and contains approximately 15,000 bases (26). There are six structural proteins, which are located on the genome in the order 3'-NP-P-M-F-HN-L-5' (5). NDV-infected cells contain, in addition, two virus-coded non-structural proteins, 36K and 33K, that are probably encoded by the same mRNA as P (4, 7). The genomic RNA is bound to three proteins to form the viral nucleocapsid. These proteins are the nucleocapsid protein NP (34), the phosphoprotein P (3, 56) and the large protein L (15). The nucleocapsid is contained within a lipid envelope derived from the host cell membrane (19, 20), on the inner side of which is a shell of membrane or matrix protein M (44, 55). Analysis of the nucleotide sequence has revealed that the M protein is composed of 364 amino acids and does not contain extensive hydrophobic regions (28). On the outer surface of the viral envelope are spikes that are formed by the two viral glycoproteins HN and F.

The HN glycoprotein contains both the hemagglutinin and neuraminidase activities of the virus (49) which are responsible for the initial attachment of the virus particle to its cellular receptor

and receptor destroying activity, respectively. As the receptor destroying enzyme, HN has a substrate specificity for 2→3, 2→6, and 2→8 linked neuraminic acid (9) and is responsible for the absence of neuraminic acid on the viral glycoproteins (22). As indicated by nucleotide sequencing the HN gene of the Beaudette C strain codes for a protein of 577 amino acids with a molecular weight of 63,149. Analysis of the amino acid sequence reveals six potential glycosylation sites and shows the major hydrophobic region to be close to the N-terminus (33). The latter observation confirms the previous finding that HN is inserted with its amino-terminal end in the lipid layer of the viral envelope (53).

The F glycoprotein is involved in virus penetration, polykaryocytosis or fusion from within and hemolysis. Hemolysis reflects fusion between the viral envelope with the erythrocyte membrane. Since only marker substances smaller than 30 nm are released during NDV-induced hemolysis, it is thought that hemolysis does not result in the formation of membrane lesions, but rather in an alteration of membrane permeability (57). The F glycoprotein consists of two disulfide linked polypeptides, F_1 and F_2, which are derived from the precursor F_0 by posttranslational proteolytic cleavage, as has been shown for NDV (36) as well as for other paramyxoviruses (16, 46, 48). The nucleotide sequence of the F gene has been elucidated with a number of different NDV strains (6, 29, 62). The gene encodes a polypeptide about 550 amino acids long which has three regions of high hydrophobicity: an N-terminal signal peptide, the N-terminus of F_1 supposed to be the fusion peptide of paramyxoviruses (13, 50) and a C-terminal membrane anchor, where fatty acid is covalently bound (52). There are several N-glycosidic glycosylation sites, of which one is in F_2 and the others in F_1 polypeptides.

SIGNIFICANCE OF PROTEOLYTIC CLEAVAGE OF NDV-GLYCOPROTEINS FOR INFECTIVITY

Cleavage of the F-protein is performed by cellular enzymes and presumably takes place when the maturing protein passes on its way to the plasma membrane from the endoplasmic reticulum via the Golgi

apparatus or through post-Golgi transport vesicles (37). Cleavage is not necessary for virus particle formation. Whether or not cleavage occurs depends on the virus strain and the host cell. Certain strains of NDV produce, in appropriate host cells, virus particles with the F protein present in the cleaved form, whereas if grown in other host systems the virions contain the uncleaved protein. On the other hand, in a given host cell the glycoproteins of some strains may be cleaved, whereas those of others are not cleaved, and it could be shown that these differences in cleavage are based on structural differences of the glycoproteins (24, 36, 62).

Virus particles containing uncleaved F-protein can be cleaved by in vitro treatment with trypsin or trypsin-like enzymes (36). This observation is in agreement with subsequent sequence studies which demonstrated that cleavage occurs at arginine-containing sites. Cleavage of F is paralleled by a charge shift suggesting that arginine at the cleavage site is removed by a carboxypeptidase (24), as has been observed with the hemagglutinin of influenza virus (12). These observations taken together indicate that the F protein is cleaved by the sequential action of a trypsin-like endoprotease and a carboxypeptidase, both provided by the host cell.

The HN glycoprotein is not thought to undergo the modifications of post-translational cleavage except in the case of some apathogenic NDV strains, such as Ulster, Queensland, and D26. These strains encode a larger precursor HN_0 (35, 36, 39), that is converted by posttranslational removal of a 9K glycopeptide (10) from the carboxyterminal end (53) into HN. In contrast to F_0, HN_0 is cleaved by a series of proteases of different specificities. Why HN_0 is observed only with some strains, but not with others, is not yet completely understood. However, it is interesting to note that nucleotide sequence analysis of the HN gene of the Beaudette C strain, which does not exhibit the precursor, revealed the presence of a relatively long non-coding region at the 3'-end of the mRNA having the potential to code for up to 55 extra amino acids. This region contains a glycosylation site in phase with the open reading frame. It is speculated that in strains expressing HN_0 the stop codon

before the non-coding region has been lost by mutation thus allowing the formation of a larger translation product (33). Sequence analysis of these strains will provide definitive proof of whether this is the mechanism by which HN_0 is generated.

Cleavage is essential for the biological activity of both NDV virus glycoproteins and for the infectivity of the virus particle. The availability of inactive precursors that were susceptible to *in vitro* activation with defined proteases was of the utmost importance for establishing the specific role for each glycoprotein in the initiation of infection. It could thus be shown that HN is responsible for adsorption and that the F-glycoprotein is involved in penetration by triggering fusion of the NDV envelope with the cell membrane (35, 36), as it is also the case with other paramyxoviruses (16, 48).

HN-specific antisera (54, 63) and monoclonal antibodies (see 41) inhibit fusion, which indicates that adsorption of the virus is a precondition for fusion and, thus, penetration, to occur. This was also found with reconstituted viral envelopes. Liposomes containing the cleaved F-glycoprotein alone, were shown to be unable to adsorb and initiate fusion (17). If on the other hand, the liposomes contain, in addition to the F-glycoprotein, another adsorbing, but nonfusing viral protein, the HA_0 of influenza viruses, fusion occurs (18). These studies also indicate that the F-glycoprotein is responsible for membrane fusion. Evidence that fusion of the envelope of the infecting virus with the plasma membrane is the mechanism of virus penetration has been obtained also from electron microscopic studies (2). Further insight into the mechanism underlying the fusion process has been employed by nuclear magnetic resonance studies of chicken embryo cells exposed to NDV (40). The mechanism of the pertubation induced by the F-glycoprotein appears to be a biophysical rather than a biochemical event, since the chemical composition of the membrane lipids is not changed in the fusion process (8).

NDV GLYCOPROTEINS AS DETERMINANTS OF PATHOGENICITY

Formation of sufficient amounts of infectious virus and rapid spread in the host organism is a precondition of pathogenicity. It is fair to assume that the more cells of a given organism that produce virus in an infectious form, the greater is the chance of the virus reaching the target organ(s) to exert pathogenic action. It has been pointed out above that activation of the viral glycoproteins by proteolytic cleavage is determined by the structure of these molecules as well as by the disposition of appropriate enzymes by the host. Depending on the virus strain and the host cell, NDV forms either infectious virions with cleaved glycoproteins or particles with reduced infectivity and uncleaved glycoproteins. Thus, there are differences in host range, and these differences proved to be of high importance for the pathogenicity of NDV.

Highly pathogenic and low pathogenic NDV strains that were grown in different host cell systems (36) have been analyzed. It was found for lentogenic strains that only a few special types of host cells were permissive, i.e. they produced infectious virus and allowed replication under multiple cycle conditions; most host cells are non-permissive for these strains. In contrast, all cell types tested were permissive for pathogenic strains. Therefore, there are considerable differences in the host spectrum of NDV which can be traced back to the differential proteolytic activation of their glycoproteins.

It is interesting to note that the pathogenic strains always contained both glycoproteins in the cleaved form regardless of the host cell. On the other hand, there were differences among the non-pathogenic strains with respect to the cleavability of either glycoprotein. While the precursor F_0 was found in all lentogenic strains, the precursor HN_0, in addition to F_0, could be observed only with the strains Queensland, Ulster and D26. The observation that these strains have the lowest pathogenicity indices among all NDV strains underlines the general significance of proteolytic activation for pathogenicity and it indicates that, in addition to F, HN is also a determinant.

The differences in cleavability of the F_0 glycoprotein are due to differences in the structure of its cleavage site. The F_1- and F_2-fragments of the F-molecule of the lentogenic NDV strains are linked by a single arginine. This is in contrast to the velogenic strains which have a sequence of several basic amino acids at the cleavage site. This concept was first suggested by electrofocussing studies (23, 24) and has now been confirmed by sequence analyses (62) as shown in Table 1. Although the F-glycoproteins of all NDV-strains are cleaved by the same general mechanism resulting of the elimination of basic amino acids, it has to be assumed that differences exist in the specificities of the endoproteases that recognize either a single arginine or the paired basic residues of arginine-arginine or lysine-arginine and that these differences are key determinants associated with pathogenicity.

The N-terminus of F_1, exposed after the cleavage reaction, is highly conserved among several NDV strains (Table 1) and other paramyxoviruses (13, 50). It is interesting to note that the strains Queensland, Ulster and D26 have Leu instead of Phe at the F_1 N-terminus (62). Since these strains replicate efficiently through multiple growth cycles and exhibit fusion and hemolytic activities if their F_0 is activated by trypsin (36), the F_1 N-terminal Phe may not be essential for fusion activity. It is not clear whether it is a mere coincidence that this amino acid substitution is paralleled by the occurrence of the HN_0 precursor in these lentogenic viruses.

The data, taken together, underline the important role of proteolytic activation of the glycoproteins in pathogenicity. Spread of the lentogenic strains of NDV in the organism is inhibited as soon as the virus infects cells which are unable to activate the glycoprotein. The result is a local infection, which usually does not result in the manifestation of an overt disease. On the other hand, cleavability of the F-glycoprotein in a wide spectrum of different cells, as is the case with the velogenic strains, permits a rapid production of infectious virus in all organs to high amounts. This allows spread of the virus throughout the organism and results in a fatal systemic infection (43).

Table 1. Cleavage sites of the F-glycoprotein of different strains of Newcastle diesease virus.

Virus strain	Pathogenicity for chicken	Viral glycoprotein	Cleavage site
Italien	velogenic	$F_{1,2}$, HN	NH_2--Gly-Gly-Arg-Arg-Gln-**ARG-ARG**▼Phe-Ile-Gly--COOH
Miyadera	velogenic	$F_{1,2}$, HN	NH_2--Gly-Gly-Arg-Arg-Gln-**ARG-ARG**▼Phe-Ile-Gly--COOH
Herts	velogenic	$F_{1,2}$, HN	NH_2--Gly-Gly-Arg-Arg-Gln-**ARG-ARG**▼Phe-Ile-Gly--COOH
Victoria	velogenic	$F_{1,2}$, HN	NH_2--Gly-Gly-Arg-Arg-Gln-**LYS-ARG**▼Phe-Ile-Gly--COOH
Beaudette C	mesogenic	$F_{1,2}$, HN	NH_2--Gly-Gly-Arg-Arg-Gln-**LYS-ARG**▼Phe-Ile-Gly--COOH
La Sota	lentogenic	F_0, HN	NH_2--Gly-Gly-Gly-Arg-Gln-Ser-**ARG**▽Phe-Ile-Gly--COOH
D26	lentogenic	F_0, HN_0	NH_2--Gly-Gly-Gly-Lys-Gln-Gly-**ARG**▽Leu-Ile-Gly--COOH
Queensland	lentogenic	F_0, HN_0	NH_2--Gly-Glu-Gly-Lys-Gln-Gly-**ARG**▽Leu-Ile-Gly--COOH
Ulster	lentogenic	F_0, HN_0	NH_2--Gly-Gly-Gly-Lys-Gln-Gly-**ARG**▽Leu-Ile-Gly--COOH

▼ Cleavage site of endoprotease recognizing pairs of basic residues,

▽ Cleavage site of endoprotease recognizing a single arginine.

Amino acids eliminated by the sequential action of endoprotease and carboxypeptidase are indicated by capitals. The sequence data of Victoria and Beaudette C strains are taken from McGinnes and Morrison (29) and Chambers et al. (6), the other sequences are from Toyoda et al. (62)

The concept of this relatively simple mechanism of pathogenicity could be verified by experiments in an intact organism, the chicken embryo (38). After infection of the chorioallantoic membrane the glycoproteins of the velogenic strains were activated in each germinal layer of this membrane, regardless of the route of infection, whereas the glycoproteins of the lentogenic viruses were cleaved only in the endodermal cells. Accordingly, there were differences between velogenic and lentogenic strains in their spread through the membrane. Multiplication of non-pathogenic viruses was restricted to the cell layer which was inoculated. Spread of newly synthesized virus was inhibited as soon as the virus reached the barrier of non-permissive cells. On the other hand, the pathogenic virus spread through the whole membrane and gained entrance into the blood vessels, and the embryo died.

The replication patterns of the apathogenic strains, displaying inefficient production of infectious virus in a wide spectrum of non-permissive cells, combined with occasional bursts of infectious virus after infection of permissive cells, suggest that viruses of this type rather than those of pathogenic NDV strains could play a role in the development of persistent infection.

CHANGES IN PATHOGENICITY BY MUTATION OF THE F-GLYCOPROTEIN

It can be assumed that the pathogenic and non-pathogenic NDV strains arose from each other by spontaneous mutation which, if the concept mentioned above, is correct, should affect the susceptibility of the viral glycoproteins to proteolytic cleavage. Indeed, a mutant has been obtained after exposure of the non-pathogenic wild-type La Sota strain to nitrous acid which acted like the pathogenic strains, and was able to produce plaques in non-permissive cells unlike its non-pathogenic parent. Furthermore, like the pathogenic viruses, the mutant induced membrane fusion and did not require trypsin treatment for full expression of infectivity if grown in non-permissive cells in contrast to the parent virus. Polyacrylamide gel electrophoresis revealed that in contrast to the wild-type, the mutant contained the F-glycoprotein synthesized in the cleaved form in all cells tested as did the pathogenic strains.

The increase in host range of this mutant was in turn paralleled by a significant increase in pathogenicity for chick embryos and for chickens (11).

NDV mutants with an alteration in pathogenicity from pathogenic to non-pathogenic viruses have also been isolated (14, 51, 61). In one instance, evidence has been obtained that the F-glycoprotein became resistant to proteolytic cleavage (30), which again supports the concept that pathogenicity is determined by the protease susceptibility of the virus glycoproteins.

When coupled with the work of Scheid and Choppin (49) on the generation in the laboratory of Sendai virus mutants activated by different proteases with different host ranges, there may be the possibility that such mutants may arise in nature also with NDV. Such mutants could exhibit organ or species specificities which differ from those of the wild-type virus.

OTHER VIRAL FACTORS INVOLVED IN PATHOGENICITY

There is no doubt that, besides cleavability of virus glycoproteins, infectivity and pathogenicity depend on a functional genome which permits optimal growth of the virus in the infected organism. Any change in the genome by a process of random mutation might give rise to virus variants with altered biological properties. Investigations carried out by Madansky and Bratt (30, 31, 32) may be taken as examples of the many *in vitro* studies which have been performed with a number of avirulent NDV mutants. These authors found that alterations in viral RNA transcription or translation could modulate growth and cell to cell spread of the virus and/or cytopathogenicity. Some of the lesions found to be responsible for the altered polypeptides, such as P and F, could be genetically separated from those responsible for the non-pathogenic phenotypes. These alterations also contributed to pathogenicity as determined by extended mean embryo death time, but did so independently of the alterations resulting in the non-cytopathic phenotype.

Similarly, Schloer and Hanson (51) and others (14, 61) found evidence for a correlation between plaque size and pathogenicity of variants and mutants of NDV, although the correlation may not be absolute. Certainly, the fact that mutants selected for a decrease in cytopathogenicity exhibit phenotypes similar to naturally occurring non-pathogenic strains suggests that reduced cytopathogenicity may account in part for their pathogenic properties (30). The question remains, however, whether this type of experiment will be able to define the factors on the viral side responsible for pathogenicity. One has to take into account that pathogenicity is primarily determined by optimal growth of the virus at a site of primary replication which then enables the virus to reach the clinically important target organ(s).

It is conceivable that the so-called mesogenic strains have acquired a mutation which prevents optimal replication and spread in the organism. In this way the defense mechanism of the host would be able to keep the virus under control. This notion is supported by the observation that these strains produce mainly small plaques and have lower pathogenicity indices than the velogenic strains. However, there are often no discrete border lines between mesogenic and velogenic strains. Unfortunately very little is known about the molecular biology of the mesogenic strains. The only exception is the Beaudette C strain which, although of relatively low pathogenicity, has a pair of basic amino acids at the cleavage site of the F-glycoprotein as have the pathogenic strains (Table 1).

CONCLUSION

Pathogenicity, i.e. the ability of a virus to induce disease in the infected organism, is the result of a complex interplay of a multitude of factors that are determined by the biological, biochemical, and genetic characteristics of the virus on the one hand, and the reactivity of the host on the other hand. Thus, a molecular basis for viral pathogenicity is not easy to define. It is reasonable, however, to assume that disease becomes manifest, if the infecting virus has the ability to multiply to high amounts and to

spread in the organism, and if cells containing vital functions are altered or killed by the virus. Since cell tropism represents primarily an interaction between the surface components of the virus and receptors of the host cell, it is obvious that the surface structure of a virus determines pathogenicity.

With NDV it could be clearly shown that infectivity, spread and pathogenicity of the virus depend, in addition to an optimally functioning viral genome, on the proteolytic cleavability and activation of the viral glycoproteins in a broad range of different host cells. Rapid multiplication and spread of the virus in the host are the most critical factors in the induction of a systemic infection caused by the velogenic NDV strains. With the lentogenic NDV strains proteolytic activation of the F-glycoprotein, and in a few instances also of the HN-glycoprotein, is restricted to very few cell types, so that spread of the virus is prevented as soon as the newly synthesized virus reaches non-permissive host cells. It can be excluded with some certainty that the differences in pathogenicity are based on the induction of different degrees of cytopathogenicity, because the apathogenic strains are as cytopathogenic as the pathogenic ones if the environmental conditions provide an appropriate proteolytic enzyme for activation of the viral glycoproteins.

Common characteristics are evident between the development of disease in chickens, infected by various strains of NDV and avian influenza viruses. The latter viruses, like NDV, comprise a whole series of strains differing in pathogenicity. Here again differences in pathogenicity could be correlated with host cell-specific cleavability and activation of the main viral glycoprotein, the hemagglutinin. Sequence analyses of hemagglutinins of different avian influenza virus strains have revealed that the apathogenic strains contain single arginine residues and the pathogenic strains paired basic residues at their cleavage site, exactly as has been observed with the F-glycoprotein of NDV (21, 45).

Thus, the available evidence indicates that differences in pathogenicity of viruses other than NDV are also the result of structural variations in the glycoprotein and that these are con-

fined to the cleavage site, i.e. to a small but functionally important part of the molecule (see 21). This concept of proteolytic activation of viral glycoproteins in the pathogenicity has recently obtained further support from experiments with influenza viruses and co-infecting bacteria (58-60). It was shown that proteases of Staphylococcus aureus are able to activate the hemagglutinin of influenza viruses by proteolytic cleavage in the respiratory tract and thereby to promote the development of influenza pneumonia. It would be interesting to see whether similar proteases could mediate a synergism between non-pathogenic NDV strains and a second relatively harmless and ubiquitous microorganism, and in this way could influence the manifestation of a disease.

ACKNOWLEDGMENTS

Work by the authors was supported by the Deutsche Forschungsgemeinschaft (SFB 47). The expert secretarial help of Mrs. M. Seitz is gratefully acknowledged.

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7

HETEROGENEITY WITHIN STRAINS OF NEWCASTLE DISEASE VIRUS: KEY TO SURVIVAL

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Both wild-type isolates and laboratory cultured strains of Newcastle disease virus contain several subpopulations that are often distinguishable by their plaque morphology. When cloned, these subpopulations may differ significantly from each other in their ability to infect and induce disease in several avian species. They may also differ in physical properties, in their ability to be bound by monoclonal antibody and in their oligoribonucleotide arrays. The supopulation complexes which presumably arose through mutation can be transmitted from host to host and appear to persist as complexes for many transfers both in nature and in the laboratory. The stability of the population complexes raise questions about interactions among the subpopulations and the possible role of the population complexes in the evolution and survival of the virus.

HETEROGENEITY OF WILD-TYPE NEWCASTLE DISEASE VIRUS

INTRODUCTION

Three to five subpopulations, distinguishable by means of their plaque morphologies on monolayers of chicken embryo fibroblasts, were found in over forty cultures of a group of 1971-72 isolates of Newcastle disease virus (1,2). These isolates, still in the first passage, had been obtained from chickens and exotic birds during an intensive state-federal campaign to eradicate a highly virulent form of the disease from California. Similar heterogenous populations have been found in newly isolated and in established laboratory stocks having different histories before and since that time (3,4).

Although genetic heterogenicity is regularly observed in NDV and is often observed in other viruses (5,6), there have been few attempts to census the kinds of virions found, their frequency in the population or to determine their persistance. Variant viruses that are isolated are usually described as mutants, and considered to be transient entities of very recent origin.

The assumption that the variant viruses are transient populations is not supported by examination of new isolates, or established stock cultures of Newcastle disease virus. Instead, the types of plaques obtained and their relative frequency appear to be fixed. This has been true even of some cultures that have been maintained apart for many years (7, Fig. 1).

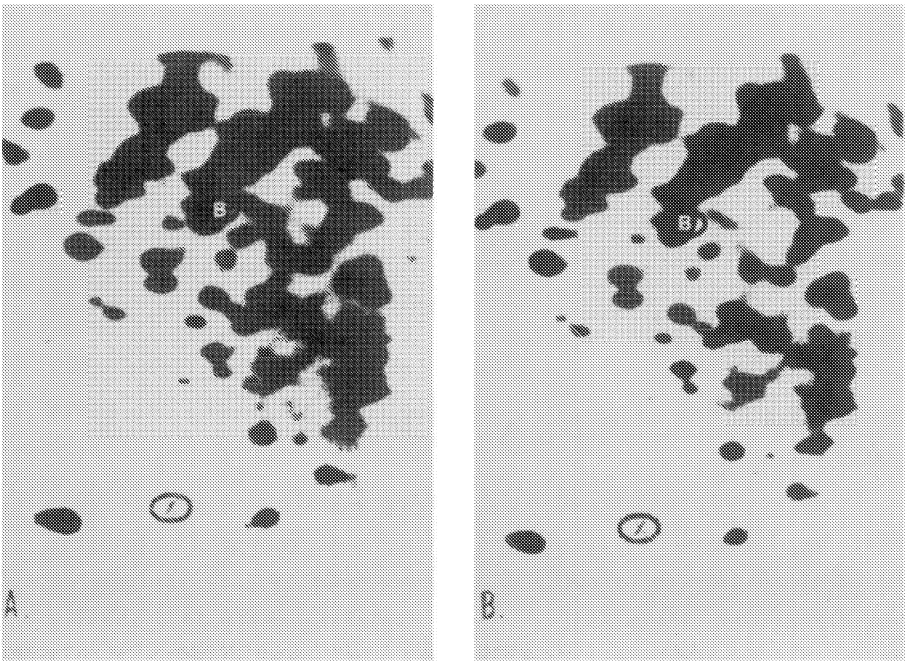

Fig. 1. Oligonucleotide fingerprints of low egg passage CG179 (A) strain (less than 5 passages) and high egg passage CG179 (B). 224th passage. Reprinted with permission from reference 7.

This paper is concerned with the origin and significance of the genetic diversity found in populations of Newcastle disease virus. Does heterogenicity play a role in survival and evolution of the virus, or is heterogenicity merely the result of a nonadaptive accumulation of mutants of a reputedly unstable virus? An attempt will be made to answer these questions.

Plaque clones

Sterner (8) recently confirmed that clones can be isolated from a virus strain that are representative of its plaque population, that each clone has a set of distinguishing characteristics (Table 1) and demonstrated that: 1) all of these clones can be maintained pure to plaque morphology for more than six passages in cell culture and 2) some of them will remain pure for six passages in chicken embryos and chickens.

Table 1. Characteristics of Plaque Clones of the Hickman Strain[1] (adapted from Sterner)

Clone	Plaque Morphology[2] type/size (mm)	Mean embryo death time (hours)	Chicken lethality dead/ exposed	Hemagglutinin		Monoclonal antibody affinity[4]	
				elution[3]	pattern	B.P.	R.A.
10	C/5	40	30/35	R		N	K
11	C/2	59	0/14	S		0	L
22	C/1	72	0/15	R		N	L
1	R/3	52	1/20	S		0	L
2	R/3	56	0/14	S		0	L
13	R/2	53	14/20	S		0	L
18	R/2	53	0/15	S		P	K

[1]Estupinan (9) previously isolated a chicken-lethal large clear plaque and a chicken apathogenic small red plaque from the Hickman strain and estimated that the two occurred at a frequency of approximately one percent in the original population and that the four other intermediate sized plaques occurred at a frequency of 10% or greater in the population. [2]Type: C = clear, R = red. [3]Elution: R = rapid, S = slow. [4]B.P. = Bratt-Peeples 17 MAb, RA = Russell-Alexander 9 MAb. Each letter signifies a shared binding pattern, however, none of the clones had identical binding patterns for the Bratt-Peeples MAb, although they did for the Russell-Alexander MAb.

Viral subpopulations are cloned by selecting and propagating distinguishable, well-isolated plaques that are representative of the subpopulations. However, unless a chelating agent (EDTA) is incorporated into the diluent and the dilution is sonicated, the frequency of spontaneous clumping makes cloning of some virus subpopulations difficult (8), and if heterozygotes are present (two genotypes combined within a single envelope 10,11), cloning will not be successful.

When ten clones are obtained from a strain by picking 10 plaques that appear to be identical in one cell system, they may not be identical in another system and they may also differ in other properties. The ability of one clone to agglutinate erythrocytes may be destroyed in 10 minutes by exposing it to a temperature of 56°C, and the hemagglutinin of another clone with a plaque that appears to be identical may resist this treatment for 120 minutes. However, plaques representative of clones that differ in pathogencity, eg. ability to kill chickens, to induce infection without death, or even to infect chickens are usually morphologically distinguishable (8,11,12,13,14,15). The multiple differences in behavior that exist among clones obtained from a viral population leads one to conjecture that the clones differ from each other by more than one mutation. This assumption is supported by a study of their oligoribonucleotide arrays (Fig. 2) (16,17).

Using an Elisa binding assay to examine his clones with 17 monoclonal antibodies (MAb) supplied by Mark Peeples and Michael Bratt (18), Frank Sterner (8) was able to clearly differentiate seven Hickman clones. Only one of the 10 MAb preparations directed to the hemagglutination-neuraminidase protein bound all of the clones and one failed to bind any of the clones. Three of the MAb preparations directed to the virus membrane protein bound all of the clones and one failed to bind any of the clones. The two MAb preparations directed to the fusion protein and the single preparations directed to the phosphoprotein and the nucleoprotein failed to bind all of the clones. Of the seven clones, clone 11 bound 15 of the 17 MAb's and clone 18 bound only 4; the other clones (clones 13 and 14) bound 10 MAb's, (clones 1 and 10) bound 12 MAb's and clone 2 bound 13 MAb's.

Seven monoclonal antibody preparations selected by Russell and Alexander (19) for use in placing over 100 Newcastle disease virus strains into nine affinity groups found the Hickman clones to be more closely alike. These preparations gave identical results for four of the clones and placed them in Alexander's group C. Two clones, 10 and 18 failed to bind two MAb's bound by the other four clones and one clone, 22, failed to grow in the assay system. Immuno-peroxidase test was used to measure binding of antibody to virus infected bovine kidney cells (19). The Peeples-Bratt MAb's appear to be directed to antigenic determinants that are subject to more variation than are the Russel-Alexander MAb's, and were therefore useful in showing antigenic differences.

NATURAL TRANSMISSION OF HETEROGENOUS POPULATIONS

Dynamics of aerosol transmission

The probability that natural infections are initiated by transfer of virions which represent all or most of the genotypes in a virus population appears reasonable if one examines natural spread of the disease. Transfer between chickens is by virus-bearing, airborne droplets that are generated in the respiratory tract of infected chickens over a period of two to four days (20). Studies with other agents have shown that the number of airborne particles released per unit of time is increased if respiration becomes labored and if paroxysms of sneezing or coughing occur (21). These variations in breathing may also change the ratio of very small droplets capable of penetrating deeply into the respiratory tract to those of larger droplets that lodge in the mucus membranes of the upper nasal passages or in the eyes (22,23). The site of initial infection, determined in part by the size of the infective droplet, has some effect on the course of the resulting infection and the eventual release of virus, (24). Virus is also released in feces which contaminates the litter, feed and water and subsequently this virus may also become airborne. However, airborne virus is continually lost from the air by gravitational forces on aggregated particles which bring them in contact with surfaces where they become trapped and the virions which remain suspended by action of

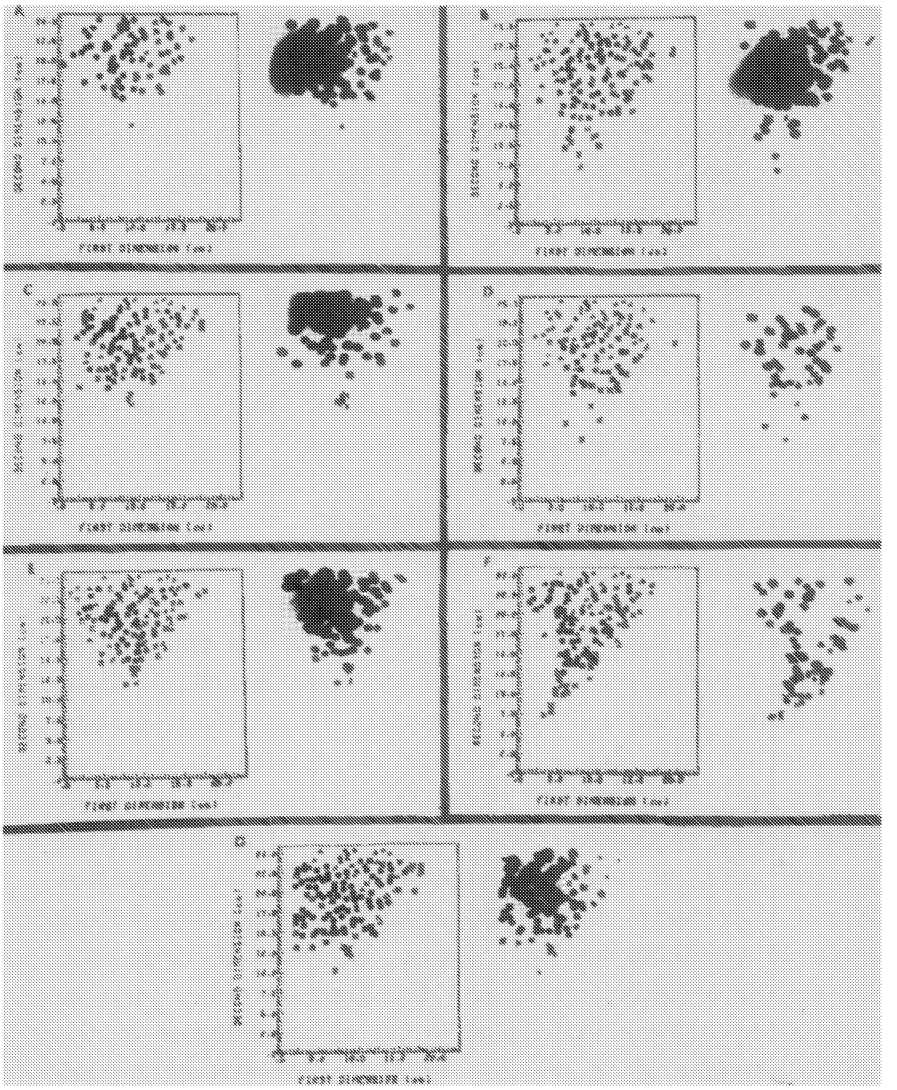

Fig. 2. On the right is the olignucleotide fingerprint of (A) the parent strain Hickman Hi-O and (B-D) the 6 clones: (B) Hi/LC, (C) Hi/MC, (D) Hi/SC), (E) Hi/LR, (F) Hi/SR, and (G) Hi/TR. On the left is the schematic (A) for Hi-O; (B) compares Hi/LC with Hi-O when they were co-electrophoresed, dots (•); represent oligonucleotides common to both, squares (□) represent oligonucleotides present only in the clone, and triangles (△) represent oligonucleotides present only in the parent Hi-O; (C) compares Hi/MC with Hi-O, (D) Hi/SC with Hi-O, (E) Hi/LR with Hi-O, (F) Hi/SR with Hi-O, and (G) Hi/TR with Hi-O. Reprinted with permission from reference 17.

turbulence (25) are subject to the denaturing effects of heat and light and other virucidal forces.

Nonetheless, within 48 hours after infection, the air surrounding the infected bird has become charged with infective virus and as the epizootic develops the infective cloud envelopes the entire flock. Similar discharges of virus into the air of structures housing animals are characteristic of Marek's disease (26) and of foot and mouth disease (27,28). If measured samples of the air are drawn into a container in which the virus can be trapped, the number of virions per liter of air can be counted (29,30). Counts have shown that air of buildings housing Newcastle disease virus-infected birds contains enough virus (18) so that each and every bird breathing the air, at a normal rate of approximately 2 1/2 liters per minute, captures several virions in its respiratory tract every minute and thousands of virions in a period of 24 hours.

Colonization of the respiratory tract

The number of virions required to initiate a respiratory infection in a chicken is dependent upon the age of the chicken and environmental factors (31). Many virions are trapped on nasal hairs and in mucus of the upper respiratory tract and removed by ciliary action before reaching a susceptible cell (32). Other virions are inactivated by natural inhibitors. Sensitivity of virus subpopulations to these inhibitors varies. For example, approximately one hundred times more virus in a stock of Newcastle disease vaccine (Table 2), was neutralized by a normal nasal inhibitor than was true of a clone obtained from the same vaccine stock (33). The result was that chickens shedding the sensitive virus failed to infect separately caged, susceptible roommates while chickens shedding the resistant clone readily spread virus to separately caged, susceptible roommates.

It is also important to know how long newly arriving virions can continue to find cells in which they can replicate and produce virus after the first arriving virion has infected a cell in that respiratory tract. If all of the arriving virus is identical, the progeny of the latter arrivals simply augment virus introduced by the early arrivals. In that situation over a period of time the host receives a large rather than a small infecting dose.

Table 2. Comparison of properties of a stock culture of the LaSota strain with a stock derived by cultivation of plaques that grew in the the presence of a nasal inhibitor from normal chickens. (Gustafson and Hanson, 1982)

Property	Original culture inhibitor sensitive	Derived culture inhibitor resistant
Percent reduction of plaque titer by nasal inhibitor	88%	0
Stability of infectivity		
at 56°C	15 minutes	8 minutes
at 4°C	200 hours	12 hours
Transmissibility:		
virus present in trachea of oronasally infected chickens	4/4	4/4
virus present in trachea of chickens exposed to air from cages of inoculates	0/4	3/4

Experimentally, a large virus dose can be delivered in seconds by placing a chicken in a chamber containing a cloud of high titered virus, but short-term exposure to either high or low titered aerosols rarely or never occurs under natural conditions. Good quantitative information on the effect of rate of delivery of infective virus on the course of infection and the length of the receptive period is lacking (34). However, it is known that the course of disease in chickens that have been in contact with severely diseased birds for a few hours can be altered if they are then inoculated with an avirulent vaccine virus. Li (35) has shown that intranasal instillation of an avirulent strain of NDV will prevent development of clinical disease in chickens that had been exposed by the same route to a lethal strain of virus 6 hours earlier, and will have some sparing effect at 12 hours and none at 24 hours. The replication of both viruses can be demonstrated if the titer of infecting viruses is low (about 100 infectious doses) but it is obscured if the titer is high (greater than a million infecting doses). A low titer inoculum most closely approximates natural conditions. The important point is that a relatively long

period of receptivity and the abundance of cellular receptors make it possible for the subpopulations of wild type strains to be transmitted to a new host and to replicate with little loss of their population diversity. Since strains and clones of NDV differ in their ability to bind to various species of mammalian erythrocytes (36) and to cells from different chicken tissues (37) and virus attachment to chicken embryo fibroblasts can be blocked by specific lectins (38), it appears that a heterogenous poulation might utilize different attachment sites in the cells of the respiratory tract or interact in different ways with the cells.

INTERRELATIONSHIPS OF SUBPOPULATIONS

Tolerance and interference

Most subpopulations in a culture exist at ratios to each other that range from 1:1 to 1:50. One or two subpopulations usually dominate, and when there are more than four subpopulations, the remaining subpopulations are so small that their detection becomes more difficult. Among the virulent velogenic strains, up to 7 distinguishable plaque types have been found (9) and among less virulent strains seldom more than two (39).

While it is more difficult to accomplish, subpopulations which are not distinguished by plaque markers can be isolated in a state of considerable purity. A thermostable subpopulation was isolated (40) (Table 3) from a thermolable strain by a stepwise reduction of the thermolable population using heat shock. Before being subjected to heat shock (56°C for 30 minutes), 99 percent of the virus was inactivated in only 30 minutes or less. When isolated, the thermostable culture remained infective after more than 150 minutes of heating at 56°C. Some investigators would explain the origin of Goldman's culture as evidence of a high rate of induced mutation. However, Piraino and Hanson (37), obtained a very similiar separation using a selection process that has no effect on the mutation rate (Table 4). A strain of Newcastle disease virus of moderate pathogenicity was subjected to several cycles of adsorption on brain cells. One line was established in chicken embryos with virus that bound to chick brain cells and one line with virus that would not bind to these cells. After three selection passages in

Table 3. Comparison of original Najarian strain (NAJ-O) with a derived heat stable strain (NAJ-H)[a] (Goldman and Hanson, 1955)

Minutes heated at 56°C	Original Strain		Derived Strain	
	Hemagglutinin % of samples positive	Infectivity % embryos killed	Hemagglutinin % of samples positive	Infectivity % embryos killed
15	82	100	-	-
45	24	100[b]	-	-
90	0	19	100	100
120	0	13[b]	100	71
180	0	0	100	66
240	0	0	100	16

[a]The heat stable strain was derived by heating the original culture to 56°C for 30 to 45 minutes, propagating the surviving virus in chicken embryos and repeating this treatment and the propagation of the treated virus three times.

[b]In 100% (6 of 6), trials the 45 minute preparation yielded virus which after propagation had hemagglutinin that was stable 120 minutes. In 40% (6 of 15) trials the 120 minute preparation yielded virus which after propagation had hemagglutinin stable for 120 minutes.

Table 4. Reduction of the neurotropism of virus strain (NDV-Iowa 125) by embryonated egg passage of virus which failed to adsorb to a 10% suspension of washed day-old chick brain cells.* (Piraino and Hanson, 1959)

Passage	Hour to death of chicken embryo	Intracerebral neurotropic index** for day old chickens of the passage inoculum	Number of 5 week old chickens showing neurotropic signs of the number innoculated
original Iowa 125	46	1.8	28/28
1	-	1.6	-
2	-	1.2	-
3	51	1.1	-
4	81	0.4	-
5	78	0.3	0/28

*Approximately 50% of the hemagglutinin present in the allantoic fluid of each passage was removed by brain cell adsorption.

**Based on the rate of development of signs and the occurrence of death in 10 chickens a day of age inoculated intracerebrally (range 0-no neurotropism to 2-high neurotropism).

which the cell-binding line retained and apparently increased its pathogenicity the nonbinding line became apathogenic for chickens.

Tolerance or the ability of each subpopulation to resist restraints on its rate of viral replication, release from cells, or ability to enter susceptible cells in the presence of other subpopulations appears to be essential for the persistence of the subpopulations. Limited attempts in our laboratory to assemble mixed populations using clones obtained from different strains have failed within a few passages. Lack of tolerance among many NDV strains is directly demonstrated by the exposure of a monolayer of chicken embryo fibroblasts first to an avirulent non plaquing strain (41) followed by a virulent plaquing strain (42) (Table 5). The

Table 5. Reduction in plaque titer (Log_{10}) of plaquing virus in monolayers infected one hour earlier with a non-plaquing virus (from Jones and Hanson, 1976)

Challenge Strain	Incubation temperature	Titer of challenge strain pfu/0.1 ml	Percent reduction in plaque titer of challenge strain in monolayers previously infected with nonplaquing virus				
			LaSota	V4	Eng F	Bl	Ulster
70711	37°C	7.3	100	98	87	75	100
	42°C	7.7	0	88	62	88	0
70181	37°C	7.5	100	100	94	97	100
	42°C	7.5	98	99	97	99	34
1083	37°C	7.7	100	100	83	99	89
	42°C	8.3	96	94	92	98	99

interval of time between the initial and second exposure and the temperature at which the cells were incubated had measurable effects on the result. However, particular avirulent strains induced a striking reduction in the plaque titer of particular virulent strains and some avirulent strains completely suppressed the development of plaques by certain virulent strains. Evidence of interference by Newcastle disease virus has been widely observed by investigators using differing criteria (43,44,45,46,47).

Interdependence

The relative ratio of one clone to another in a cultured population is determined in part by the multiplicity of infection, with the ratio usually remaining unchanged on serial passage at low multiplicity and often changing at high multiplicity, presumably because of differing degrees of tolerance for defective virus (48). Some changes in virulence of virus stocks on serial passage may be explained by the multiplicity of the virus in the inoculating dose selected (4). This may be analogous to experience with vesicular stomatitis virus (49). Alice Huang (50) has suggested that the cyclic changes observed in the titer of defective and interfering vesicular stomatitis virus in cell cultures may also occur in the field disease and explain changes in the epizootiological behaviour of vesicular stomatitis (51). The relative ratio of substrains may also be determined in part by the availability of cells with appropriate sites for binding groups and initiation of infection. The availability of many niches in hosts that can be differentially exploited by viral subpopulations has been suggested by differential isolation of subpopulations from body sites, such as a large clear plaque of the Hickman strain from the brain (9) and a slow eluting subpopulation of the Ulster strain from the cloaca (39). However, it is unlikely that in each new host each subpopulation replicates to a titer determined only by its cellular niche, free from significant conflict with other subpopulations.

Interdependence may exist between some clones (8). These are clones representative of subpopulations isolated from diseased chickens which can be readily passaged in cell culture and chicken embryos but which when introduced alone are unable to initiate a productive infection in chickens (Table 6). Clone 22, a small plaque clone, and clone 10, a large plaque clone of the Hickman strain, are examples of clones that replicate in chickens but appear not to be shed from chickens. However, when inoculated together into chickens, the two clones induced a productive infection that was communicable to contact chickens. Whether such dependent clones exchange genetic information as has been observed for La Crosse encephalitis virus (52) or utilize a product produced by the sister clone is unknown. The latter is suggested by the observation that

Table 6. Comparison* in adult chickens of the properties of the parent, (Hickman strain) two of its clones given alone and in combination (Sterner, 1986)

	Lethality oronasal exposure	Lethality induced in room contacts	Immunity induced in room contacts
Clone 10	30/35	0/20	0/20
Clone 22	0/15	0/10	0/10
Clone 10 and 22	2/15	0/10	10/10
Parent	15/15	10/10	-

*Reactors/exposed

certain small plaquing Newcastle disease virus populations can be stimulated to produce large plaques if bacterial neuraminidase is added to the culture medium (personal communication, McMillan, 1985). The failure of mutants that arise in chicken embryos to appear when the virus is cultured in chickens is consistent with the failure of certain cell cultured clones to survive in chickens. The complex nonspecific defense system of a mature chicken may not be readily evaded by some spontaneous mutants that arise during replication of the virus even though some of these mutants are able to replicate in the more permissive system of an immature host.

In following these events in the mature host, it is important to separate virus communicability from pathogenicity. Communicability includes the ability to escape from the infected host, to utilize some means of transfer between hosts, to evade the nonspecific defenses of the new host, and to initiate infection. Pathogencity consists of events involved in viral replication, avoidance of induced defenses, and spread within the host so as to eventually escape. A mutant such as clone 10 can be pathogenic and not communicable. Whether the host is seriously impaired and its life endangered by the infection or not appears to be relevant to the parasite only when the severity of the induced disease limits the escape and perpetuation of the virus.

SIGNIFICANCE OF HETEROGENOUS POPULATIONS

Survival of the virus

During an epizootic, heterogenous viral populations are transferred from infected to susceptible chickens, and this transfer to each host of many genotypes rather than a single one increases the chance that a genotype with a minor antigenic difference (Table 7)

Table 7. Antigenic differences among clones of the Hickman strain as measured by their neutralization constant (NK) (Estupinan and Hanson, 1971) (Also see Table 1)

Viral Clone	Antisera to the Clone		
	LC	SC	SR
LC	100	76	43
SC	60	100	95
SR	30	33	100

will be present and can exploit a partially immune host (1,2). If a new host species is encountered, the heterogenous population may provide a genotype capable of initiating a productive infection in that host. Pathogenic organisms that are able to transfer to susceptible hosts potentially successful mutants along with the more abundant parental genotype could have a greater chance of surviving rapid changes in the epidemic climate. Both the genotype of the chicken (increased rate of growth and narrowed genetic base) (53), and its environment (tailored diet and high density confinement) (54), changed rapidly between the nineteen twenties and the nineteen fifties. Some of the changes, particularily the increased flock size and confinement at high density, modified the epidemic climate for respiratory infections and greatly facilitated their spread. The concept of parasitism as an evolutionary struggle between populations of hosts and parasites first conceived by Theobald Smith (55) appears to be true of this paramyxovirus and its primary hosts.

The initiation of viral infection by the transfer of virions representing several subpopulations rather than by the introduction of virions representation of one genotype and the infection of many cells over a large surface or of cells at several anatomical sites rather than the infection of one cell at a single site has many

implications. A virus population containing several genotypes may more easily evade specialized defenses and find a larger number of binding sites than can a population consisting of one genotype. The heterogenous population has a greater chance of replicating and producing large number of progeny that will be shed into the environment. It follows that the nature of the virus population recovered at different sites and stages of the developing infection may be different and success in recovering all components of this population depends in part upon the laboratory host system used. Recovery of a representative wild-type population also depends upon conditions of storage of the culture during the isolation procedure. A subpopulation in an avirulent NDV strain that was resistant to an inhibitor in normal mucus was destroyed by overnight storage of the culture at 8°C (33). The inhibitor resistant subpopulation was more transmissible so its epidemological behaviour was unlike that of the original virus.

Control of disease

The diversity of the invading subpopulations, as well as the increased genetic base for mutation this diversity brings, increases the possibility that the infected host will receive a broader antigenic stimulus than if it had been infected by a virus clone or inoculated with a synthetic antigen (56). A heterogenous virus population in which new mutants arise and compete for an opportunity to place their progeny into the population that is shed, could be expected to undergo changes in virulence and shifts in antigenic properties. These changes have been observed during natural epizootics of NDV (1) and other viruses. Vaccination of host populations with a vaccine that contains only part of the antigens expressed by the virus (57), even though that subset of antigens or antigenic segments is capable of inducing a good immunity to representative isolates of wild-type virus, provides a situation in which antigenically different mutants within a heterogenous population can rapidly rise to a position of dominance in the wild-type populations. The shift in subpopulations would create a wild-type virus which would be increasingly resistant to the vaccine. There is a significant possibility of failure if measures for control of virus diseases do not take into account the shifting

polygenic nature of wild virus populations.

Unanswered questions

I have presented evidence that isolates of NDV consist of several related subpopulations that exist in some sort of an association and that this relationship has implications for disease control. The demonstration of mutualistic behaviour in an RNA virus raises questions that have not been discussed. One concerns the function of dependent subpopulations and the other, the nature of processes involved in mutualistic behaviour.

One can conceive of conditions, and probably create them experimentally, in which only one of the invading subpopulations can become established and subsequently be shed to continue the chain of infection. The usefulness of that subpopulation is justified by its survival, but before this event the allocation of resources to it or to any of the other subpopulations of differing functionality is not evident. Gould (58) has asked this question in respect to the selection process involved in the development of the avian wing, a structure which must have been aerodynamically useless during most of its evolution. The shift from one function to another which Gould calls "exaptation" can not be studied in higher forms of life but the existing and potential functions of virus subpopulations can and should be studied.

In higher forms the diversion of resources to ensure the survival of the genes of siblings or other kin at the expense of ones own genes is called altruism. At the molecular level F.N.C. Crick (59) and others (60) have suggested quite the opposite type of behaviour, that DNA is selfish, ready to abandon its own created structures and independent existence for a free ride through the insertation of its nucleotides into another working strand of nucleic acid. Within a NDV population, clones that appear to be incapable of independent existence are provided with resources that permit them to move from host to host with the independent clones. This has the appearance of altruism on the part of the independent clones or conversely of selfishness on the part of the dependent clones. Whether it is either, it should be possible to study the mutualistic mechanisms that are involved in perpetuation of the virus of Newcastle disease.

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8

MONOCLONAL ANTIBODIES IN RESEARCH, DIAGNOSIS AND EPIZOOTIOLOGY OF NEWCASTLE DISEASE

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INTRODUCTION

Monoclonal antibodies (Mabs) can be used to show that variable and conserved antigens exist on all Newcastle disease virus (NDV) proteins (Table 1) and unequivocally separate NDV from other avian paramyxovirus serotypes.

The association between epitopes and function e.g. virus neutralisation (VN) and virulence has also been investigated. Most of the exposed epitopes on HN and F are associated with VN.

This review of Mab will use these subheadings: Conserved and variable epitopes; Virus neutralization *in vitro* and *in vivo*; Diagnosis and epizootiology.

CONSERVED AND VARIABLE EPITOPES ON NDV

By 1980 several laboratories were starting to make Mabs to NDV in order to look for antigenic variation (successfully) and virulence markers (largely unsuccessfully). They used the techniques particularly applied to study antigenic drift of the H3 subtype of influenza virus haemagglutinin (HA). Laboratories working on NDV produced 4-17 Mabs to the NDV spike proteins HN or F and defined regions by competitive blocking, their results are summarised in Table 1 (refs 1 - 8). A region was divided into epitopes if Mab(s) to one epitope still neutralized mutants immunoselected by Mab(s) to a second epitope in the same region. Alternatively, and more simply, a region was divided into epitopes when the Mabs to that region bound to different panels of field isolates. Thus Iorio and Bratt's 17 Mabs to the HN of NDV Australia-Victoria (A-V) defined four regions by competition blocking

Table 1. Regions and epitopes on NDV.

Virus isolate	Protein	No.of Mabs made	Methods used	No.of regions	No.of epitopes	No.of conserved epitopes	Key Ref. (includes earlier refs.)
Australia-Victoria	HN	17	a,b, c,e	4	9/10	6	(1)
JapDuck 26 (D26)	HN	11	b,c, d,e	4	4	1	(2)
Sato	HN	11	a,b, d,f	2	6	2	(3)
La Sota	HN	18	c,d	?	4	1	(4)
Miyadera	HN	3)	c,e	?	3	1	(5)
Taka	HN	1)		?	1	0	
Ulster	HN	4	a,b,c d,e	2	2	0	(6)
Ulster	F	1		1	1	0	(6)
La Sota	F	1)	a,c	?	5?	2	(7)
Italien	F	4)		?			
Sato	F	12	a,b, c,f	4	6	4	(7)
JapD26	M	8	b,c	2	6	4	(8)
JapD26	P	5	b,c	4	4	1	(8)
JapD26	NP	3	b,c	2	2	2	(8)
Ulster	NP	2	b,c	1	2	1	(6)

Methods used to enumerate regions and epitopes. a = Binding of Mabs to their mutants; b = Cross-blocking; c = Binding to different field isolates; d = Stability and glycosylation of the antigen; e,f = Functional properties, haemagglutination inhibition, haemolysis inhibition, VN, neuraminidase inhibition with neuraminlactose and fetuin as substrates (e) or fetuin only (f) Proteins

with evidence that a zone on region 1 overlapped region 4 (1). Within region 3 there were three variable epitopes because the three Mabs to this region bound to different field isolates. Regions 1, 2 and 4 were all conserved but could be subdivided into a total of six epitopes by mutant selection data and non-reciprocal blocking data (1).

Whether Table 1 lists all the epitopes in a particular NDV protein or not is unknown but unlikely because the number of epitopes increases with the number of Mabs and techniques employed. How the epitopes and regions described by one laboratory correspond to those described by a second laboratory is also not known because there has been little exchange of Mabs and viruses between laboratories. The HN and F of NDV, like the HA of influenza, seem to comprise four main exposed antigenic regions. Confirmation and siting of these NDV regions will have to await three-dimensional maps and knowledge of where amino-acid substitutions immunoselected by Mabs occur.

All laboratories have reported conserved epitopes of HN and F. Whether the conserved epitopes reported by one laboratory are duplicated by another is unknown. With this caveat the F protein appears to have a higher ratio of conserved epitopes, 6/11, as compared to 11/29 on HN (see Table 1).

The definition of a conserved epitope is dependent on the choice of isolates tested. Russell and Alexander (6) considered the HN-1 epitope of Ulster to be largely conserved because it occurred on 36/40 isolates which they had examined in 1982, and the other four which lacked HN-1 were all lentogenic viruses isolated from feral ducks as exemplified by isolate MC110. Within a year of their publication 23 outbreaks of NDV in poultry had occurred in Britain and 20 of the 21 viruses isolated lacked this HN-1 epitope in common with feral and racing pigeon isolates (9). The conserved epitopes on the HN and F recognised respectively by Mabs 8C11 and 1C3 from Meuleman's group were more widespread and did occur on pigeon and MC110-like viruses (4). Such conservation of epitopes on NDV explains both the monotype nature of NDV by conventional serology and vaccinal cross protection between isolates.

Variable epitopes have been discovered on the internal viral proteins, matrix, polymerase and nucleoproteins, as well as the external HN and F proteins (Table 1) of both virulent and avirulent strains. Nishikawa et al., (8) give this as evidence for such variation occurring spontaneously rather than because of immunological selection. When yolk sac immunity is present it is almost entirely IgG (10) and unable to prevent aerosol infection of day-old chicks by La Sota (11). The survival of NDV, with a large number of conserved epitopes, may therefore be because there is a continual supply of susceptible hosts e.g. chickens and wild birds.

Some epitopes of NDV, particularly the more conserved VN epitopes on HN, resist denaturation by sodium dodecyl sulphate (SDS), reduction of disulphide bonding by 2-mercaptoethanol and are independent of glycosylation (8, 12, 13) as if they are linear antigens. Mabs to the above epitopes are the most frequent to be made by mice and represented 8/18 (12), 3/4 (13) or 6/9 (14) of anti-HN clones as if they are the immunodominant HN regions of NDV for mice. Linear conserved, immunodominant epitopes make good candidate subunit vaccines. Nucleotide sequences which code for such epitopes can now be localised because HN and F genes of NDV have been sequenced (see Chapter by P.T. Emmerson) and mutations affecting such epitopes could be mapped by sequencing wild type virus and its Mab-immunoselected mutants. Mabs could also be used to detect whether an epitope is eventually reproduced as a synthetic peptide or expressed gene product.

VIRUS NEUTRALIZATION IN VITRO AND IN VIVO

The ability of a Mab to neutralize has been determined by assessment of neutralization titre (NT) or neutralization index (NI). The last dilution of Mab to neutralize a known amount of virus e.g. 50% or 90% plaque-forming units (pfu) is the NT. The maximum drop of viral infectivity after reaction with the Mab is the NI.

The method of neutralization used affects the results and this is most apparent for Mabs to HN. Nishikawa et al., first considered their Mab to the site 3 region of NDV Jap D-26 was unable to completely neutralize 100 pfu in a microwell VN test (14) but later found its titre to be 1/25,000 when assessed by a 50% drop in fluorescent focus staining at 8 hr post infection before virus invaded all the microwell (2).

Mabs to F neutralized better than Mabs to HN in vitro and in vivo (see later). Although the different Mabs to HN and F had equivalent NT they varied greatly with respect to NI. Thus the NT exceeded 10^3 for 14/17 Mabs to F (7, 15, 16) and for 15/18 Mabs to HN (2, 3, 13, 15). The NI, by contrast, exceeded 10^3 for 4/6 Mabs to F and were less than 10^3 for all 20 Mabs to HN (Table 2).

Table 2. Comparative neutralization by Mabs to HN or F

Neutralization index (NI)+ (max. drop in infectivity)	Percentage of Mabs in this NI range	
	Mabs to HN	Mabs to F
$10^5 - 10^6$		17%
$10^4 - 10^5$		17%
$10^3 - 10^4$		32%
$10^2 - 10^3$	20%	17%
$10^1 - 10^2$	35%	17%
$10^{\cdot 3} - 10^1$	40%	
0	5%	
	100%	100%

+Neutralization indices obtained for 20 Mabs to HN and 6 Mabs to F from references 1, 2, 15 and 16.

Neutralization via HN could be improved by several methods so as to allow the selection of mutants. These included leaving Mab in the growth medium i.e. the plaquing overlay (16) or allantoic fluid (3) and adding rabbit anti-mouse IgG (17) or guinea-pig complement (Russell, unpublished data) to the reaction medium.

This weaker VN with Mabs to HN compared to those against F was not due to certain particles resisting neutralization via HN because pairs of Mabs to HN neutralized additively (18) or even synergistically (15, 16). Mabs to HN could, however, bind to different NDV strains identically, but vary with respect to how

well they neutralized, e.g. Mabs to the HN-3 region of NDV (A-V) reduced homologous pfu by 80-90% but caused little or no reduction of several virulent or avirulent NDVs (1). Similarly some immuno-selected mutants of NDV are still bound but no longer neutralized by the selecting Mab (14, 17).

The stronger neutralization via F was suggested to be a direct block of viral penetration (which occurs within 90 seconds at 37^0C using Ulster and MDBK or MDCK cells) whereas the more variable neutralization via HN could represent an indirect block of penetration (16, 19). Mabs to HN with haemagglutination inhibition (HI) but no haemolysis inhibition (HLI) activity and which prevent the uptake of fluorescein-labelled virus into BHK-21 cells (2) obviously block adsorption. Mabs to HN with HLI but no HI activity have been considered either to sterically hinder fusion protein (2, 3, 20) or to block secondary HN attachments (21). Russell considered a block of secondary attachment likely because neutralization by both types of Mab to HN was enhanced pre or post adsorption when the number of sialic acid receptors for HN were reduced by pre-treatment of the cell cultures with bacterial neuraminidase (21). Influenza virus gathers together sialic acid receptors before endocytosis (22) and NDV may require a similar step between adsorption and fusion.

The protection of six-week old chickens by neutralizing Mabs to Italien has been reported by Meulemans *et al.*, (15) and related to *in vitro* VN results. Protection via F was regular (5/5 Mabs) and protection via HN was less certain, (1/3 Mabs), when Mabs were given one day after 100 LD_{50} of virus. Protection was not absolute because although the above Mabs prevented birds from dying by six days post infection, only one of five Mabs, (No. 12C4 to F), significantly reduced deaths by 15 days post infection. By 15 days virus replication had presumably re-commenced because the mouse Mabs had become denatured rather than mutant virus breaking through the Mabs. On the basis of the above results Meulemans suggests that the F protein would be a more suitable candidate than HN for genetically engineered vaccines (15). Russell earlier commented that because Mab-VN via HN was synergistic, HN vaccines might do

better if they included more than one HN epitope (21).

The best of the above anti-F Mabs (12C4) protected better than did chicken antiserum to whole virus (15). In separate work, passive protection against the Italien strain of NDV using conventional antisera protection required both anti-HN and anti-F antibodies because chicken antisera to either protein gave partial protection (23). Protection became almost complete and comparable to that of anti-whole NDV serum when the two antisera were combined. The chicken antiserum to F, unlike the mouse Mabs, failed to neutralize or even retard plaque growth and was presumed to to protect in a complement-dependent manner. Whether mice and chickens differ in the immunodominant epitopes which they recognise on F, or their antibodies to F differ with respect to affinity is unknown. This, however, becomes important when selecting 'good' vaccine epitopes on the basis of functional tests with mouse Mabs.

Some Mabs to HN inhibit neuraminidase only when a large molecular weight substrate (i.e. fetuin) is used. Others inhibit with both small (i.e. neuraminlactose), and large, substrates. This has enabled Iorio and Bratt to make a functional map of NDV epitopes relative to the haemagglutination and neuraminidase sites of the HN molecule (20). However Colman et al (24) recently found that whilst a complete Mab molecule inhibited the neuraminidase of influenza N9 on both substrates, its Fab fragment only inhibited activity on the larger substrate as if substrate specificity is not dependent on epitope but rather on binding characteristics e.g. perturbation of the enzyme.

DIAGNOSIS AND EPIZOOTIOLOGY

Traditionally, confirmatory NDV diagnosis is done by HI testing of putative haemagglutinating agents isolated from infected birds or of chicken sera from recovered birds. In both cases NDV isolates have been considered as monotypic. Use of Mabs have now demonstrated that NDV does carry conserved antigens on the HN (11/30 on Table 1). A combination of two such Mabs (8C11 and 4D6) from Meulemans' laboratory (4) have been found to be suitable for the identification of any known NDV isolates by HI testing. Whether

these Mabs would be suitable for detecting NDV antibodies in sera from recovered birds by competition assays was not reported. Russell and Alexander (6) earlier considered that Mabs to variable NDV epitopes were competed for by chicken antibodies to conserved epitopes of NDV in indirect immunoperoxidase (IIP) tests and so it seems likely that Mabs to conserved NDV regions could be specifically blocked by chicken sera.

Conventional HI testing has presented evidence for antibodies that bind to antigens which are shared either between avian paramyxoviruses, serotypes 1 and 3 (25) or between serotypes 1, 3, 4, 7 and 9 (26). Mabs to antigens shared between PMV serotypes, have been demonstrated for both HN and F. Thus Mab 161/617 which is specific to pigeon NDV (see later) also has HI activity to psittacine but not turkey PMV-3 isolates (Collins and Alexander, in preparation) and Mab 244/1 to a conserved epitope on site IV of NDV (Sato) binds prototype viruses of PMV-2, 3 and 4 but not 6 or 7 (7). Three non-specific Mab to the HN of NDV have been produced (3) and bind not only to avian paramyxoviruses but also to influenza. This emphasises how shared and host-specific antigens existing on NDV may cause problems of non-serotypic reactivity in certain batches of conventional antisera. Mabs to conserved epitopes on NDV, in contrast, usually do not bind to other avian PMVs and can be produced indefinitely from hybridomas in culture.

The majority of Mabs to NDV fail to bind to some field isolates and therefore recognise variable antigens of NDV (Table 1). The ability of a panel of Mabs to bind to field isolates can be used to identify similar viruses and to monitor the spread of a distinctive epizootic virus. This antigenic fingerprinting approach for NDV was first reported by Russell and Alexander in 1983 using nine Mabs (6). The continuing use of this approach was updated during a large survey of NDVs received by the International Reference Laboratory for NDV, Central Veterinary Laboratory, England (27). Russell and Alexander infected Madin-Darby bovine kidney (MDBK) cells with 10^{-3} or 10^{-4} allantoic fluid overnight and were then able to distinguish infected cells which bound Mabs by their brown colouration by IIP staining (6). Binding by their three Mabs

to the HN-1 epitope was largely corroborated by HI testing which could be directly performed on allantoic virus. The groupings of NDV identified in these studies by IIP using ten Mabs are listed in Table 3.

Russell and Alexander first placed viruses into eight groups, A-H, based on their Mab binding profiles using anti-Ulster Mabs to HN-1, HN-2, F (showing cell membrane staining), POL, NP (inclusion body staining), and 424, an unidentified antigen located in the cytoplasm of infected cells. It was found that isolates and strains grouped on this basis shared biological and epizootiological properties. Groups A and B encompassed 18 viscerotropic virulent isolates from the 1970-1974 panzootic (group A) or other outbreaks (group B). Group C was a mixture of lento-and velogenic viruses, which included a 1968 Kuwait isolate from poultry. Group D consisted of four neurotropic velogenic isolates typical of early "pneumoencephalitis" viruses seen in the USA. The remaining groups contained lentogenic isolates from a wide range of hosts, notably La Sota and B1 were in group E which lacked the HN-2 and F epitopes of Ulster 2C in group G. Queensland V4 isolates were in group G with Ulster 2C. Group H, which comprised four European isolates from feral ducks was the only group not to react with the three Mabs to the HN-1 epitope and this was also a property of subsequent pigeon NDVs which became group P. Group L included three USA isolates from aquatic birds: loon/83, duck/unspec/Calif/1972, coot/Virginia/1977, (Alexander, unpublished results) (Table 3).

The use of additional Mabs has indicated possible subdivisions of the above groupings. For instance one Mab of Meulemans (Mab 12C4 to F) separates Eastwood 70 and Essex 70 within group A because it binds to Eastwood extremely poorly by IIP as compared to any other virus tested (10^{-3} compared to 10^{-5} or 10^{-6}) (Russell and Meulemans, unpublished results). Conversely a new Mab to the HN of Ulster 2C (CVL,11) binds to Eastwood 70 but not Essex 70 (Alexander, Collins and Russell, unpublished results).

Table 3. Summary of the Newcastle disease virus groupings based on binding of ten monoclonal antibodies to infected Madin-Darby bovine kidney cells.

Group	Current features of group	Example	Mabs bound	Mabs not bound
A	Viscerotropic, velogenic 1970-74 panzootic	Essex '70	14,32,86 38,479	445,481 688,424, 161
B	Other viscerotropic, velogenic before 1970, after 1972	Herts '33	14,32,86, 481,38,479	445,688 424,161
C1	Velogenic psittacine & Middle East isolates	Kuwait 256	14,32,86,481 38,424,479	445,688 161
C2	Lentogenic, duck isolates	1092/81	14,32,86,481 38,424,479	445,688 161
D	Neurotropic velogenic USA isolates	GB Texas	14,32,86,445 38,688,424, 479	481,161
E	Lentogenic, vaccinal	Hitchner B1	14,32,86,38 688,424,479	445,481 161
F	Lentogenic, miscellaneous	F strain	14,32,86,38 424,479	445,481, 688,161
G	Lentogenic, aquatic birds, passerines, fowl - worldwide	Ulster 2C	14,32,86,445 481,38,688 424,479	161
H	Lentogenic, aquatic birds - worldwide	MC110	481,38,424 479	14,32,86 445,688, 161
L	Lentogenic, aquatic birds - USA	Loon/83	14,32,86, 481,38,688 424,479	445,161
P	"Mesogenic", pigeon PMV-1 chickens, pigeon & other species worldwide	561/83	481,38,479 161	14,32,86 445,688 424,161

From references 6 and 27; Alexander, Collins and Russell (unpublished data).

Mabs were particularly useful in determining the epizootiology of the pigeon virus. Isolates of this virus were rapidly separable from other NDVs of poultry because their haemagglutination was not inhibited by Mabs to the HN-1 epitope of Ulster. They were positively identifiable by IIP of infected MDBK cells when they bound only three of nine anti-Ulster Mabs (481 to F, 38 and 479 to NP) which defined group P (28). A more recent Mab from mice which had been immunised with a Central Veterinary Laboratory isolate of the pigeon virus, Mab 161/617, binds to and inhibits haemagglutination of all viruses from group P but none from other groups (27). The haemagglutination of group P viruses is also inhibited via the conserved group 1 HN epitope recognised by Meulemans' Mab 8C11 and they are bound/neutralized by the Meulemans'six Mab to fusion protein (29; Meulemans and Russell, unpublished data). The presence of their conserved epitopes, particularly on F, vindicates the usage of conventional NDV vaccines against pigeon virus (30, 31).

All the viruses recovered from racing and feral pigeons during 1981-1985 from Europe, N. America, Japan and Israel were group P (27, 32). The 1982 isolate from a Sudanese food pigeon was, however, noticed to be different because whereas Mabs to HN-1 failed to inhibit its haemagglutination, they did bind by IIP (32). The Sudan isolate has recently been confirmed to be distinguishable from other group P isolates because it does not bind the group P specific Mab 161/617 (Alexander and Collins, unpublished data).

By way of digression, the binding of Mab to HN-1 without HI was reported later in group G for some, but not all, laboratory stocks of Queensland V4, for all five isolates from feral Australian birds (32) and also for the Loon virus of group L (27). It is therefore more straightforward to use the IIP test for antigen detection if possible. This reveals epitopes whose tertiary conformation, or perhaps receptor binding profile, may render them inaccessible to HI using certain isolates. Interestingly enough, the converse was reported for NDV Beaudette-C which was the only virus to be active in HI tests using Mab 445 to Ulster's HN-2

epitope (6). Beaudette-C was purposely selected as a heat stable variant of Beaudette (B) by Granoff in 1959 (34) and this presumably altered the conformation of its HN molecule.

The effectiveness of the Mab approach to strain identification was strikingly demonstrated by Kaleta et al (35) when they showed the Iraqi 1978 agent of contagious pigeon paralysis may have been the earliest known isolate of group P pigeon NDV. Pigeon paralysis was widespread in this Mesopotamian region and the infection spread westwards into Italy by 1982 which coincided with its introduction into racing pigeons and feral pigeons. In Great Britain unvaccinated poultry were fed untreated food which had been infested by feral pigeons at Merseyside docks. Group P NDV was isolated from 20 of the 21 viruses isolated from the 23 outbreaks during 1984 (9). Group P viruses were later isolated from a captive kestrel (Falco tinnunculus) and feral sparrow (Passerine sp.) (27) demonstrating the potential for other feral birds to introduce the virus into chicken feed or accommodation.

The one virus of the 23 outbreaks in Great Britain which was not of group P came from outbreak 2 which had not known connection with feral pigeons (9). This virus had the binding properties of a group E virus with an intracerebral pathogenicity index (ICPI) of 0.7 indicating it was similar to the vaccine strain LaSota (27). In the large survey of international NDVs received by the International Reference Laboratory for NDV at the Central Veterinary Laboratory similar group E viruses with ICPI of 0.2-0.8 were isolated from a total of 14 poultry flocks showing clinical signs resembling ND (27). This raised three possibilities: (i) vaccinal virus may be isolated independently of the pathogenic agent (possibly due to administration once the birds started to show clinical disease), (ii) vaccinal virus was responsible for clinical disease either as reported in day-old chicks using certain vaccinal strains of La Sota (11) or in combination with secondary infections, e.g. mycoplasma or bacteria, (iii) virulent NDV was present but was not isolated because it was overgrown by the vaccinal strain. This is unlikely because virulent strains replicate in a wider range of organs than lentogenic strains (36). However, with regard to the

third point three Israeli 1985 isolates of turkeys and one from pigeons which all had high ICPI could not be adequately grouped. They were suspected as being a mixture of viruses because whereas the majority of the cells stained as if they contained a group P or H virus, a minority of the cells were bound by additional Mabs suggesting a minor virus population was also present (27).

The reported Mab groupings of isolates received by the Central Veterinary Laboratory during 1982-1986 were useful in three other main respects: (i) confirming the identification of vaccinal/lentogenic strains; (ii) discovering the re-emergence of a group C Middle East strain of NDV including spread to Europe; (iii) distinguishing two types of NDV in Australia (33).

(i) In the above survey Mab grouping was done in parallel with IVPI and ICPI tests. The virulence characteristics and also the serological identity of vaccinal strains of La Sota, Roakin or Mukteswar from Spain or Pakistan were thus confirmed. No Mab exists which unequivocally separates lentogenic NDVs from other types of the virus and this may be because the virulence of NDV is primarily, but not entirely, associated with the cleavability of its F protein in different cell types (37). However, there is no reason why additional antigenic markers for virulence should not be present even if they have no known function, as yet. Meulemans reported one Mab, 5AI, in his HN group III which bound to Italien but did not inhibit haemagglutination by Italien or any other virulent viruses (13 tested) (4). This Mab did cause HI of 36 of 38 lentogenic isolates with Ulster (group G) and MC110 (group H) being the exceptions. This emphasises how the tertiary structure of the HN as defined by its haemagglutination properties may be associated with avirulence. Meulemans also described a Mab, 7D4, which bound to La Sota but not Hitchner B1 or Ulster by HI tests (4). He could therefore separate La Sota which bound 5A1 and 7D4, Hitchner B1 which bound 5AI and Ulster which bound neither by HI testing (4). Srinivasappa et al (38) selected one Mab (AVS-1) which bound to the four commonly used lentogenic vaccinal strains (Ulster, Hitchner B1, La Sota and Queensland V4) by HI and ELISA as compared to ten velogenic or mesogenic viruses. The Mab did not define

avirulence however, because it failed to bind two other lentogenic chicken isolates, the international reference F strain and a field isolate (NEB GOL), as well as four pigeon viruses which were classed as lentogenic (38).

(ii) The Central Veterinary Laboratory survey showed how five recent European poultry isolates (three Italian and two Austrian) in group C, were indistinguishable from recent isolates from the Mauritius and Middle East, e.g. Saudi Arabia, Kuwait. Velogenic group C isolates were also isolated from exotic and quarantined birds but not from migratory birds. Thus two velogenic NDV were present in Europe at the same time; group P viruses in poultry in Great Britain and worldwide in pigeons and the group C Middle East virus in quarantined psittacines and poultry from some European countries. The group C virus was also present in Hong Kong food pigeons but has not been reported in feral or racing pigeons (27).

(iii) Two NDVs in Australia. The Australian prototype of NDV is Queensland V4 which was placed in group G with Ulster (6). All the viruses isolated by cloacal swabs in 1980 from one location, Kununurra, were also in this group G. The 1979 isolates from wading birds in a variety of locations in Western Australia were all of group H, which previously had only been reported in European feral ducks and was not known to be present in Australia (33). Group G and H viruses have also been isolated from feral waterfowl and wading birds in North America (Alexander, unpublished results).

This re-emphasises how lentogenic NDVs (e.g. of groups G, H and L) appear to circulate amongst feral aquatic birds. Velogenic viruses have not been isolated from this source, although these birds can carry velogenic NDV in domesticity, e.g. Swiss Duck isolate 2V164 of group P (27) and Hong Kong duck or goose isolates (39). Until now it has been trade in captive birds, e.g. psittacines and, more recently, the mixing and racing of pigeons that have allowed the spread of virulent isolates from endemic areas. When velogenic viruses do shift host or country then antigenic fingerprinting by Mab is an extremely robust method for helping to detect the origin and spread of the virus.

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9

NEWCASTLE DISEASE DIAGNOSIS

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INTRODUCTION

The diagnosis of Newcastle disease (ND) has never been straightforward. In the the first descriptions of ND, differentiation was made from a number of other diseases that produced basically similar signs. It is surprising therefore that, with what we would regard today as relatively unsophisticated tools, researchers and diagnosticians such as Doyle (1) were convinced from the outset that they were recording a disease that had been unreported prior to their observations.

Greater understanding of viruses in general and the development of modern virus culture and immunological techniques has not necessarily helped simplify the diagnosis of ND. For example, the discovery of ND virus (NDV) strains showing a spectrum of virulence for poultry and the use of viruses of low or moderate virulence as live vaccines has meant that demonstration of infection with NDV is usually inadequate for unequivocal diagnosis of ND. As modern virology has developed the poultry industry throughout the world has expanded explosively and this has led to enormous trade in poultry products. The need to control the spread of ND by such trade and the legislation imposed on trade ostensibly to control the spread of ND (which may not always be the same thing) have resulted in more stringent requirements of the diagnostician. In general terms this means that there are no universally accredited methods for the diagnosis of ND in that, for example, virus that may be considered an acceptable "vaccine" in one country may represent a serious threat to the poultry industry in another, not necessarily due to disease

losses but by the effect on trade to other countries.

CLINICAL SIGNS AND LESIONS

The clinical signs and pathology of ND in poultry and other birds have been described in other chapters of this book (2,3). These may vary with the infecting virus, the species of bird, the age of the host, the immune status of the host and environmental conditions and, as a result, none may be regarded as pathognomonic for ND.

Beard and Hanson (4) have classified ND viruses into five pathotypes based on the clinical signs induced in infected chickens : i) viscerotropic velogenic (VV) - high mortality with intestinal lesions, ii) neurotropic velogenic (NV) - high mortality following nervous signs, iii) mesogenic - low mortality, respiratory and nervous signs, iv) lentogenic - mild or inapparent respiratory infections, v) asymptomatic enteric - inapparent intestinal infection. Even under constant controlled laboratory conditions these groupings may not be obvious (5) but give an indication of the variable nature of the disease and serve as a guide to clinical signs suspicious of ND.

Full and accurate diagnosis of ND almost always involves the isolation and characterisation of the virus.

ISOLATION OF NDV

Samples

Successful isolation of NDV has been most frequently obtained from samples taken from the respiratory or intestinal tracts and cloacal swabs, or faeces, and tracheal swabs should always be included regardless of clinical signs. Other samples taken at post-mortem examination should relate to the clinical signs seen and organs obviously affected. Infection of susceptible hosts with highly pathogenic viruses usually results in wide spread dissemination of the virus throughout the carcass (6) whereas infections with viruses of lower virulence or in less susceptible hosts may result in localised infections. For example most NDV infections of waterfowl tend to result in the presence of virus

only in the intestinal tract.

Most NDV isolates are fairly stable in non-putrifying tissue and organ samples or faeces providing they are not exposed to high temperatures. In countries with generally low ambient temperatures transport of material for virus isolation does not usually present any problems, although packaging in ice, or equivalent, or even freezing is usually a worthwhile precaution. In tropical countries deterioration of samples is a serious problem especially where transport is slow and refrigeration often unavailable. Omojola and Hanson (7) suggested that bone marrow may be a useful sample for virulent viruses as they were able to demonstrate the presence of virus after several days at 30°C.

The use of antibiotic transport medium appears to be of secondary importance to protection of samples from excessive heat.

Virus isolation

Most virulent strains of NDV will grow in a wide range of cell culture systems and it is possible that in some instances, due to local conditions, inoculation of cell cultures may be the best method for attempting NDV isolation. However, the most widely recommended method is the treatment of samples with antibiotics and the inoculation of embryonated fowls' eggs (4,8,9).

The choice of eggs is important, these should be obtained from a "specific pathogen free" flock or, at least, eggs from hens free of NDV antibodies, and used at 9 to 10 days old.

Tissues and organ samples should be finely minced and 10-20% w/v suspensions of these and faeces samples made in phosphate buffered saline (PBS) containing antibiotics and at pH 7.0-7.4. Swabs should be placed in sufficient antibiotic PBS to allow full immersion. The antibiotics used and their concentrations may vary from laboratory to laboratory, often due to local conditions and availability, and do not appear to be critical. A typical example is: 10,000 units/ml penicillin, 10mg/ml streptomycin, 250ug/ml gentamycin and 5,000 units/ml mycostatin. In addition 50mg/ml oxytetracycline may be included if the presence of Chlamydia is a

possibility. Samples should be held at room temperature for about two hours before centrifugation at 1000g. Supernatant from the samples is then inoculated in 0.2ml volumes into the allantoic cavity of each of at least five 9 to 10-day-old eggs. Inoculated eggs should be placed in 37°C and candled daily, eggs dead or dying or all eggs 5 to 7 days after inoculation should be chilled to 4°C, the allantoic/amniotic fluid harvested and tested for haemagglutination of chicken red blood cells (8) and the absence of bacteria. Haemagglutination (HA) activity in bacteria-free fluids will, almost certainly, be due to either NDV, one of the other avian paramyxoviruses or an influenza virus. HA negative fluids should be subjected, undiluted, to at least one further passage through embryonated eggs.

Virus identification

The isolation of NDV may be confirmed by a haemagglutination inhibition (HI) test on the haemagglutinating fluids using known positive antiserum. Methods for HA and HI titrations are given in this chapter under Serology. Some viruses, particularly other avian paramyxovirus serotypes, may show some inhibition in HI tests with NDV antiserum. These relationships are reviewed in another chapter in this book (10). The most important cross relationship is seen with PMV-3 viruses, especially those isolated from exotic birds. In addition, NDV variants have been reported which may show lower titres with diagnostic sera than more classical strains (11-13). The possibility of misdiagnosis may be kept to a minimum by the use of control antigens and sera. Some laboratories now have monoclonal antibodies available which may be used in routine HI tests to confirm the identification of NDV isolates (14-17).

Virus characterisation

The isolation of NDV from birds showing disease signs, even those typical of ND, does not confirm NDV as the causative organism. This point was emphasised by Alexander et al (14) who described several viruses submitted to an International Reference

Laboratory as apathogenic despite their isolation from birds with clinical signs similar to those produced by virulent NDV. The isolation of vaccinal strains of NDV from birds infected with virulent virus may also occur from time to time due to the routine prophylactic use of live vaccines or vaccination as a panic measure in the face of suspicious clinical signs.

In addition to these parameters, which point to the necessity for further virus characterisation, there may be an international or local requirement for an assessment of virulence before ND is confirmed (18).

Currently accepted virus characterisation involves an assessment of virulence by one or more of the following in vivo tests:-

i) Mean death time in embryonated fowls' eggs (MDT). This test was originally described by Hanson and Brandly to differentiate NDV strains (19). Minor variations in the test have been reported by some workers but in summary the test is as follows. Fresh infective allantoic fluid is diluted in a tenfold series (10^{-5} to 10^{-9} is usually adequate) in sterile saline. Five 9 to 10-day-old embryonated specific pathogen free fowls' eggs are inoculated with 0.1ml of each dilution, the time of inoculation recorded and the eggs placed at $37^{o}C$. Eight to ten hours later (morning and late afternoon are usually used) a further five eggs are inoculated with 0.1ml of each dilution (dilutions can be held at $4^{o}C$). Embryos are examined twice daily for seven days and the time of death recorded. The minimum lethal dose is the highest dilution at which all eggs die and the MDT is the mean time in hours for the minimum lethal dose to kill those embryos.

NDV strains and isolates have been placed into three groups on the basis of their MDT (19):- VELOGENIC - MDT less than 60 hours; MESOGENIC - MDT 60 to 90 hours; LENTOGENIC - MDT greater than 90 hours. These terms are often used to describe viruses of high, moderate or low virulence regardless of the method of assessment.

Although the MDT may give a useful guide to virulence on most occasions, it has been considered to be imprecise, particularly

when used to assess viruses isolated from hosts other than chickens.

ii) Intracerebral pathogenicity index in day-old chicks (ICPI). A typical methodology for this test is to dilute fresh infective allantoic fluid (HA titre must not be less than 2^4) 1/10 in sterile isotonic saline (antibiotics must not be used) and to inject 0.05ml intracerebrally into each of 10 one-day-old chicks hatched from specific pathogen free fowls' eggs. The birds are then examined every 24 hours for eight days. At each observation each bird is scored 0 if normal, 1 if sick and 2 if dead. The ICPI is the mean score per bird over the eight day period. The most virulent NDV isolates give indices close to 2 while the avirulent viruses given values close to 0.

Some variation has been reported in the methodology for ICPI tests. The USA National Academy of Science (9) and Allan et al (20) recommend the inoculation of 0.05ml volumes of 1/10 diluted allantoic fluid, while Hanson (21) used 0.1ml of undiluted fluid. The European Pharmacopoeia (22) suggested titrating the virus before testing and inoculating $10^{5.7}$ 50% egg infectious doses in 0.05ml for screening vaccinal viruses. Although titration and inoculation of the same amount of infectious virus may be the most accurate method of comparing different virus strains, such time consuming procedures are rarely practicable in routine diagnosis.

The ICPI test is a sensitive measure of virulence. The main drawbacks to the test are that it does not distinguish between virulent viruses very well, moderately virulent strains for older birds, such as Komarov and Mukteswar, may give ICPI values in excess of 1.5, and minor variations in the number of birds sick and time of onset may result in marked differences for viruses of low virulence.

iii) Intravenous pathogenicity index in six-week-old chickens (IVPI). This test involves the inoculation of 0.1ml of fresh infective allantoic fluid (HA titre greater than 2^4) diluted 1/10 in sterile isotonic saline intravenously into each of 10 six-week-old specific pathogen free chickens. The birds are then examined every 24 hours and scored 0 if normal, 1 if sick, 2 if paralysed and 3 if dead at each observation. The IVPI is the mean score per

bird per observation over the 10 day period.

Virulent strains and isolates of NDV give IVPI values close to 3 whereas viruses of low virulence and most of intermediate virulence will give values of 0.

Occasionally, variations to these tests have been suggested to meet specific requirements. For example, Hanson (23) used swabbing of the conjunctiva and cloaca of 8-week-old chickens with undiluted allantoic fluid to replace intravenous inoculation in an effort to detect viscerotropic viruses.

Final diagnostic significance placed on tests of pathogenicity will depend on local conditions and legislation. In many European countries where no or only mild live vaccines are used infections with viruses showing ICPI values of 0.7 or more are regarded as reportable and requiring control measures, whereas in many other countries of the world it is the practice to use mesogenic vaccines such as Komarov, Mukteswar or Roakin which may have ICPI values as high as 1.5.

Values obtained in ICPI, IVPI and MDT pathogenicity tests for some well characterised strains of NDV are shown in Table 1.

TABLE 1

PATHOGENICITY INDICES OF STRAINS OF NEWCASTLE DISEASE VIRUS

STRAIN	ICPI[a]	IVPI[b]	MDT[c]
Ulster 2C	0.0	0.0	>150
Queensland V4	0.0	0.0	>150
Hitchner B1	0.2	0.0	120
F	0.25	0.0	119
La Sota	0.4	0.0	103
Komarov	1.4	0.0	69
Roakin	1.45	0.0	68
Mukteswar	1.4	0.0	46
Beaudette C	1.6	1.45	62
GB Texas	1.75	2.7	55
Italien	1.85	2.8	50
Essex 70	1.85	2.5	60
Herts 33	1.9	2.6	48
pigeon/England/561/83	1.5	0.0	120
chicken/England/702/84	1.9	2.1	60

a : Intracerebral pathogenicity index in day-old chicks
b : Intravenous pathogenicity index in six-week-old chickens
c : Mean death of infected embryos in hours for one minimum lethal dose

Ultimately, the objective of diagnostic virus characterisation is to distinguish between epizootic and enzootic virus and while this normally means distinction between viruses or high or low virulence, other techniques may also be employed. For example, Hanson (4,23) used a battery of tests including plaque type and size, haemagglutinin elution rate, haemagglutinin thermostability and the ability to haemagglutinate equine red blood cells to characterise and distinguish between various strains of NDV.

A relationship between plaque size in chick embryo cells and virulence for chickens is well known (24) and relates to whether or not post-translational cleavage of HN and F polypeptide precursors occurs in the host system (25). Rott (26) has suggested this can be used to differentiate between viruses of high and low virulence for chickens.

Specific biochemical tests such as oligonucleotide (27) and polypeptide (28) fingerprinting have also been used for strain characterisation and may be useful techniques for tracing specific epizootic virus.

Several groups have prepared mouse monoclonal antibodies to NDV strains which have been used to characterise NDV strains and isolates (14-16, 29-33). This approach has enabled distinction between viruses on the basis of epizootiological and other biological groupings and has already made a significant contribution to the understanding of the epizootiology of NDV which has been reviewed in a chapter of this book by Russell (34). The usefulness of monoclonal antibody typing was particularly apparent in the study and understanding of the panzootic amongst pigeons during the 1980s where monoclonal antibodies were used to show the unique variant nature of the virus involved and to confirm its spread throughout the World (35).

A further important finding for the characterisation of NDV isolates was demonstrated by the variant virus from pigeons. On initial isolation from pigeons such viruses tended to show ICPI values of about 1.4 and IVPI values of 0.0; however, on passage through chickens marked increase was seen in IVPI values to over

2.0 while ICPI values rose to over 1.7 (36). The latter values were comparable to isolates of the pigeon variant virus from natural infections of chickens (37). These findings demonstrate the importance of not relying on a single laboratory test for accurate diagnosis.

DIRECT DETECTION OF NDV ANTIGENS

Occasionally, for the purposes of diagnosis, it is sufficient to demonstrate that infection with NDV has taken place and in this situation the infecting strain and its virulence are of no significance. Under such circumstances it is possible to employ rapid methods for the detection of antigen in organs likely to be infected. There have been two reports of routine use of such techniques both employing labelled antibodies of NDV in immunofluorescence tests, one on longitudinal tracheal sections (38) the other on impression smears from organs (39).

SEROLOGY

Serological tests for NDV may be used to demonstrate infection with virus or to monitor vaccination. Most serological techniques used for the detection of antibodies to viruses have been applied to NDV. Conventionally the HI test has been the method of choice but in recent years several enzyme linked immunosorbent assay (ELISA) tests have been developed (40-44). Studies on the sensitivity, specificity and correlation to HI tests of ELISA tests have tended to produce variable results (41,45). The advantage of such tests is that they are easily semi-automated and as diagnostic laboratories increase the amount of rapid flock screening for multiple agents (46), ELISA tests may become the method of choice for the measurement of antibodies to most poultry pathogens. Under certain circumstances other serological tests may be of value and the use of single radial immunodiffusion (47), single radial haemolysis (48) and agar gel precipitin (49) tests have been evaluated for the measurement of antibodies to NDV. However, at present there is no consensus for the use of such tests and the most widely used method for the assessment of

NDV antibodies is the HI test.

Methods for the HI test

Several methods have been used for carrying out HA AND HI tests. The method most widely referred to is probably that of Allan and Gough (50) using microtitre plates. However, recently, detailed standard methods for the HA and HI tests for avian infectious bronchitis virus have been defined (51) and since these represent suitable methods for all haemagglutinating viruses it seems reasonable to adopt them as standard techniques. The tests, using V-bottomed microwell plastic plates, may be carried out at room temperature but if ambient temperature is high it is recommended that solutions are used straight from the refrigerator and plates are transferred to $4^{o}C$ during the tests. In summary the tests are as follows:-

HA test : For accurate determination of the HA titre it is necessary to titrate from an initial series of close dilutions, e.g. 1/2, 1/3, 1/4, 1/5 etc. For routine diagnosis this is not usually necessary. Twofold dilutions of 0.025ml amounts of infective allantoic fluid are made in phosphate buffered isotonic saline (PBS) at pH 7.0-7.4. To each dilution 0.025ml of PBS and 0.025ml of 1% v/v chicken red blood cells are added. After mixing gently the plates are allowed to stand for 45 minutes. The titration should be read to the highest dilution giving 100% agglutination of the red cells, this amount of antigen represents one HA unit.

HI test : Twofold dilutions of 0.025ml amounts of the serum to be tested are made in PBS and 4 HA units of antigen in 0.025ml are added to each well. After 15-30 minutes 0.025ml of 1% v/v chicken red blood cells is added and after gentle mixing left for 45 minutes. The HI titre is the highest dilution of serum causing complete inhibition of 4 HA units. Agglutination in this system is assessed by tilting the plates, only those wells in which the red cell buttons "stream" at the same rate as control red blood cells with no antigen should be recorded as showing inhibition.

Poultry sera rarely give non-specific inhibition in ND HI tests and it is not usually considered necessary to pretreat serum samples. Sera from species other than chickens may sometimes cause agglutination of chicken red blood cells which would mask low level inhibition or complicate the test. It is a wise precaution to test for such agglutination and remove it by adsorption with chicken red blood cells prior to use in the test.

Sera from birds which have not been immunised or infected with NDV invariably give HI titres less than 1/8 (titres are usually expressed as the reciprocal of the dilution, e.g. 8 or 2^3 or $\log_2 3$ [52]).

Brugh (53) studied the effects changes in the various parameters of the test may have on the titres obtained and concluded that marked variation in most of the times, antigen and red blood cell concentrations caused only minor differences in the titre obtained, but that the antigen/antiserum incubation period was more critical. A study of the reproducibility of HI titres for the same sera in 17 laboratories in 10 states of the USA produced marked variation (54) and indicates the necessity of critical monitoring if laboratory to laboratory comparability of results is required. International standard reference NDV antiserum (55) is available from the International Laboratory for Biological Standards, Central Veterinary Laboratory, Weybridge, Surrey which is intended for use in standardising the HI test for NDV in different laboratories.

Interpretation of serological response

The value of serological tests in the diagnosis of ND is limited. In the absence of vaccination, a positive HI, or other test, is clear evidence that the bird has been exposed to NDV. But gives no clue to the nature of the infecting strain.

HI titres are also used to measure the immune response to vaccination. In chickens HI titre levels of 2^4-2^6 may be obtained after a single live vaccine dose and titres as high as 2^9-2^{11} following vaccination programmes including use of inactivated vaccines. Allan et al (20) gave estimates for the expected

outcome of challenge with highly virulent virus on vaccinated flocks for birds showing different levels of immune response:- 2^2 or less - 100% mortality; 2^2-2^5 - 10% mortality; 2^4-2^6 - no mortality; 2^6-2^8 - serious egg production losses; 2^9-2^{11} - birds remain normal. These figures were obtained in laboratory experiments using strain Herts 33 as a challenge. While they are listed here to give some idea of target immune responses these levels should be regarded very much as a simplification of the field situation where many other factors may exist which would affect the response to challenge of vaccinated birds. Further assessment of convalescent HI titres as evidence of challenge of vaccinated birds has also been attempted (20) but such amnestic responses will also be greatly influenced by environmental and other factors and care is needed in making such interpretations.

One further point should always be borne in mind in relationship to diagnostic serology; levels of immunity which afford protection do not necessarily prevent virus replication and fully vaccinated birds may still represent an important source of virus in the spread of disease.

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10

NEWCASTLE DISEASE

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PATHOGENESIS

The pathogenesis of Newcastle disease virus (NDV) depends on a number of factors of which the most important are the virulence and tropism of the virus.

Other factors which may influence the morbidity, mortality and clinical signs are:-

(1) The age of the bird.
(2) The immune status of the birds.
(3) The route of exposure.
(4) The magnitude and duration of the infecting dose.
(5) The susceptibility of the host species.
(6) External factors such as social stress and temperature.

VIRULENCE AND TROPISM OF THE VIRUS

Hanson (1) divided NDV strains into 4 pathotypes:- The viscerotropic-velogenic (Doyle's form or Asiatic NDV), is a very acute and lethal form with haemorrhagic lesions of the digestive tract. In the neurotropic-velogenic (Beach's form), the main signs are neurological and respiratory (2). The mesogenic (Beaudette's form) is an acute respiratory and sometimes lethal nervous infection of young chicks. Mortality is rare in older birds (3). The lentogenic (Hitchner's form), is a mild or inapparent respiratory infection of chickens. (4). These descriptive terms for the pathotypes are based on the mean death time (MDT) of embryonated eggs inoculated with the viruses and their ability to produce nervous or enteric changes. A fifth pathotype was added (5), the avirulent alimentary tract associated virus (the Ulster type, 6).

However, these divisions were shown to be arbitrary and there was a spectrum of virus effects (7). Thus Herts would be classified as a viscerotropic-velogenic strain but it also produces severe respiratory signs. Periorbital oedema was thought to be a good marker for VVNDV strains (8), but the Lamb Essex 70 strain produced marked oedema yet rarely caused enteric lesions (7).

Although outbreaks of Newcastle disease in Western Europe in the early 1970s were associated with high mortality and severe respiratory distress, Alexander and Allan were unable to induce respiratory distress with Lamb-Essex '70 unless infection was by aerosol. By contrast Beaudette induced respiratory signs even when given by cloacal swab (7).

Recently, monoclonal antibodies have allowed the classification of 40 isolates into 8 distinct groups (9). This work has been extended (10) and it has been demonstrated that monoclonal antibodies are a powerful tool to enable isolates to be quickly and accurately classified into groups sharing epizootiological and biological properties.

There appears to be two main reservoirs of NDV. The avirulent virus mainly associated with waterfowl and the highly virulent viruses associated with tropical birds, such as psittacines. Both are associated with growth in the intestine. The origins of the highly virulent respiratory form and the mesogenic and lentogenic strains are less clear. It is possible that they are derived from the above strains. Thus strains isolated from exotic birds have been found to contain different sub-populations, some of high and some of low virulence (11).

Avirulent or low virulent viruses which have been isolated either from domestic fowl or from wild birds, especially ducks and geese appear to form a distinct group. These viruses generally have an ICPI of 0 (6, 12), but some have low but detectable pathogenicity (13). Their effects on chick embryos vary from those with MDT of the minimum lethal dose of virus (MLD) of 114-168 hr (14) to those where the MDT of the MLD cannot be established because all embryos are not killed (6, 13). Viruses of

this group normally have very stable haemagglutinin. Thus Rosenberger et al. (14) found that of 4 isolates from Canada geese the haemagglutination activity was removed after 15 min and 30 min at 56°C for two viruses, while for the other two, haemagglutinin activity survived heating for more than 2 hr at 56°C. Similar results were found for isolates from ducks (15). Some Ulster isolates from fowl were found with haemagglutination activity which was removed after 6 hr while others required 48 hr to remove the activity (McFerran, unpublished). All of these isolates grow predominantly in the intestine. In natural infections 11 isolates were made from faeces and only one from the respiratory tract (6). Experimental work using these isolates confirmed the tropism for the alimentary tract. Following oral infection virus could only be recovered from the pharynx, small and large intestines (McFerran, unpublished). Kono et al. (16) killed 2 chicks on each of days 1, 3, 5 and 7 after oral inoculation of $10^{7.5}$ $TCID_{50}$ avirulent Ishic NDV. Virus was recovered from the trachea of one bird on one occasion but not from the spleen. Virus was, however, constantly recovered from the alimentary tract, especially the distal part of the small intestine, caecum and large intestine.

There appears, therefore, to be a reservoir of avirulent viruses infecting ducks and geese and possibly other sea birds, as demonstrated by the isolation of avirulent viruses from Comorants (Phalacrocorax aristotelis) (17), which, on occasion, can infect domestic poultry.

There also appears to be a pool of highly virulent viruses maintained in exotic birds such as Psittacines. In the USA of 2.9 million quarantined birds examined, NDV was recovered from 173 lots, ie 26.3%. Of these isolates VVNDV has obtained from 141 lots, non-viscerotropic velogenic NDV was recognised in 6 lots, mesogenic in 3 lots and lentogenic NDV from 23 lots. The majority of velogenic isolates were from Psittaciformes, with Passeriformes next in importance (18). It appears that some species can become carriers of infection. Thus whilst canaries appeared to eliminate

the virus, nuns and conures excreted virus up to 84 days post exposure and parrots excreted for up to 376 days (19).

AGE OF THE BIRD

In general the younger the bird the more severe the disease following either natural or artificial exposure.

Thus highly pathogenic strains kill a large percentage of adult fowl and all young chickens. Mesogenic strains kill few adults but still kill a high percentage of young chickens, whilst lentogenic strains may kill none or only a small proportion of very young chickens (20).

However there is a much better spread of virus between birds aged 21 and 63 days than between 7 day old birds. It is not established if this is because the older birds excrete more virus (21).

Resistance to disease increases with age in turkeys (22, 23).

Mallards are most susceptible to infection at 6 days old (24).

IMMUNE STATUS OF THE BIRD

The role of immunity is considered elsewhere, but both passively derived antibody and actively derived immunity can markedly affect the pathogenesis and clinical signs. In general antibody will prevent the virus growing in the visceral organs and will keep the bird clinically healthy. However it will not prevent virus replication at mucosal surfaces and therefore virus can still be excreted. It requires local replication of virus to stimulate local immunity which reduces or eliminates growth of virus at the mucosal surface (16, 25).

There is evidence that there is a genetic basis for resistance (26) but this appears to be of little importance in commercial birds.

ROUTE OF EXPOSURE

Kohn (27) showed that whilst 3 to 6-week-old chickens could be infected with 100 50% egg lethal doses (ELD_{50}) of virus by aerosol or the intratracheal route, it required 4,000-5,000 ELD_{50} to infect intranasally and 20,000 ELD_{50} when injected into the

crop. If birds were anaesthetised to eliminate the swallowing reflex, the amount of virus to establish infection intranasally or by aerosol were similar. This suggests that the natural site of primary replication is the lower respiratory tract.

However, not all strains are normally able to infect the lower respiratory tract. Thus, when the B1 vaccine strain spreads by contact, virus is usually only found in the upper respiratory tract. However, if fully susceptible chicks are given B1 virus by aerosol, quite severe disease can ensue, with lesions in the air sacs. If B1 is given by aerosol it will produce higher antibody titres than in birds inoculated by the intramuscular, conjunctival or nasal routes, and will stimulate local immunity in the trachea (28). Furthermore, whilst the lentogenic B1 given by aerosol reaches 10^5 EID_{50} in the trachea, it cannot be recovered from the trachea following intramuscular (I/M) inoculation, in contrast with the virulent GB strain which grows to 10 or 100 fold higher titres in the trachea after I/M or aerosol infection (28). The reason NDV is less infectious when given orally appears to be because the gizzard contents (around pH 2.6) reduce the infectivity 1,000 fold (29). This effect can be reversed by inoculating the virus into the crop, feeding in a gelatine capsule or inoculating into the rectum (30).

Ulster-type avirulent viruses replicate only in the intestine and in spite of the apparent insensitivity of the oral route, spread effectively. However, the relative insensitivity of the oral route may be important for vaccination. Thus, birds given vaccine in water also get vaccine in their nasal passages as they plunge their beaks into the water. If drinking from nipples the upper respiratory tract may not be contaminated.

THE MAGNITUDE AND DURATION OF THE INFECTING DOSE

A strain which is lethal at high titre may not cause mortality at limiting doses and in some cases no illness may be produced and infection only demonstrated by the development of antibody (25).

Cheville et al., (31) also demonstrated the effect of virus dose. Birds died as soon as 6 hours after the onset of signs with

high titre inocula whilst at lower titres of the same strain signs lasted for 96 hours and in some cases the birds survived.

A pneumontropic velogenic strain (Antrim 73) produced severe tracheal lesions in an outbreak in Northern Ireland. Experimentally, one intranasal (I/N) inoculation of high titre virus caused death, with minimal signs or lesions apart from facial oedema and CNS lesions. When birds were given repeated I/N inoculations over a 24-48 hr. period or were placed in contact with infected birds, signs and lesions typical of the natural cases occurred. Furthermore if the airflow in the isolators were too high, instead of all the in-contacts developing severe disease and dying, a proportion showed minor signs or only developed antibody (McCracken and McFerran, unpublished).

THE SUSCEPTIBILITY OF THE HOST SPECIES

There is considerable variation in the susceptibility of birds to disease as discussed under signs but less is known about the susceptibility to infection. Thus ducks are often infected with NDV and are clearly susceptible, but rarely show clinical signs, and thus have been considered insusceptible. Canaries (Serinus canarius) appear to be fairly resistant (19).

Some species (eg Cranes and Parrots) may be infected and shed velogenic virus for weeks, without showing signs (15, 19).

EXTERNAL FACTORS

External factors such as humidity, rainfall, hours of sunlight, ambient temperature and wind speed have a major influence on virus spread by air and offer an explanation for better virus spread in autumn and winter in Europe (32). However external factors appear to play a minor role in the pathogenesis and course of the disease. Newcastle disease is present in both temperate and tropical areas and it occurs in all seasons. However it has been demonstrated that at high ambient temperatures chickens appear more susceptible and more frequently develop neurologic signs (33). However Shrikrishnan (34) demonstrated that nervous signs were more common when there were low ambient temperatures and high levels of carbon dioxide in the atmosphere.

Social factors, inducing stress, result in the virus being more invasive and growing to higher titres (34).

SEQUENCE OF ORGANS INVOLVED

The virus replicates at the site of entry. This is usually the conjunctiva, respiratory or alimentary tracts. Thus 18 hr. after intranasal inoculation with a mesogenic strain there is destruction of mucus cells in individual acini in the middle turbinate. There is rapid spread to neighbouring ciliated and goblet cells and acini. By day 2 there is continuing destruction of the mucosa, inflammatory cell infiltration and loss of cartilage basophilia. The inflammatory reaction decreases by day 5 and by day 8 the epithelium is normal (35). There is then viraemia, followed by multiplication of virus in many organs. Thus by 44 hours post infection titres as high as 6.9 $\log_{10}$/g are found in the kidney, spleen, lungs and bursa and substantial amounts of virus are present in the duodenum, trachea, pancreas and brain (36). Basically, virulent strains are pantropic and replicate in the reticuloendothelial system and to a lesser extent in most other organs including the brain and heart. Many of the effects are due to replication in endothelial cells. Anaemia appears to be due, in part at least, to NDV replication in erythrocytes (31). Mesogenic strains generally behave in a similar fashion, but some may not invade the brain (36). Karzon and Bang (37) compared the growth of a velogenic and a lentogenic strain following intramuscular inoculation. Both grew equally fast in extraneural tissue. Titres in brain lagged behind visceral titres, even though intracerebral inoculation demonstrated that brain cells were very sensitive. The virulent strain caused a greater destruction of brain tissue per infectious unit and indeed the lentogenic strain grew poorly in the brain. Sinha et al. (20) however produced evidence that the viraemia is longer and had higher titres with lethal than with non-lethal strains. They however confirmed that the ability to pass the blood-brain barrier was not the explanation for virulence as two of the lethal strains studied were not regularly isolated from the brain. By contrast when avirulent or lentogenic viruses are

inoculated into the brain they do not kill and this is the basis of the currently utilised intracerebral pathogenicity index.

There are a number of differences from this basic pattern, depending on strain involved. Thus avirulent strains grow only or mainly in the alimentary tract, reaching titres between 4 and 8 $\log_{10}$/g in the small intestine on the 3rd day after infection. Virus grew in the cytoplasm of the epithelial lung cells of the intestine and especially the lower portion of the small intestine (16). The source of virus in the faeces is obviously from the growth of virus in the epithelial cells of the intestine. However the virus also grows very well in the bursa and more virulent strains grow to higher titres in the kidneys. Virus from both these sources will add to that produced in the intestine.

The lentogenic viruses may only show limited growth and with a minor or no viraemia. Thus B1 and F strains are usually limited to growth in the upper respiratory tract and only appear in the lower tract when the virus is given by aerosol (36,38).

SIGNS

FOWL

The signs seen in fowl can be considered under 5 headings; the generalised effects and those on the reproductive, respiratory, enteric, and nervous systems. The signs produced are not pathognomonic and they are very much influenced by the virulence and tropism of the virus. They are also affected by a number of other factors, discussed above.

Generalised signs include loss of appetite progressing to failure to eat and abnormal thirst, and severe dehydration and emaciation may occur in association with fever.

Ruffled feathers, huddling, listlessness, somnolence, progressing to complete depression are features. Birds often sit on their hocks with their eyes half to fully closed. Oedema of the face (especially the eyelids) is sometimes seen with velogenic strains and therefore can no longer be considered diagnostic for Fowl Plague (7). Change of voice, becoming harsh, is sometimes followed by complete silence in the house due to the depression of the birds. Diffuse congestion of the skin with localised areas of

petechiation, especially in the wattles, can be a feature. Combs and wattles can become cyanotic and oedematous due to a combination of respiratory and circulatory involvements. Pallor, resulting from anaemia, may be a sign with less virulent viruses. In outbreaks involving velogenic virus, birds may be found dead without any signs.

The effect on egg production is usually marked. There can be a reduction in egg numbers associated with smaller eggs, misshapen and rough shelled eggs and shell-less eggs and a decrease in the quality of albumen. In other outbreaks the egg production can rapidly cease or fall to very low levels. This is usually preceded by the production of shell-less eggs. Egg production often returns to normal levels after 3-4 weeks, but in some outbreaks it never returns. In some cases surviving birds may go into moult.

Respiratory signs may occur as mild rales and snicks which only can be detected with careful observation.

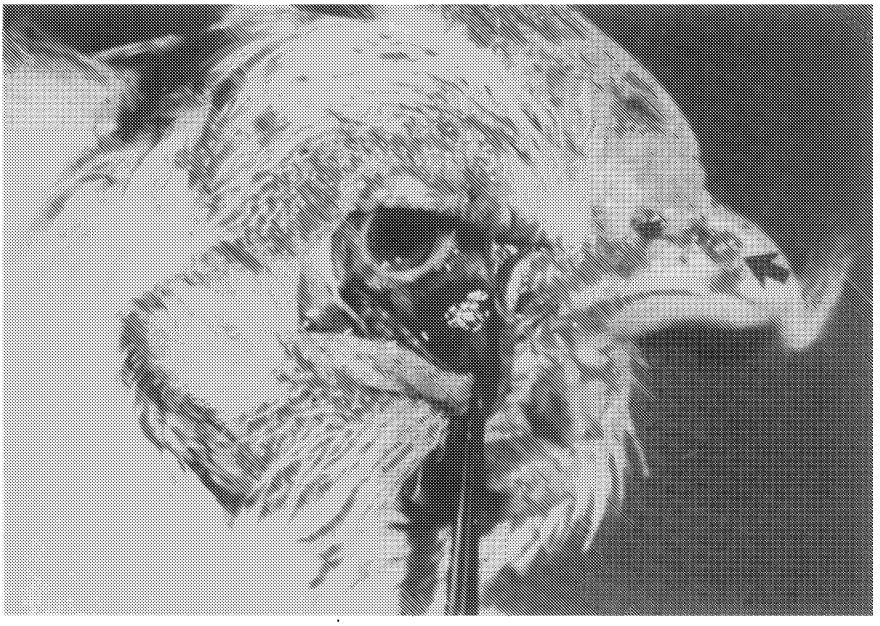

Fig. 1 Lesions of conjunctivitis are present and oedema and haemorrhage are evident.

This is best heard at night when the birds are settled. Signs may be more severe with sneezing, coughing, nasal discharge (Fig 1) and laboured breathing to frank respiratory distress with open mouthed breathing. Inspiration can be accompanied by a rattling sound. Head shaking, with the birds trying to dislodge mucus from the respiratory passages can be a feature. There may be a uni or bilateral mucopurulent conjunctivitis.

Greenish-yellow diarrhoea is a feature of some outbreaks but is by no means a universal sign.

Nervous signs are very variable and usually are not seen until the disease is advanced. They include tremors, torticollis, opisthotinus, convulsions which are steady and rhythmic, incoordinated movement and paralysis of wings or legs.

Attempts have been made to classify these signs into various forms - e.g. Doyle's or Beach's. Due to the gradation of effects produced by different viruses, together with other factors such as duration and intensity of exposure, route of exposure and age of bird, this is not a useful exercise.

TURKEYS

Generalised signs are normally less severe with respiratory and nervous signs predominating. Egg production can be affected, with the development of soft shelled and misshapen eggs and poor quality albumen. In some outbreaks infections are subclinical and the only signs seen are partial or complete motor paralysis of one or both legs (39). In other outbreaks peracute cases with high mortality may occur (40).

DUCKS AND GEESE

Even when infected by mesogenic or velogenic viruses, infections are normally subclinical (41). However disease can occur. Thus Raszewaka (42) recorded 2 natural outbreaks of NDV in 11 to 14-day-old goslings, Higgins (43) described acute ND in 6 duck flocks in Hong Kong and Estudillo (44) found that a few members of the Anatidae and Anseriformes showed paralysis of legs and wings. No respiratory signs were observed. Morbidity in geese, swans and ducks was 10% or less, with about 10% mortality in the ducks and geese and no mortality in the swans.

PIGEONS

Pigeons can either be infected with traditional strains of NDV or with the newly described pigeon PMV-1 variant (45). Velogenic NDV produces conjunctivitis, rhinitis, dyspnoea, diarrhoea, tremors of the neck and wings, torticollis and incoordination. Mortality can reach 40% in infections with the pigeon PMV-1 isolates in which nervous signs and diarrhoea are the dominant features.

WILD BIRDS

The signs seen in wild birds are very variable. Estudillo (44) described an outbreak in a large aviary in Mexico of a highly virulent virus. Galliformes (pheasants, peafowl and guinea fowl) were very susceptible and had a high mortality. Nervous signs were predominant, green diarrhoea was present and respiratory signs were a feature in the peafowl. Psittaciformes (parrots, and macaws) were also highly susceptible, but signs were almost always confined to those of nervous disease with abnormal attitudes and movement, ataxia, torticollis and paralysis. Columbiformes (doves and crowned pigeons) were less susceptible and nervous signs were most evident. Other breeds such as Ramphastidae (toucans, toucanet), Anatidae (ducks), Anseriformes (geese, swans) Phoenicopteridae (flamingos), Gruiformes (swan, cranes), rhea, carrowary and sparrows all were susceptible, but with much less severe disease. In all these birds nervous signs were often the only evidence of infection.

An experimental study (19) confirmed the susceptibility of Psittacidae to infection and the predominance of nervous signs. Nuns (Lonchura melacca) showed much milder signs. Canaries (Serinus canarius) and mynahs (Gracula religiosa) were susceptible but the only signs observed were of progressive mortalities. Mortalities ranged from 21 to 29% except in the case of the half moon conures (Aratinga canicularis) where it reached 55%. Whilst up to 100% of budgerigars, conures and parrots and about 75% of the nuns became infected when placed in contact with infected birds of the same species only 25% of canaries and 45% of mynahs

were infected. In addition canaries, mynahs and nuns had a poor transient serological response.

PATHOLOGY

The pathology of PMV-1 infection varies considerably from strain to strain but there are inadequate comparative pathological studies to provide definite descriptions of lesions associated with each form. Some experimental studies have attempted to investigate the comparative pathology of the various strains of PMV-1 (8) but such studies are few and there is a necessity for detailed comparative pathological studies of NDV infections to be carried out.

GROSS PATHOLOGY

If birds infected with a virulent strain die, or are killed, pathological changes will include the presence of a fevered carcase, anaemia and dehydration.

Gastro-intestinal tract: Whilst some avirulent strains (e.g. Ulster) often replicate in the intestines, gross lesions are not present with this pathotype. Lesions have been recognised in the gastro-intestinal tract especially with viscerotropic viruses where outstanding lesions are haemorrhagic foci associated with necrosis in the intestinal wall. Such lesions have been described in the proventriculus, caeca and small intestine but are especially prominent in the posterior half of the duodenum and in the jejenum and ileum (5, 46). The lesions are most evident when the intestine is opened and individual lesions may be 15 mm or more in length. The intestines are generally hyperaemic and empty following infection with viscerotropic pathotypes (31) and probably are a reflection of ante-natal diarrhoea. Alexander and Allan (7) compared the intestinal lesions in chickens, following infection with 9 different isolates and, whilst intestinal lesions were most commonly reproduced with viscerotropic pathotypes, similar lesions were occasionally also reproduced following infection with other pathotypes.

Central nervous system: Gross lesions of the central nervous system are not recognised as a feature of PMV-1 infection, irrespective of pathotype involved and even though vasculitis

frequently occurs in the central nervous system following infection with viscerotropic, velogenic and mesogenic pathotypes.
Respiratory system: Gross lesions are evident in the respiratory system, especially trachea and air sacs, of birds infected with velogenic and mesogenic pathotypes (7). Air sacculitis may also be induced by lentogenic strains following aerosol vaccination of fully susceptible birds (McCracken, unpublished) with B1 virus. Tracheal changes can vary from excess catarrhal exudate to severe haemorrhage. The lungs are less consistently involved but may be grossly enlarged and congested. Pericarditis may also be observed especially where the air sacs are involved and these lesions tend to be more severe in the young non-vaccinated bird.

Fig. 2 Ovules are degenerating and yolk material is lying free (arrows) in the abdominal cavity.

Reproductive system: In a study of the reproductive tract, Biswal and Morrill (47) infected pullets intranasally with the California strain 11914 and demonstrated that egg production was depressed

during the 2nd and 3rd weeks of infection and egg shell weight and thickness were affected up to 56 days. Twenty-seven per cent of the infected birds showed degeneration of the follicles (Fig. 2) with atresia being the most commonly observed lesion. Such changes were most prominent between 7 and 14 days post-inoculation. Other gross changes included resorption of yolk, roughness of external follicular outline, congestion, haemorrhage and dark brown discolouration. Yolk material was also occasionally present in the peritoneal cavity. Oviducts appeared shrunken and some oedema was evident at 5 and 7 days post-inoculation. The oviduct mucosa lacked the normal glistening appearance but no other obvious changes were recorded.

Skin and eye: Skin and eye lesions (Fig. 1) have been recognised especially with velogenic pathotypes (7, 8). Gross changes consisted of swelling of the face tissues and eyelid, especially at 2-7 days post-inoculation and were associated with oedema.

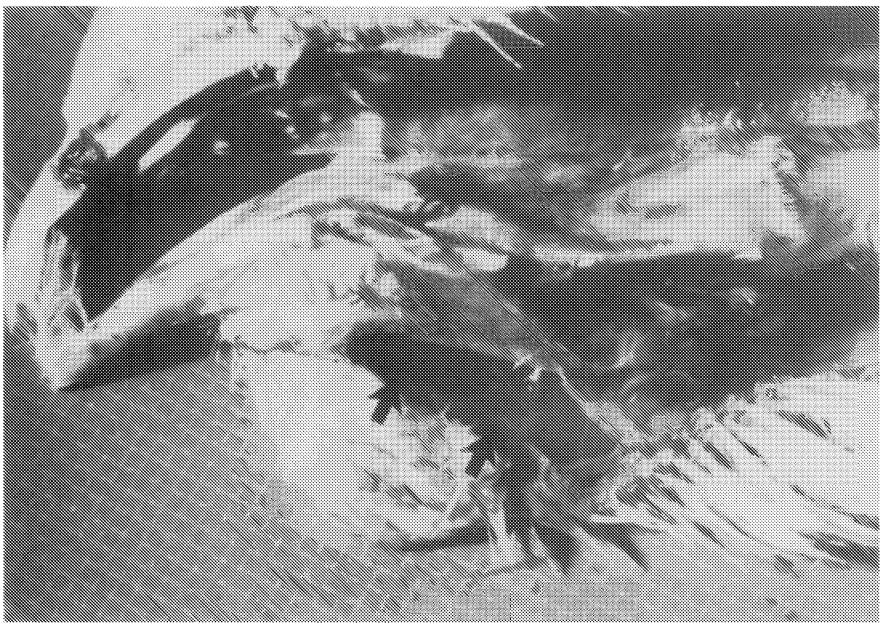

Fig. 3 Oedema fluid (arrows) is evident in the subcutaneous tissues of the neck.

This oedema fluid may extend throughout the connective tissue of the neck especially around the trachea (Fig. 3).

Haemorrhage has also been seen in the conjunctivae (Fig. 1) and tiny bleeding ulcers may be present in the skin. Large fluid-filled bullae have less commonly affected the combs but congestion and petechiae of the combs and wattle are more common. Spalatin et al. (8) found that oedema of the eyelid and face could be reproduced most consistently by applying virus to the eye with a swab and this is supported by the present authors' observations that in-contact birds showed such lesions much more commonly than birds inoculated intranasally with the Antrim'73 isolate.

Other viscera: Gross lesions are not infrequently observed in other organs and tissues and are generally a result of vascular damage; oedema and haemorrhage being the most common. Such lesions have been recorded in the spleen and bursa of Fabricius and with viscerotropic pathotypes. The heart may be enlarged and contain white streaks (31) associated with myocardial necrosis.

HISTOPATHOLOGY

Gastro-intestinal tract: Gastro-intestinal lesions are commonly seen following infection with viscerotropic pathotypes and it has been suggested that these lesions are due mainly to 4 mechanisms; diffuse necrosis of reticulo-endothelial cells, intravascular lysis of erythrocytes and subsequent erythrophagocytosis, foci of necrosis in parenchymal tissues and general hyperaemia, oedema and haemorrhage throughout interstitial tissues. It would appear that the majority of necrotising haemorrhagic intestinal lesions develop in lymphoid aggregates (46) but detailed descriptions of the nature and development of lesions are not published. Other lesions described include hyalinization of capillaries and arterioles, hyaline thrombi and necrosis of endothelial cells of blood vessels (5) with the development of vasculitis. These vascular lesions are also recognised in many other tissues and organs.

Central nervous system: Lesions most consistently described in the central nervous system are those of a non-purulent encephalomyelitis (5) and include neuronal degeneration, gliosis,

hypertrophy and proliferation of the endothelial cells and perivascular lymphocytic infiltration (Fig. 4). Such lesions are invariably present in birds infected with the neurotropic velogenic pathotype (48, 49, 50), commonly with viscerotropic and mesogenic pathotypes (31, 51) and probably not at all with lentogenic and avirulent strains (49, 50). Lesions appear to be most frequently encountered in the cerebellum, brain stem, mid brain and spinal cord but none are pathognomonic of PMV-1 infection. However, endothelial cell hypertrophy and proliferation are less commonly encountered in other diseases and its presence should be carefully assessed. Virus antigen has been

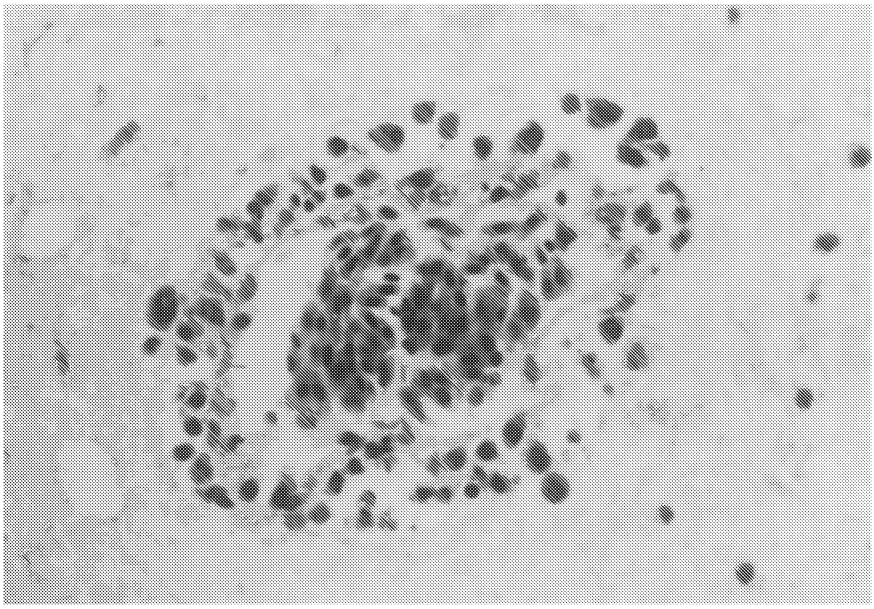

Fig. 4 Showing a cellular accumulation in the perivascular region of a vessel in the cerebellar white matter

demonstrated in vascular endothelium, neurons (particularly Purkinje cells) and glial cells by 3-5 days post-inoculation (50, 51) but by 6-9 days post-infection virus antigen can no longer be detected in the central nervous system. Wilczynski et al. (50)

demonstrated the replication of virus in Purkinje cells by ultrastructural studies. Coincident with the appearance of virus antigen, perivascular cellular accumulations and neuronal degeneration are seen and by 9 days post-inoculation Purkinje neurons and other neurons may be degenerated and replaced by glial tissue. Lesions in the central nervous system may not however maximise in intensity until 20 days post-inoculation but by 30 days post-inoculation resolution is more obvious and only perivascular lymphocytic infiltration and gliosis may be evident. Necrotic foci, frequently observed in avian influenza, are not a striking feature of NDV infection.

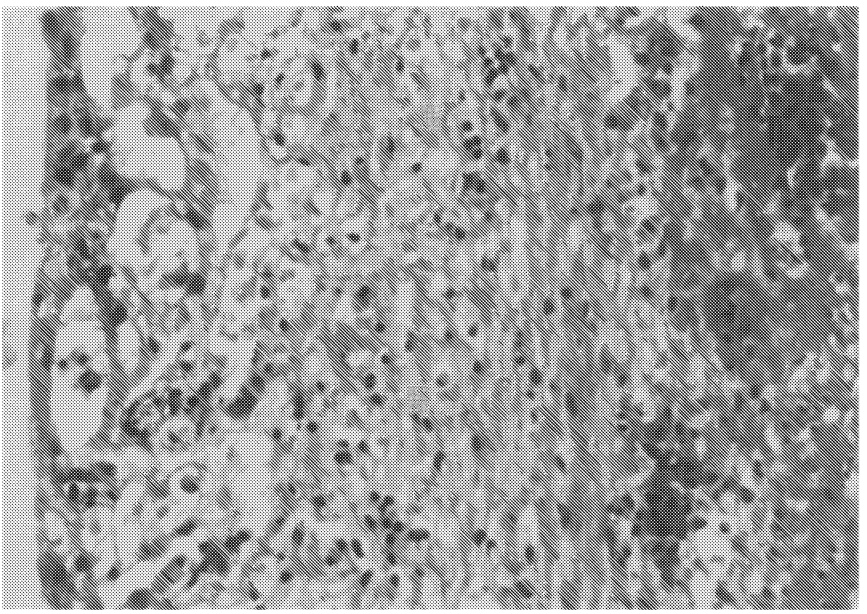

Fig. 5 The tracheal epithelium is hyperplastic and the lamina propria is distended with infiltrating inflammatory cells.

Respiratory system: In its most severe form, trachea lesions are both necrotic and haemorrhagic and may extend throughout the entire trachea. Cilia may be lost by 2 days post-infection (52)

and the epithelial cells are often degenerate and desquamate. Subsequent proliferation of epithelial cells is accompanied by an intense cellular infiltration of lymphocytes and macrophages into the submucosa and lamina propria (McCracken - unpublished) (Fig. 5). By 10 days post-inoculation the cilia may be regenerated and the cellular aggregates are almost entirely lymphocytic. Vasculitis, with haemorrhage throughout the tracheal mucosa, is often present in cases of haemorrhagic tracheitis but detailed descriptions of respiratory lesions are very limited. Beard and Easterday (52) demonstrated that, following the aerosol exposure of young chicks to a lentogenic or a velogenic virus, histopathological changes did occur in the tracheal mucous membrane and were equally severe in both cases. However the lesions were of a degenerative and proliferative nature and severe haemorrhagic lesions were not evident. This is in contrast to the lesions seen with other isolates such as the Antrim'73 isolate. Lesions in other respiratory tissues are less common but have been recorded (31, 46, 53). However, as with tracheal lesions, limited studies have been published. Air sacs may be oedmatous and infiltrated with heterophils and lymphocytes. Epithelial cell degeneration and proliferation are evident in severe cases and similar lesions may be found in the nasal cavity and sinuses. Cheville et al. (31) infected birds with the Texas 219 and Florida Largo viscerotropic pathotypes and found lesions in the lungs. Whilst Texas 219 isolate resulted in severe hyperaemia and oedema of the parabronchi, the Florida Largo isolate induced more extensive lesions of haemorrhage and erythrophagocytosis in the deep alveolar areas of the parabronchial tubes.

Reproductive system: Histopathological changes in the ovary are variable, depending upon the duration of infection (47) and were first noted at 5 days post-inoculation. Degeneration was evident in both mature and immature follicles especially between 7 and 21 days post-inoculation. Degenerative changes included vacuolation of yolk material, separation of granulosal cells from the yolk and subsequent necrosis. Haemorrhage occurred into these necrotic follicles. The cortex was infiltrated with eosinophils and a few

heterophils and between 7 and 28 days post-inoculation the infiltration was sometimes so marked as to mask the architectural pattern of the cortex. Large numbers of lymphocytes and plasma cells infiltrated the ovary by 21 to 56 days post-inoculation and appeared to spread out from the cortex to other regions of the ovary. Lymphocytes were also occasionally observed in the sympathetic nerve trunk.

The infundibulum of the oviduct contained lymphocytic aggregates in the loose connective tissue but were most prominent at 28-56 days post-inoculation when egg production was almost back to normal. Heterophils were seen to invade the epithelial lining of the magnum, isthmus and vagina and focal necrosis and cystic dilation of the glands of the magnum and isthmus were also present. In conjunction with these lesions, oedema was present in the submesothelium especially at 7-14 days post-inoculation. In later stages of infection, lymphocytic aggregates were evident. Similar epithelial changes were observed in the uterus but the most marked changes in the oviduct were in the tubular uterine glands. These were widely separated by oedema fluid and haemorrhages were evident in the glandular mucosa. Necrosis, cyst formation and atrophy of the glands were observed. As in other regions of the oviduct, lymphocytic aggregates subsequently appeared in the uterus.

Skin and eye: Some strains induce a massive diffuse oedema of the eyelids and subcutaneous tissues of the head and neck. Vascular lesions have included a severe necrotising vasculitis and thrombosis due to hyaline proteinaceous material (8) and presumably account for the oedema fluid accumulation. Hydropic degeneration of epithelium and microvesicle formation have also been described (31).

Other viscera: Histopathological lesions have been recorded in many other organs including the thymus, spleen, bursa of Fabricius, kidney, liver, pancreas, adrenals and heart and have been associated with foci of degeneration or with haemorrhagic lesions (31, 46, 51). Lymphoid tissues frequently exhibit degeneration and necrosis of the lymphocytes. Following infection

by natural routes, the spleen and thymus show focal vacuolation and destruction of lymphocytes in the cortical areas and in germinal centres from 5 days post-inoculation. Similar changes were also observed in the bursa with degeneration being most marked in the medullary region (51). Whilst lesions have been recorded in many viscera, necrotic foci in the spleen are the type most commonly observed.

Whilst it is well recognised from natural outbreaks of disease that the immune status, through prior vaccination, can have dramatic effects on clinical signs and mortality there is only limited work published on pathological lesions (nature and distribution) in immunized birds following challenge with virulent PMV-1 viruses.

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11

PANZOOTIC NEWCASTLE DISEASE VIRUS IN PIGEONS

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INTRODUCTION

Until 1971, no case of natural infection by avian paramyxovirus type 1 (PMV-1) (Newcastle disease virus, NDV) had been observed in pigeons.

From 1971 to 1973, an epizootic of Newcastle disease (ND) decimated aviculture in Europe. During this period, the first cases of natural infection were reported in racing pigeons in Holland, Great Britain, Belgium and Germany (18, 27, 30, 33). It was assumed that the pigeons which were affected by intestinal and nervous disorders had been infected as a result of contact with diseased domestic poultry. The strains of isolated virus were classical velogenic PMV-1 strains (NDV) indistinguishable from the epizootic virus in poultry (33).

In 1980, classical lentogenic PMV-1 strains were isolated from pigeons affected only by respiratory disorders (35). Serological investigations made at this time showed that 7% of Belgian racing pigeons and 19% of French racing pigeons had been infected by the virus but without showing any clinical illness (22, 34, 35).

In 1981, a disease in Mediterranean racing pigeons with clinical signs resembling the neurotropic form of ND was first seen. The earliest published report related to two racing pigeons imported into Belgium from Italy in 1981 (36). The virus isolated from these diseased pigeons was characterised as a PMV-1 strain (37). In 1983, the infection spread rapidly in the racing population

throughout Europe, presumably due to the mixing or birds during races and extensive trade in these birds. By 1984, the disease reached panzootic proportions and since 1985, the infection may be considered as worldwide (5).

PROPERTIES OF THE "PIGEON" PMV-1

Antigenic properties

The virus was first isolated in 1981 from diseased Italian pigeons and was characterised as a PMV-1 strain (36, 36). In 1984, Alexander et al, confirmed the classification of the "pigeon" strains within the serotype 1 of avian paramyxoviruses (1). However, the "pigeon" viruses can be distinguished from more classical PMV-1 viruses by the significantly different titres obtained in haemagglutination inhibition tests (1, 10), the failure of mouse monoclonal antibodies directed against the HN1 epitope of NDV Ulster 2C to inhibit their haemagglutinating activity and a unique binding pattern seen with nine mouse monoclonal antibodies raised against Ulster 2C (1). Two years later, Meulemans et al, showed that polyclonal and monoclonal antibodies directed against the HN and F proteins of chicken velogenic Italien NDV reacted with 21 "pigeon" isolates showing a close relationship between chicken velogenic and pigeon viruses. These authors therefore suggested that pigeon isolates were probably derived from chicken velogenic strains (24).

Biological properties

The "pigeon" PMV-1 strain isolated from Italian pigeons in 1981 and majority of Belgian, French and German strains isolated in 1983 and 1984 were characterised as mesogenic for chickens (16, 17, 37, 40). Alexander et al, (5) have shown that the intracerebral pathogenicity indices (ICPI) in day-old chicks of 51 "pigeon" PMV-1 strains from 15 countries present a fairly compact distribution around the mean of 1.44 whereas the intravenous pathogenicity

indices (IVPI) in six-week-old chickens showed a much more diffuse distribution over a wide range from 0.00 up to 2.44.

"Pigeon" PMV-1 strains thus display some virulence for chickens and Alexander and Parsons (2) demonstrated that "pigeon" isolates increase in pathogenicity by intravenous inoculation when passaged throughout chickens. The existence of velogenic isolates among "pigeon" viruses represent, therefore, a real source of infection for commercial poultry flocks. In 1981 vaccination against NDV infection had been made illegal in Great Britain and the poultry flock in that country was fully susceptible to NDV. During 1984, 23 outbreaks of NDV were confirmed in domestic fowls in Great Britain. Twenty of the 21 viruses isolated from the outbreaks were shown to have identical monoclonal antibody binding patterns to the "pigeon" PMV-1 viruses. In addition, these viruses were not inhibited in HI tests with monoclonal antibodies directed against the HN1 epitope (4, 6).

EPIZOOTIOLOGY

Distribution

The earliest report of the disease suggests it reached pigeons in Europe in 1981 when virus was first isolated from diseased Italian pigeons (36). Appearance of an epizootic of ND in pigeons in the Mediterranean countries was afterwards confirmed (8, 26). It seems probable that infection had been present in Iraq in 1977 but was confused with herpesvirus infection and that the clinical disease was seen in Egypt in early 1981 (19, 25, 36).

Infection spread across continental Europe and Great Britain during 1983-84 and since 1984, in addition to the European countries, PMV-1 infections of pigeons have been reported in countries representing Asian (Japan), Middle East (Iraq, Egypt, Israel), Africa (Sudan) and North America (USA) (1, 5, 6, 28, 31).

Transmission

The incubation period is variable: from a few days to several weeks. In the experimental infection, the incubation period varied from 4 to 18 days. In field outbreaks, new clinical cases may appear in an infected loft up to 5 weeks after the beginning of the disease.

Infected pigeons eliminate virus in the laryngeal secretions and faeces from the second day after infection for 10 to 15 days (10, 40). Infection can thus be transmitted through direct and indirect contact with oro-nasal secretions and faeces even during the incubation period. it has been shown that in experimental infection of pigeons with velogenic NDV, virus persists for not more than 3 weeks in the intestine and 5 weeks in the brain (33). After they have been ill for 6 weeks, pigeons may be considered as no longer carriers of virus and thus are unlikely to transmit the infection. Important mechanical vectors are men and contaminated objects (baskets, trucks).

Under natural conditions, the virus may be carried through the air in the form of virulent dust propelled by the wind. It has been demonstrated that NDV will survive for period of six months or more in avian faeces under normal temperatures (21).

Epizootiological tracing of the first outbreaks of infection with "pigeon" virus in domestic fowls in Great Britain revealed that constituents of the rations fed to the birds came from stores in Merseyside docks that were infested with diseased pigeons. PMV-1 viruses were isolated from pigeon carcases taken from the food and from samples of the food itself (3, 4, 6).

DISEASE SIGNS, PATHOLOGY

In the classical clinical picture, the intestinal signs appear first and are followed by nervous signs. In the current form of the disease, the respiratory and ocular symptoms are practically non-existent. The intestinal disorders on the other hand are very

marked. They are expresed as watery or haemorrhagic diarrhoea. Diseased pigeons will consequently show a marked increase in water consumption.

If pigeons become infected during moulting, remiges or coverts may be badly-grown, barbes and barbules may be poorly developed and feathers may be broken.

The nervous disorders are very characteristic (Fig 1): tremor of the head, torticollis, paralysis of one or both wings and/or of the legs, disordered balance (tottering step, tendency to fall over backwards or on the side), disordered vision (pigeons peck alongside the grains of the food).

Less typical forms of the disease may be observed such as the initial appearance of nervous disorders without any previous diarrhoea; or the appearance of diarrhoea without nervous signs.

Morbidity varies from 30 to 70%. Mortality remains low, scarcely exceeding 10% if uncomplicated by secondary bacterial or parasitic infections. Percentages of morbidity and mortality may, or course, increase or regress in the future depending on the pathogenic capacity of the virus.

At necropsy, few lesions are visible except for catarrhal or haemorrhagic enteritis during the acute phase of the infection.

The main clinical signs reported for egg-laying chickens infected with "pigeon" virus are characteristically egg production problems, beginning with white and soft-shelled eggs and gradually progressing to complete cessation of egg production. Diarrhoea may be present with elevated mortality and nervous signs are seen occasionally. In almost all outbreaks in laying birds spread of the disease is remarkably slow both within a house and from house to house. In infected broiler flocks, diarrhoea is first reported. Disease is noticed as elevated and rising mortality associated with nervous signs consisting of difficulty in moving and eating, leg paralysis, weakness and lethargy (4, 6).

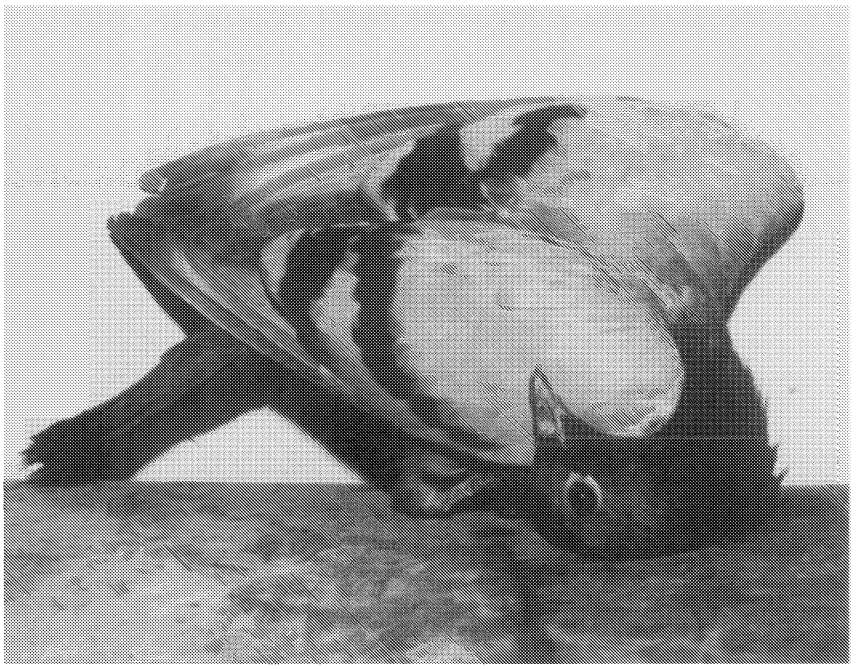

FIGURE 1 : NERVOUS SIGNS ASSOCIATED WITH PANZOOTIC PMV-1 VIRUS IN PIGEONS

Nervous signs consisting of head tremor, torticollis, paralysis of one or both wings or legs, imbalance and inco-ordination were characteristic of the disease. Birds frequently appeared healthy until disturbed when nervous signs become apparent.

DIAGNOSIS

The aetiologic diagnosis can be obtained in the laboratory by virus isolation or by titration of specific antibodies.

"Pigeon" PMV-1 strains can be distinguished from classical NDV strains by differences between titres obtained in haemagglutination inhibition reactions, especially using pigeon antisera instead of conventional chicken antisera (10).

PROGNOSIS

Pigeon-fanciers are apt to dispose of their sick pigeons once paramyxovirosis has been confirmed in the pigeon-lofts. This is a mistake if valuable birds are affected. Pigeons affected by nervous disorders, even if very marked, may return to health after a convalescence of 2 to 6 months, provided they are fed and watered. If necessary, the pigeon-fancier will have to hand feed them with soaked grains and administer water. After they recover, these pigeons will retain their sense of direction and retain some value for sport. We personally know several fanciers who won a number of prizes in 1984 with pigeons which showed severe nervous disorders at the end of 1983, but were kept alive by daily attention.

However, some pigeons may suffer from chronic enteritis for several months (persistent diarrhoea) and consequently their racing performances will be diminished.

PROPHYLAXIS

Early reports of pigeon PMV-1 infection have suggested that protection could be conferred on racing pigeons by standard chicken NDV-live or -inactivated oil-emulsion vaccines (16, 17, 23, 32, 35) but results are difficult to compare because of the challenge route and the virus strain used. The authors have examined protection afforded by live vaccines (La Sota and B1) and oil-emulsion inactivated vaccines using either intranasal/ocular, contact or parental

routes of challenge.

The first attempts to vaccinate pigeons against NDV infection were made by Lüthgen (23) using inactivated virus parentally injected or the La Sota strain administered by drinking water or aerosol. Only pigeons vaccinated with inactivated virus showed a seroconversion and were protected against an intramuscular challenge with a virulent NDV strain.

Since then (23, 32, 34, 35), it has been demonstrated that, for pigeons, NDV-like vaccines such as La Sota must be administered by means of nasal or eye drops and each birds has to be vaccinated with minimum 10^7 EID_{50} of virus. Seroconversions will remain very weak and duration or protection must not be expected to exceed 2 months. Administration of the vaccine in drinking water gives hazardous results. Alexander et al, (7) have also shown that 2 doses of live NDV-B1 vaccine given 4 weks apart induced a poor immune response and little protection from challenge with a "pigeon" virus injected intravenously. Moreover, it has been proven that lentogenic NDV strains display some virulence for pigeons and are excreted for several days after infection. Inoculated pigeons may develop conjuctivitis, mild respiratory disorders and play a role in the dissemination of NDV lentogenic strains (34, 35). For all these reasons, in our opinion, vaccination by means of live-NDV does not meet the pigeon-fanciers requirements and inactivated vaccines are preferable for racing pigeons.

In Great Britain, an oil-emulsion inactivated vaccine based on the Ulster 2C strain (OEa) was licensed for use in pigeons in 1983 (7). In the same year, an inactivated aqueous-suspension vaccine (ASa) was developed for pigeons (10, 38). This vaccine contains purified inactivated La Sota strain suspended in an aqueous adjuvant (10^9 EID_{50}/0.2ml; 0.2ml pro dose 1). In laboratory vaccination trials, after subcutaneous injection of one ASa dose, antibodies were detectable after 7 days, reached their maximal titres after 3 weeks and persisted 12 months. Significant differences were observed between the antibody titres according to whether the virus strain used in the haemagglutination inhibition reactions was

a classical or a "pigeon" PMV-1 strain. Titres were one or 2 $\log_2$ less when reactions were performed against "pigeon" virus. Ninety five to one hundred percent of ASa vaccinated pigeons resisted a challenge performed 1, 6 and 12 months after the vaccination with a "pigeon" PMV-1 strain administered intramuscularly. In comparison with control pigeons, vaccination also significantly reduced the virus shedding in the laryngeal secretions and the faeces after challenge (10, 11, 13).

The efficacy of the ASa vaccine was compared to that of 3 other inactivated vaccines (12): ASb (an aqueous-suspension vaccine based on the Terumo strain, a velogenic Italian chicken NDV strain), OEa (based on the Ulster 2C strain) and OEb (a standard chicken oil-emulsion vaccine based on the Poletti strain). In single-dose experiments (ASa and b: 0.2ml; OEa and b: 0.5ml), it has been shown that the immune response measured by HI tests was the highest with the 0.2ml ASa dose. In comparison with control pigeons, the different vaccine procedures significantly reduced virus shedding in the laryngeal secretions and in the faeces after an intramuscular challenge performed one month postvaccination but morbidity/mortality rates were lower in groups vaccinated with the AS vaccines than with the OE vaccines. No challenged ASa vaccinated pigeon developed clinical signs and only one ASb vaccinated bird presented nervous signs, whereas morbidity/mortality reached 30 and 35% in the OE vaccinated groups. These results show that better protection is afforded by the inactivated aqueous-suspension vaccines. The vaccine prepared from NDV-La Sota strain performed very well, even though La Sota virus may be distinguished from the "pigeon" isolates with monoclonal antibodies prepared against tne NDV-Ulster 2C strain (1, 29) and even if polyclonal and monoclonal antibodies directed against the HN and F proteins of chicken velogenic Italien NDV react with all pigeon isolates showing a closer relationship between chicken velogenic and pigeon viruses than the latter and La Sota (24). Alexander et al, (7) have suggested that the degree of protection given by any particular vaccine may vary with the number of antigenic sites shared with the challenge virus and that the

extent to which vaccine and challenge virus share epitopes involved in neutralising may reflect the ability of that vaccine to protect against the challenge virus. However, it is not clear how many sites on the virus particle may be involved in the neutralising process. The results of Duchatel and Vindevogel (12) suggest that, in practice, severe challenge remains the most stringent test of immunity and it is not evident that standard inactivated NDV vaccines normally used in chickens are good candidates for vaccinating pigeons as suggested by Meulemans et al, (24).

In conclusion, one 0.2ml ASa dose affords good protection for one year from a "pigeon" virus intramuscular challenge. In contrast one 0.5ml OEa or OEb dose is not sufficient to induce high antibody levels and resistance to a severe challenge is inferior to that induced by the ASa vaccine. As shown by Alexander et al, (7) and Box et al, (9) good protection with OEa requires two doses given 4 weeks apart. Young pigeons have to be vaccinated when 4 and 8 weeks old and they may thus be infected during this period. In contrast, within 10 days of injection of one 0.2ml ASa dose, pigeons are protected from an intramuscular "pigeon" virus challenge (15).

A homologous inactivated oil-emulsion vaccine derived from the cloned pigeon strain PMV-1/pigeon/Munich/14/83 has been recently developed in Germany (20). But so far, very few results are available to compare the efficacy of that vaccine to ASa and OEa vaccines. One subcutaneous injection of the inactivated oil-emulsion homologous vaccine (0.25ml) or 2 injections at 4 weeks interval should confer protection for one year.

There is some evidence that inactivated oil-emulsion vaccines are not well tolerated by racing pigeons (39, 41). After a first subcutaneous injection, one pigeon in 10,000 may die of shock and 1.5% of birds may develop a granuloma at the site of injection. In addition oil-emulsion vaccine injection may be followed by reactivation of herpesvirus (Columbid herpesvirus 1) which latently infects the majority of pigeons (39). In contrast, subcutaneous injection of the aqueous-suspension ASa is painless and does not induce any

secondary reaction, mortality by shock, granuloma forming, moulting and egg-laying problems, or any decrease in flying performances (10).

TREATMENT

No specific treatment for PMV-1 infection of pigeons exists but symptomatic treatment may help the birds to recover.

Pigeons affected by diarrhoea will have to be rehydrated by the addition of electrolytes to their drinking water. Their feed should include a vitamin A, B, C supplement and essential amino acids.

Duchatel and Vindevogel (14) have shown that vaccination with the inactivated aqueous suspension ASa of previously PMV-1 infected pigeons has no beneficial but no disadvantgeous effects on the disease while vaccination with live-La Sota significantly increases both viral excretion in the faeces and the number of pigeons with diarrhoea at the beginning of the clinical phase of the infection. Consequently, when first clinical signs are appearing in an infected loft, vaccination with ASa may be attempted without heightening the disease. As in natural infection new clinical cases may appear up to 5 weeks after the beginning of the disease and since a single ASa dose has been shown to afford good protection after 10 days, a beneficial effect may be obtained in some animals.

The severity of the infection will also depend on the general health of the pigeons and the presence of any other diseases in the pigeon loft such as salmonellosis, trichomoniasis, coccidiosis, infestation with worms. If present these secondary bacterial or parasitic infections have to be treated.

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12

NEWCASTLE DISEASE IN FREE-LIVING AND PET BIRDS

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INTRODUCTION

Newcastle disease (ND) has economic and ecologic impact on pet and free-living as well as on domestic birds. Virtually all of the approximately 8,000 species of birds seem to be susceptible to infection with Newcastle disease viruses (NDVs).

A precise and detailed review of the abundant literature published since the first report on ND by DOYLE (54) is impeded by several obstacles:

Casuistic reports contain quite frequently only anecdotal information on clinical signs and pathological lesions. Transmission experiments using the same species of birds from which the isolate was recovered are either not performed at all or are done with birds of unknown immune status with respect to NDV and/or other agents.

Quite a number of articles have been published in languages for which an authorative translation is not easily available. This review will deal with publications in the official languages of the World's Veterinary Poultry Association. If available, English abstracts of papers in other languages were also considered.

The identification of affected birds is sometimes given only as the vernacular name or specified in latinized names and/or systematic categories, which are no longer in use or have been modified often since the publication of original papers. Nomenclature and taxonomy of birds has been subject to change in the past and probably will be modified in the future. Volatile terminology of names and taxons creates difficulties in asigning hosts to current established systematic categories. This paper makes use of the systematic list of birds published by WOLTERS (241).

Strains which are highly pathogenic for the domestic chicken are evaluated in most of the reports. It is only in the last two decades that NDVs are described which are not pathogenic for chickens or any other species of birds. These viruses were mostly obtained within the frame work of avian influenza A surveillance studies.

Many papers contain limited experimental data which fail to unequivocally prove that the isolated agent is indeed NDV, eg. paramyxovirus (PMV)-1 and not a member of the other proposed serotypes 2 to 9.

Most of the early isolates are lost or have been passaged unknown times in ill-defined culture systems making a detailed and meaningful re-examination with currently available techniques impossible. This fact demands again the foundation and maintenance of an internationally operating reference laboratory.

Despite of these shortcomings it is attempted in this review to summarize briefly the epizootiology and the main characteristics - with respect to the host species of birds - of the various clinical forms and pathological manifestations of ND in birds. For questions relating to virus isolation and characterization, disease prevention and control by immune prophylaxis the reader is refered to the respective chapters of this monograph.

EPIZOOTIOLOGY

Significant spread of NDV may occur along three major routes: (a) Movement of live domestic poultry, poultry products (meat, eggs, feathers etc), offal and manure; (b) movement of pet and captured free-living birds; (c) intra- and intercontinental migration of birds.

In exporting countries, captured free-living birds may be temporarily kept in contact with domestic chickens and waterfowl. This enables mutual transmission of NDV and spread/shedding along the major routes of export. Increased NDV-associated mortality soon after arrival at port of destination indicates that infection probably occured just prior to departure of these birds.

Several reports suggest that the duration of virus excretion depends on both (patho)types of NDV and species/group of birds. Virus shedding is usually a short episode in galliforme and some species of song birds (127) but might last several to many months in psittacines (137).

In Columbiformes and some Passeriformes the kidneys seem to be chronically infected which results in long-lasting shedding of NDV with the excreta of the kidneys. However, examination of whole droppings or cloacal swabs does obscure this observation. Upon necropsy examination of individual specimes is superior to a heterologous mixture of various tissues.

In the literature, most attention is devoted to NDV which might be, or is in fact, pathogenic for chickens. Much less information is available on the epidemiology of meso- and lentogenic NDV. Within the framework of avian influenza A surveillance studies many PMV-1 strains have been isolated from obviously healthy individuals. Most, but not all, of these isolates seem to be apathogenic for chickens which are considered most susceptible among the species of domesticated birds.

Recent evidence suggests that defined species or groupings of birds harbour predominantly distinguishable strains of NDV. RUSSELL and ALEXANDER (184) used a panel of monoclonal antibodies to differentiate 40 strains of different origin into eight groups. These strains appeared identical using polyclonal antisera and conventional methods.

The survival of NDV in the environment is influenced by the kind of strain and the physical and chemical properties of the surrounding material. In general, NDV is not considered as a very resistant virus and its infectivity can be readily destroyed by commonly used disinfectants.

Epizootiology of NDV in free-living birds

Free-living birds developed a number of different behavioural traits which can be generally interpreted as means to escape or prevent infection with various agents.

Migration between continents or different regions within a continent, results (a) in separation from an environment which could be contaminated with potentially infectious droppings and (b) the physical strain of migration separates healthy birds from the weakened or diseased, the latter being captured and eliminated by predators.

Upon arrival in the breeding habitat birds tend to separate from each other and try to establish and defend their territory for the breeding time. At pairing time birds seem to select an appropiate, healthy partner. Prior to mating two birds get close contact by mutual pruning and other rituals. In most species this phase is long enough to exchange/transmit infectious

agents which may be present and to produce antibodies against them. In case of synthesized humoral antibodies these will be transmitted via yolk to the offspring.

The tendency of birds to build a new nest each year or to use nests made in the preceding year by birds of another species can be interpreted as an evolutionary development to avoid contact with potentially contaminated material.

Birds take care to remove the droppings of their offspring from the nest site thus preventing contamination of the young. In addition, parents rigorously remove underdeveloped weakened offspring from the nest.

The feeding time of young nestlings coincides exactly with that period in which abundant mainly proteinaceous food is available. This enables the parents to provide the offspring with food of a high level of protein and energy but with a low level of undigestable fibres etc.

Fledglings circulate with their parents within their territory and apparently avoid living for long periods at the same site.

All this species-specific behaviour can be interpreted as evolutionary strategies for better survival by escaping infections and by strengthening or maintaining health due to appropriate and constant food supply.

The rapid inactivation of NDV by ultraviolet light precludes accumulation of infectivity in natural habitats. This fact, in combination with the various inherited behavioural strategies for better survival, results in an extremely low incidence of NDV in natural habitats of birds.

In contrast, caging of free-living birds results in a breakdown of the inherited behavioural mechanisms making accumulation of infectious agents more likely and build-up of a high infection pressure possible. Therefore NDV occurs more often in captive birds than in birds of the same species in nature.

Epizootiology of NDV in captive birds

The majority of outbreaks of ND occurs in birds kept in captivity (quarantine, exotic bird parks, zoological gardens, private bird collections etc). Frequent characteristics for these establishments are (a) high population density of one or more species; (b) constant flow of arriving and departing birds; (c) few large and open feeders furnished with food in abundance; (d) few open drinkers; (e) accumulation of droppings which are ac-

cessable to birds on the floor; (f) care-takers move their appliances from one unit to the other; (g) flow of air and water from unit to unit is not prevented. Unlike in domestic poultry rearing, hygiene and sanitary conditions are often questionable. This results in easy introduction and unnoticed spread of NDV within and between these establishments.

General health and natural resistance to infection are also compromised by social stress. Lack of appropriate environmental and social stimuli narrows down the inherited behaviour to merely survival. In most cases regular pairing, nesting, mating, egg laying and breeding do not occur. Instead, abnormal traits occur which are indicative of frustrated behaviour (stereotype motion, feather pecking etc).

Continued stress, both environmental and social, results in debilitation of resistance and enhanced susceptibility to a large variety of infectious agents. In conclusion, both environmental/social factors and suppression of inherited behaviour contribute to the high prevalence of ND in caged birds.

The source of NDV is, in most cases, connected with introduction of latently-infected, virus-shedding birds. Circumstantial evidence suggests that newly captured birds acquire their infection with NDV in collecting centres, during transport and transit or in quarantine stations. Many countries in which birds are trapped for export purposes have a history of ND in fowl kept in back-yards and maintained in close vicinity to birds for export. Massive losses in newly imported birds point in that direction.

The frequency of reported isolations of NDV from pet and free-living birds parallels that of the domestic chicken. Also, the geographic location of isolations coincides for domestic and wild birds. The panzootic ND in 1969/73 in Europe and North America was linked to imported virus-excreting parrots from some South American countries. Anecdotal evidence suggests that these parrots acquired their NDV from infected back-yard chickens in local collecting centres in these countries.

CLINICAL SIGNS

Evaluation of clinical signs of ND requires, as a paramount prerequisite, fundamental knowledge not only of species-dependent, but also of sex-, age- and environment-associated normal behaviour. General activity varies with seasons of the year as well as periods of the day and weather conditions. Some birds display the highest level of activity at early morning, some at high noon, some at dawn or at night. Feeding-associated

behaviour is also characterized by great variability.

Of the 50 orders described by WOLTERS (241) natural or experimental exposure to various strains of NDV has been described in 27 orders, yet birds of the other orders where ND has not been recorded might be susceptible.

Table 1 contains data on all available species with a record of ND along with their taxonomic status in the class Aves and in brief information on the mode of infection (natural or experimental) as well as clinical signs and pathological lesions observed by the authors. The host range of NDV represents approximately 236 species of pet and free-living birds in addition to domestic avian species (chicken, turkey, goose, duck and pigeon).

A comparative evaluation of the progression of ND in birds of different species shows a great variability of clinical manifestations. The severity of signs may differ within species of the same genus. In general, families and/or orders of birds tend to display a similar degree of susceptibility to infection and to disease. For practical reasons the following descriptions of major clinical signs are sublimated from the reports published. Only the frequently examined orders are considered.

The development of clinical signs of ND in pet and free-living birds parallels that in domestic fowl. This means that any form of ND occuring in chickens may be observed in other species of birds. In general, degree, duration and outcome of the disease depend on (a) pathotype, dosage and way of entry of the virus, (b) age and age-associated natural resistance, (c) level and type of immunity, if any, and (d) degree and duration of environmental and social stress.

A high level of susceptibility can be found in Phasianiformes (which include gallinaceus birds) but also in Psittaciformes, Struthioniformes (e.g. ostrich) and Columbiformes. A somewhat lesser level of susceptibility occurs in birds of the Sphenisciformes (penguins), Strigiformes (owl), Falconi- and Accipitriformes (falcons and eagles), Ciconiiformes (storks), and the large group of Passeriformes (sparrows and song birds). As the least susceptible group Anatiformes (waterfowl), Cuculiformes (koels), Pelecaniformes (shags), Lariformes (gulls), Ralliformes (coots) and Cariamiformes (cranes) are to be mentioned. However, this grouping mirrors more a tendency than a valuable prediction of susceptibility.

Following natural or experimental infection with NDV pathogenic for susceptible chickens almost all birds will respond more or less with general signs such as apathy/lethargy and anorexia for various periods of time. More severe signs are usually associated with defined taxons of birds (Table 1).

All birds which lay coloured or tinted eggs react upon infection by NDV with the formation of shells with pale to white colour. Thinned shells with fragile shell membranes are associated with pronounced clinical signs.

Enteritis due to inflammatory reactions of the intestine is quite common. Watery discharge due to renal failure can be misinterpreted as enteritis. In addition, faeces vary from liquid to solid; carnivorous and fish-eating birds excrete more urates than birds feeding on vegetable matter.

Functional disorders of the central nervous system or peripheral nerves begin with abnormal motion or flight and may result in complete lameness of wings and legs. Abnormal position of the neck and head is visible. Opisthotonus und torticollis represent final stages of the disease.

PATHOLOGICAL LESIONS

The severity of lesions does not necessarily correspond to the degree of clinical signs. Depending on duration of anorexia the general condition of the carcass varies from good to moderate to poor. Prominent petechia and haemorrhages are present in gallinaceous birds, ostriches and in some of the psittacines, falcons, storks and passerines (Table 1). The same birds develop intestinal button-like lesions or pseudomembranous to diphtheric alternations in the gut. In more acute cases an enlargement of liver, spleen and kidney is to be found.

Histopathologically a more or less distinct disseminated non-purulent encephalomyelitis and degenerative changes in peripheral nerves are present. In lame birds loss of axon sheets and perivascular cellular infiltrations are likely. Focal spongiform changes are frequent in the brain and ventral roots of the medulla.

Clinical signs as well as gross and microscopic lesions suggestive of ND need confirmation by virus isolation and/or serology. In chronic cases virus isolations tend to be negative. However, antibodies can be detected in

serum or blood clots (obtained from heart or large blood vessels) using the haemagglutination inhibition test.

EXPERIMENTAL ND

A limited number of transmission experiments with psittacine, passerine and other arbitrarily selected birds are described (Table 1). The results of these trials seem to confirm the observations made on spontaneously infected birds in terms of clinical signs, gross and microscopic pathology, sero-conversion and virus excretion. Parenteral inoculation of high doses of NDV which was isolated either from diseased chickens or captive birds results in an accellerated course of the disease and higher mortality as compared to natural exposure.

The limitations of such experiments are associated with the unnatural route of virus inoculation, unappropriate, mostly overcrowded housing and lack of species-specific feeding of the birds. In addition, the immune status to NDV and the immune responsiveness was not always determined at the beginning of the experiments.

OBSERVATIONS AND INTERPRETATIONS

In this contribution the authors have used the list of birds published by WOLTERS (241) for nomenclature and taxonomy. WOLTERS himself points out that his list (and any other) is in need of improvement in many aspects of phylogeny and true mutual relations of species, genera, families and orders. The list used in this study, however, allows the conclusion that birds of some taxons tend to be more susceptible to clinically overt ND than others.

Considering the natural habitat of birds in respect to ND susceptibility, another remarkable facet is evident. Birds living in close contact to sea or fresh water tend to be quite resistant to pathogenic NDV. This fact could be explained on the basis of a phylogenetic acting selection pressure on birds. This hypothesis is only valid if the presence of NDV during the evolution of the birds is anticipated. Since NDV can survive for prolonged times in sea and fresh water a significant exposure of birds seems to be likely.

Also connected with the habitat of birds and ND susceptibility is the predominant type and source of food. Fish and marine animal-eaters seem to resist NDV infection more easily in comparison to birds which live main-

ly on seeds, grain, fruit and other vegetable matter. Omnivorous birds in particular tend to succumb to ND more than others.

Gregarious birds which form temporary or permenant groups, flocks or other units are more likely to acquire ND than solitary living birds. Social communication (mutual pruning, mating rituals and use of places for feeding and resting common to all) facilitates virus transmission and persistance in such groups.

Ancient literature unfortunately makes no mention of diseases with characteristics of ND, although various species of birds populated rural and municipal settlements for centuries in high numbers. Although in those days birds played an important economic role as sources of meat, eggs, feathers (for writing utensils and temperature insulation) etc, reports on diseases suggestive of ND in birds are rare and usually vague. The lack of publications probably reflects more the ranking of avian science in the veterinary profession than the actual health situation of poultry.

CONCLUDING REMARKS

The wealth of reports on ND in free-living birds suggests, at first glance, that virtually all species are susceptible to infection. However, of the approximately 8,000 known species only about 236 species, about 2.5 %. have a record of NDV isolation.

Field ornithologists face little, if any, difficulties in species determination. In contrast, laboratory veterinarians and personnel in diagnostic centres may have problems in exact determination of species or genera. In view of this possiblity, some reports contain more vague terms like pigeons, doves, macaws, amazones, birds of prey, waterfowl etc.

Avian paramyxoviruses were regarded for a long time as a serologically uniform group of viruses differing only in virulence for chickens. It appears to be likely that some of the early isolates of "NDV" were in fact viruses of the other serotypes which are now recognized. Re-examination of these viruses including the application of monoclonal antibodies directed to various epitopes of the virion appears to be desireable.

ACKNOWLEDGEMENT

The Head of the main library of the School of Veterinary Medicine Hannover, Bibliotheksdirektor Dr. K. Baresel, and his cooperative staff members are duly honored for providing access to even remote literature.

Table 1. Host range of NDV in free-living and pet birds

Explanation of symbols.

Mode of infection. N - natural, E - experimental.

Clinical sings	Lesions
- no data	- no data
+ unspecified clinical signs	+ unspecified lesions
N no clinical signs	N no lesions
G general clinical signs (such as anorexia, lethargy of apathy)	A emaciation H haemorrhages
R respiratory signs	R rhinitis, conjunctivits, sinusitis, tracheitis, pneumonia, aerosacculitis
C CNS signs	C non-purulent encephalomyelitis
E enteric signs	E enteritis
() rare	() rare

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Struthioniformes / Struthiones			
Struthionidae			
Struthio camelus, Ostrich	N	- / -	Placidi & Santucci, 1954 b (172)
	N	G C / H	Kauker & Siegert, 1957 (114)
		G C / -	Klöppel, 1963 (121)
		C R / -	Corrado, 1966 (47)
	N	CE / HRE	Kaleta, cit. Alexander et al., 1987 (9)
Struthioniformes / Rheae			
Rheidae			
Rhea americana, Lesser Rhea	N	C / -	Estudillo, 1972 (63)
	N E	GCRE/HCRE	Nunes et al., 1975 (153)
		E(RC)/ -	López, 1976 (132)
Cariamiformes	N	- / -	Placidi & Santucci, 1954 b (172)
Gruiformes			
Gruidae / Gruinae			
Grus antigone, Sarus Crane	N	C / -	Estudillo, 1972 (63)
Grus canadensis, Sandhill Crane	N	C / -	Estudillo, 1972 (63)
	E	- / -	Vickers & Hanson, 1979 (221)
Anthropoides virgo, Demoiselle Crane	N	C / -	Estudillo, 1972 (63)
	N	GRE/ -	Kaleta & Marschall, 1981 (108)
Gruidae / Balearicinae			
Balearica pavonina, Crowned Crane	N	+ / +	Kauker & Siegert, 1957 (114)
	N	C / -	Estudillo, 1972 (63)
Ralliformes			
Rallidae / Himantornithinae			
Gallinula chloropus, Moorhen	N	- / -	Hinshaw et al., 1985 (94), Manjunath & Mallick, 1981 (145)
Fulica gigantea, Giant Coot	N	- / -	Pierson & Pfow, 1975 (170)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Ralliformes			
Rallidae / Himantornithinae			
Fulica americana, American Coot	N	- / -	Pearson et al., 1975 (164)
	N	- / -	Vickers & Hanson, 1982 a (222)
	N	- / -	Hinshaw et al., 1985 (94)
Fulica atra, European Coot	N	- / -	Chandra et al., 1973 (41); Hakim et al., 1979 (85)
	N	- /-	Ahmed et al., 1980 (4)
Podicipediformes			
Podicipedidae			
Aechmophorus occidentalis, Western Grebe	N	- /-	Hinshaw et al., 1985 (94)
Dytes auritus, Slavonian (Horned) Grebe	N	- / -	Hinshaw et al., 1985 (94)
Jacaniformes			
Jacanae / Jacanidae			
Irediparra gallinacea, Comb-crested Jacana	N	- /-	Alexander et al., 1986 (8)
Charadriiformes / Scolopaces			
Scolopacidae / Eroliinae			
Calidris, Sandpiper	N	- / -	Hinshaw et al., 1985 (94)
Heteropygia acuminata, Sandpiper	N	- / -	Alexander et al., 1986 (8)
Scolopacidae / Gallinagininae			
Limnodromus scolopaceus, Long-billed Dowitcher	N	- / -	Hinshaw et al., 1985 (94)
Scolopacidae / Tringinae			
Tringa totanus, Common Redshank/ Eastern Shank	N	- /-	Manjunath & Mallick, 1981 (145)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Charadriiformes / Scolopaces			
Scolopacidae / Tringinae			
Tringa flavipes, Lesser Yellowlegs	N	- /-	Hinshaw et al., 1985 (94)
Charadriiformes / Charadrii			
Haematopodidae			
Haematopus ostralegus, Oystercatcher	N	- / -	Blaxland, 1951 (27)
Vanillidae			
Hoplopterus spinosus, Spur-winged Plover	N	N / -	Clubb et al., 1980 (45)
Erythrogonys cinctus, Red-kneed Dotterel	N	- / -	Alexander et al., 1986 (8)
Charadriidae			
Elseyornis melanops, Black-fronted Dotterel	N	- / -	Mackenzie et al., 1984 (139); Alexander et al. 1986 (8)
Lariformes / Lari			
Laridae			
Larus argentatus, Herring Gull	N	- / -	Blaxland, 1951 (27)
Ichthyaetus ichthyaetus, Great Black-headed Gull	N	- / -	Blaxland, 1951 (27)
Chroicocephalus ridibundus, Black-headed Gull	N	- / -	Tumova et al., 1984 (216); Telbis, 1986 (212)
Sternidae / Sterninae			
Sterna fuscata, Sooty Tern	N	- / -	Mackenzie et al., 1984 (139); Alexander et al., 1986 (8)
Sternidae / Anoinae			
Anous stolidus, Brown (Common) Noddy	N	- / -	Mackenzie et al., 1984 (139); Alexander et al., 1986 (8)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Lariformes / Lari			
Sternidae / Anoinae			
Anous tenuirostris, Lesser Noddy			Alexander et al., 1986 (8)
Alciformes			
Alcidae			
Uria aalge, Guillemot (Murre)	N	- / -	Blaxland, 1951 (27)
Gaviiformes			
Gaviidae Diver, Loon	N	- / -	Blaxland, 1951 (27); Alexander et al., 1987 (9)
Sphenisciformes			
Spheniscidae			
Aptenodytes patagonicus, King-Penguin	N	GR / HR	Krauss et al., 1963 (125)
Pygoscelis adeliae, Adelie Penguin	E	- / -	Pierson & Pfow, 1975 (170)
Pelicaniformes / Pelecani			
Phalacrocoracidae			
Phalacrocorax carbo, Black Cormorant	N E	N / -	McPherson, 1956 (140)
		+ / -	Lancaster, 1977 (127)
Phalacrocorax aristotelis, Shag	N	- / -	Blaxland, 1951 (27)
Sulidae			
Morus bassanus, Gannet	N	- / H	Wilson, 1950 (236)
Columbiformes			
Gouridae			
Goura cristata, Crowned Pigeon	N	C / -	Estudillo, 1972 (63)
Columbidae			
Zenaidura macroura, Mourning Dove			Jezierski, 1950 (104)
	N	- / -	Pearson & McCann, 1975 (165)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Columbiformes			
Columbidae			
Geopelia striata, Zebra-Dove		- / -	Collier & Dinger, 1950 (46); Adler et al., 1951 (1)
Streptopelia chinensis, Spotted Turle-Dove	N	- / -	Kraneveld & Mansjoer, 1950 (124)
	N	- / -	Pearson & McCann, 1975 (165)
	N	C R / -	Hanson & Sinha, 1952 (86)
	E	- / -	Magid et al., 1965 (142)
Streptopelia senegalensis, Laughing Dove (Senegal Turtle-Dove)	E	+ / -	Kaschula, 1950 (111)
	N	C R / -	Hanson & Sinha, 1952 (86)
	E	C / +	Zied et al., 1981 (244)
	N	- / -	Mousa et al., 1982 (152)
Streptopelia turtur, Turtle-Dove	E	N / -	Kaschula, 1950 (111)
	E	N or C / -	Placidi & Santucci, 1954 a (171)
	N	- / -	Pierson & Pfow, 1975 (170)
Streptopelia capicola, Cape Turtle-Dove	E	N / -	Kaschula, 1950 (111)
Streptopelia semitorquata, Red-eyed Dove	N	C R / -	Hanson & Sinha, 1952 (86)
Streptopelia humilis, Dwarf Turtle-Dove	N	C R / -	Hanson & Sinha, 1952 (86)
Streptopelia risoria, Barbary Dove	E	C / -	Fenstermacher et al., 1946 (66)
	N	C R / -	Hanson & Sinha, 1952 (86)
	N	- / -	Pearson & McCann, 1975 (165)
Columba livia, Rock Pigeon	N	C E / -	Iyer, 1939 (101) Beach, 1942 (22)
	E	N / -	Fenstermacher et al., 1946 (66)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Columbiformes			
Columbidae			
Columba livia, Rock Pigeon	N E	(C)/ -	Schyns & Florent, 1951 (194)
	N	C R / -	Hanson & Sinha, 1952 (86)
	E	N or - / -	Kaschula, 1952 (113)
	E	N or C / -	Placidi & Santucci, 1954 a (171)
	N	+ / -	Placidi & Santucci, 1954 b (172)
	N E	GCER / N	Marastoni & Sidoli, 1959 (148)
	E	C / -	Reuss, 1961 (180)
	E	- / -	Olah & Palatka, 1963 (155)
	N	G C / H	El-Dahaby & Sokkar, 1967 (58)
	N E	- / -	Ahmed & Sabri, 1969 (3)
		- / -	Kogo et al., 1969 (122)
	N	G C / -	Stewart, 1971 (209)
	N	C / N	Richter & Goosens, 1971 (181)
	N	G C / N	Maes et al., 1974 (141)
	N E	C / -	Greuel et al., 1975 (78)
	N	- / -	Pearson & McCann, 1975 (165)
	E	N / -	Smit, 1975 (203)
	E	G C / H	Erickson et al., 1979 (62)
	N	GCER/(H)ER	Canic, 1981 (37)
	N	G C / -	Eisa & Omer, 1984 (57)
	N	- / -	Alexander, 1987 (9)
Palumbus palumbus, Wood Pigeon	E	N or C / -	Placidi & Santucci, 1954 a (171)
Myristicivora bicolor, Pied Imperial Pigeon	E	- / -	Kaleta & Marschall, 1981 (108)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Columbiformes			
Columbidae	E	C(R)/ +	Gomez, 1930 (76)
- Pigeons & Doves (without species names)	N	- / -	Konno et al., 1929 (123); Farinas, 1930 (64)
	N	GCE / -	Picard, 1934 (168); Marastoni & Sidoli, 1959 (148)
	N	C / -	Iyer, 1939 (101)
	E	C E / -	Iyer, 1939 (101)
	E	C / -	Dinter, 1944 (51); Kaschula, 1951 (112) Hupbauer, 1944 (99) Haddow & Idnani, 1946 (84)
	E	- / -	Malbrant, 1942 (144); Popovic, 1951 (176)
	E	- / -	Sassenhoff-Gylstorff, 1953 (191)
	E	CR / -	Hanson & Sinha, 1952 (86)
	N	+ / -	Placidi & Santucci, 1954 b (172)
	E	N(C)/ -	Walker et al., 1954 (232)
	N	- / -	Vrtiak, 1958 (230)
	E	GCE/ -	Zoletto, 1958 (245); Marastoni & Sidoli, 1959 (148)
	E	N or C / -	Zoletto, 1958 (245)
	E	N / -	Ahmed & Reda, 1967 (2); Smit, 1975 (203)
	N	- / -	Vindevogel et al., 1972 (226)
	N	- / -	Utterback & Schwartz, 1973 (220)
		- / -	Faddoul, 1974 (65)
	N	- / -	Burridge et al., 1975 a (34); López, 1976 (132)
	E	- / -	Jurcina & Michalov, 1980 (106)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Columbiformes			
Columbidae			
- Pigeons & Doves (without species names)	N	- / -	Landre et al., 1982 (128); Biancifiori & Fioroni, 1983 (26)
	E	CR / -	Vindevogel et al., 1982 (227, 228)
	N	- / -	Biancifiori & Fioroni, 1983 (26)
	E	R / -	Vindevogel et al., 1983 (229)
	N	GC / -	Eisa & Omer, 1984 (57)
Psittaciformes			
Micropsittinae / Loriculinae, Lovebirds	N	- / -	Grausgruber, 1972 (77)
Agapornis taranta, Black-winged Lovebird	N	- / -	Gylstorff, 1974 (83)
	E	G(C) / -	Lüthgen, 1981 (137)
Agapornis pullarius, Red-faced Lovebird	N	- / -	Malbrant, 1942 (144)
Agapornis personatus, Masked Lovebird	N	- / -	Gylstorff, 1974 (83)
Psittacidae / Forpinae			
Forpus passerinus, Green-rumped Parrotlet	N	- / -	Steger, 1974 (208)
Psittacidae / Aratinginae			
Macaws	N	CRE / -	Estudillo, 1972 (63)
		C / -	Lüthgen, 1981 (137)
		C E / -	Wachendörfer & Lüthgen, 1971 (231)
		GRE /-	Clubb et al., 1980 (45)
		- / -	Smit, 1975 (203); Senne et al., 1983 (196)
	E	GCE / -	Winteroll & Grimm, 1974 (238)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Psittaciformes			
Psittacidae / Aratinginae			
Anadorhynchus hyacinthinus/glaucus Blue Hyacinth Macaw of Brazil		C R / -	Estudillo, 1972 (63)
Ara ararauna, Blue and Yellow Macaw		+ / -	Clubb et al., 1980 (45)
Ara servera, Chestnut-fronted Macaw		- / -	Cavrini & Cabassi, 1960 (39)
Ara chloroptera, Green-winged Macaw	N	- / -	Alexander et al., 1977 (5)
Ara macao, Scarlet Macaw	N	- / -	Pearson & McCann, 1975 (165); Alexander et al., 1977 (5)
		- / -	Lüthgen & Wachendörfer, 1970 (134)
Red Arara Macaw		- / -	Estudillo, 1972 (63)
Guaruba guarouba, Golden Conure	N	N / -	Ehrsam et al., 1975 (56)
Nandayus nenday, Nanday Conure	N	C / -	Clubb et al., 1980 (45)
Aratinga solstitialis, Sun Conure	N	- / -	Smit, 1975 (203)
Psittacines	N	- / -	Francis, 1973 (67) & 1977 (68); Manjunath & Mallick, 1981 (145)
Eupsittula canicularis, Orange-fronted Conure	E	GCR / -	Erickson et al., 1975 (59)
	N	- / -	Pearson & McCann, 1975 (165)
	E	- / -	Erickson et al., 1977 (60)
Eupsittula pertinax, Brown-throated Conure	N	- / -	Clubb et al., 1980 (45)
Pyrrhura molinae, Green-cheeked Conure		C / -	Clubb et al., 1980 (45)
		- / -	Brugh & Beard, 1984 (33)
Myiopsitta monachus, Monk Parakeet		- / -	Pfow, 1972 (166); Walker et al., 1973 (233)
		C / -	Clubb et al., 1980 (45)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Psittaciformes			
Psittacidae / Brotogeryinae			
Brotogeris versicolura/chiriri, Canary-winged Parakeet	N	G / -	Grausgruber, 1972 (77)
	N	- / -	Pearson & McCann, 1975 (165)
Pionipsitta pileata, Pileated Parrot		- / -	Gylstorff, 1974 (83)
Psittacidae / Amazoninae			
Amazona finschi, Lilac-crowned Amazon	N	- / -	Pearson & McCann, 1975 (165)
Amazona viridigenalis, Green-cheeked Amazon	N	GE / -	Cavill, 1974 (38)
	N	- / -	Pearson & McCann, 1975 (165)
Amazona aestiva, Blue-fronted Amazon	N	- / -	Hirai et al., 1981 (96)
	N E	- / -	Lüthgen, 1981 (137)
Amazona ochrocephala, Yellow-crowned Amazon	N	G(C)RE/ -	Cavrini & Cabassi, 1960 (39)
		CR / -	Pohl, 1971 (173)
	N	+ / -	Lüthgen & Wachendörfer, 1970 (134)
	N	GCR/HCRE	Matzer & Mota, 1971 (149)
		- / -	Sperhake, 1974 (207)
		GC / -	Erickson et al., 1975 (59)
	N	- / -	Pearson & McCann, 1975 (165); Alexander et al., 1977 (5)
	E	GC / -	Erickson et al., 1977 (60 a)
	E	- / -	Erickson et al., 1977 (60b); Lüthgen, 1981 (137)
	N	C / H	Clubb et al., 1980 (45)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Psittaciformes			
Psittacidae / Amazoninae			
Amazona farinosa, Mealy Amazon		- / -	Gylstorff, 1974 (83)
Amazona vinacea, Vinaceous Amazon		- / -	Lüthgen, 1981 (137)
-, Amazons	N	CE / -	Lüthgen, 1981 (137)
(without species names)		CE / C	Wachendörfer & Lüthgen, 1971 (231)
	N	N or G/ -	Ehrsam et al., 1975 (56)
	N	GC / -	Erickson et al., 1975 (59)
	N	- / -	Smit, 1975 (203)
	E	GC / -	Erickson et al., 1977 (60 a)
	E	- / -	Erickson et al., 1978 (61)
Pionus maximiliani, Scaly-headed Parrot	N	- / -	Lüthgen, 1981 (137)
Pionus menstruus, Blue-headed Parrot	N	- / -	Clubb et al., 1980 (45)
Psittacidae / Pionitinae			
Pionites melanocephalus, Black-headed Caique	N	- / -	Clubb et al., 1980 (45)
Psittacidae / Psittacinae			
Poicephalus senegalus, Senegal Parrot	E	- / -	Lüthgen, 1981 (137)
Psittacus erithacus, Grey Parrot	N	GR / -	Scott & Winmill, 1960 (195)
	N	G / -	Allan, 1968 (11)
	N	- / -	Lüthgen & Wachendörfer, 1970 (134)
	N	C / -	Wachendörfer & Lüthgen, 1971 (231)
	N	- / -	Grausgruber, 1972 (77)
	N	GCR / -	Clubb et al., 1980 (45)
	N	GE / -	Onunkwo & Momoh, 1980 (156)
	N	GC / -	Lüthgen, 1981 (137)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Psittaciformes			
Psittaculidae			
Psittacula sp.	N	- / -	Alexander et al., 1987 (9)
Psittacula colomboides, Malabar Parakeet	N	- / -	Allan, 1968 (11)
Psittacula cyanocephala, Plum-headed Parakeet	N	- / -	Grausgruber, 1972 (77)
	E	GE / -	Lüthgen, 1981 (137)
Psittacula krameri, Rose-ringed Parakeet	N	GE / -	Zuydam, 1952 (247)
		- / -	Chew & Liow, 1970 (42); Lüthgen, 1981 (137)
	N	- / -	Grausgruber, 1972 (77); Pearson & McCann, 1975 (165)
	N	- / -	Clubb et al., 1980 (45)
	E	GE / H	Lüthgen, 1981 (137)
	N E	N or - / -	Vijayan & Sulochana, 1985 a (224), b (225)
Psittacula eupatria, Alexandrine Parakeet	N	- / -	Grausgruber, 1972 (77); Smit, 1975 (203)
	E	+ or N/ -	Lüthgen, 1981 (137)
Psittacula alexandri, Moustached Parakeet	E	GE / -	Lüthgen, 1981 (137)
Eclectus roratus, Eclectus Parrot		- / -	Lüthgen, 1981 (137)
Polytelidae			
Alisterus scapularis, Australian King-Parrot		- / -	Sperhake, 1974 (207), Woernle, 1974 (240)
		- / -	Ehrsam et al., 1975 (56)
Platyceridae			
Psephotus haematonotus, Red-rumped Parrot	N	- / -	Hirai et al., 1981 (96)
Platycercus eximius, Eastern Rosella	E	GE / -	April & Pearson, 1985 (13)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Psittaciformes			
Platyceridae			
Platycercus adcitus/palliceps, Pale-headed Rosella	N	- / -	Hirai et al., 1981 (96)
Melopsittacidae			
Melopsittacus undulatus, Budgerigar	E	+ C / -	Sallermann, 1973 (190)
	N	GC / -	Erickson et al., 1975 (59)
	N	- / -	Smit, 1975 (203); Pearson & McCann, 1975 (165)
	E	C / -	Erickson et al., 1977 (60 a)
	E	- / -	Erickson et al., 1977 (60 b) & 1978 (61)
	N	- / -	Clubb et al., 1980 (45)
	E	GC / -	Lüthgen, 1981 (137)
	E	C / -	Vindevogel et al., 1983 (229)
	N	G / H	Friederichs et al., 1986 (69)
Cacatuidae / Cacatuinae			
Probosciger aterrimus, Palm Cockatoo	N	+ / -	Smit, 1975 (203)
Eolophus roseicapilla, Galah	N	- / -	Chew & Liow, 1970 (42)
Cacatua goffini, Goffin's Cockatoo	N	CR / -	Clubb et al., 1980 (45)
Cacatua galerita, Sulphur-crested Cockatoo		- / -	Wachendörfer & Lüthgen, 1971 (231)
	N	- / -	Grausgruber, 1972 (77); Lüthgen, 1981 (137)
Cacatua sulphurea, Lesser sulphur-Cockatoo	N	G / -	Cavill, 1974 (38)
	N	- / -	Smit, 1975 (203); Hirai et al., 1980 (95) & 1981 (96)
Cacatua alba, White Cockatoo	N	G / +H	Cavill, 1974 (38)
Cacatua moluccensis, Salmon-crested Cockatoo		- / -	Wachendörfer & Lüthgen, 1971 (231)
	N	G / -	Cavill, 1974 (38)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Psittaciformes			
Cacatuidae / Cacatuinae			
Cacatua moluccensis, Salmon-crested Cockatoo	N	C / -	Eaves & Grimes, 1978 (55); Clubb *et* al., 1980 (45)
unspecified species of Cockatoos	N	- / -	Arnstein, 1965 (16); Woodward, 1974 (242)
Nymphicinae			
Nymphicus hollandicus, Cockatiel		- / -	Gylstorff, 1974 (83); Sperhake, 1974 (207)
	E	GC / -	Winteroll & Grimm, 1974 (238)
	N	- / -	Pearson & McCann, 1975 (165); Alexander *et* al., 1987 (9)
	N	GRE/ -	Clubb *et* al., 1980 (45)
		- / -	Hirai *et* al., 1981 (96)
	E	+ / -	Brugh & Beard, 1984 (33)
Psittaciformes (without exact species names)	N	/ E(R)	Rigby *et* al., 1981 (182)
Strigiformes			
Strigidae / Tytoninae			
Tyto alba, Barn Owl	N	- / -	Keymer & Dawson, 1971 (120); Chu *et* al., 1976 (43)
Strigidae / Asioninae			
Asio otus, Long-eared Owl	N	- / -	Gilmour, 1971 (74)
Strigidae / Strigmae			
Stix aluco, Tawny Owl	N	- / -	Gilmour, 1971 (74); Telbis, 1986 (212)
	E	G / E	Winteroll, 1976 (239)
Otus scops, (European) Scops Owl	N	- / -	Schoop *et* al., 1955 (193)
Bubo virginianus, Great Horned Owl	N	C / R	Ingalls *et* al., 1951 (100)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Strigiformes			
Strigidae / Striginae			
Ketupa ketupu, Malaysian Fish Owl		- / -	Chew & Liow, 1970 (42)
Athene noctua, Littel Owl	N	N / -	Schoop et al., 1955 (193)
	N	GCR / -	Chu et al., 1976 (43)
-, Birds of prey		- / -	Gilmour, 1971 (74); Keymer & Dawson, 1971 (120)
		R / -	Arnall & Keymer, 1975 (15)
		- / -	Winteroll, 1976 (239)
Falconiformes			
Falconidae / Falconinae			
Falcons from Pakistan		- / -	Anonymus, 1975 (12)
Hierofalco biarmicus/abyssinicus, Lanner	N	- / -	Okoh, 1979 (154)
Chicquera chiquera, Red-headed Falcon	N	- / -	Chu et al., 1976 (43)
Tinnunculus tinnunculus, Kestrel	N	GCR/ R	Keymer & Dawson, 1971 (120)
	N	C / -	Borland, 1972 (29)
	E	C / H	Heidenreich, 1976 (90)
	N	GE / -	Chu et al., 1976 (43)
	E	GC / E	Winteroll, 1976 (239)
	N	- / -	Alexander et al., 1987 (9)
Accipitriformes			
Accipitridae / Aegypiniinae			
Gyps africanus, African White-backed Vulture		- / -	Kauker & Siegert, 1957 (114)
Accipitridae / Gypaetinae			
Gypaetus barbatus, Lammergeyer (Bearded Vulture)	E	N / N	Placidi & Santucci, 1954 a (171)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Accipitriformes			
Accipitridae / Haliaeetinae			
Haliaetus albicilla, White-tailed Sea-Eagle	N	- / -	Schoop et al., 1955 (193)
		GC / -	Heidenreich, 1977 (91)
Accipitridae / Accipitrinae			
Accipiter virugatus gularis, Sparrow Hawk		- / -	Sahai, 1937 b (187)
Buteo buteo, Common Buzzard	E	GCE/ -	Heidenreich, 1976 (90)
	E	C / -	Winteroll, 1976 (239)
unspecified Shaneen Hawk	N	- / -	Pierson & Pfow, 1975 (170)
Accipitriformes / Pandiones			
Pandionidae			
Pandion haliaetus, Osprey	N	GRE/ -	Zuydam, 1952 (247)
		- / -	Ratcliffe, 1955 (178); Keymer, 1958 (117).
Sagittariiformes			
Sagittariidae		- / -	Ratcliffe, 1955 (178)
Sagittarius serpentarius, Secretary-Bird	N	GC / -	Schoop et al., 1955 (192); Chu et al., 1976 (43)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Ciconiiformes / Ciconiae			
Ciconiidae			
Mycteria leucocephala, Painted Wood-Stork	N	- / -	Manjunath & Mallick, 1981 (145)
Ciconia ciconia, White Stork	N	CE / -	Saillard, 1952 (189)
	N	G / H	Kaleta et al., 1981 (107)
	N	- or N/- or N	Kaleta & Kummerfeld, 1983 (109)
Ciconiiformes / Ardeae			
Ardeidae			
Bubulcus ibis, Cattle Egret	E	N / -	Placidi & Santucci, 1954 a (171)
Ardea cinerea, Grey Heron		- / -	Placidi & Santucci, 1954 b (172)
Casmerodius albus, Great White Egret or Great White Heron	N	- / -	Manjunath & Mallick, 1981 (145)
Phoenicopteriformes			
Phoenicopteridae	N	(CR)E/ -	López, 1976 (132)
Phoenicopterus ruber, Great(er) Flamingo	N	C / -	Estudillo, 1972 (63)
	N	GCRE/ -	Kaleta & Marschall, 1981 (108)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Phoenicopteriformes			
Phoenicopteridae			
Phoenicopterus chilensis, Chilean Flamingo	N	CR / -	Estudillo, 1972 (63)
Anatiformes (Anseriformes)			
Anatidae / Anserinae			
-, Swans		- / -	Picard, 1928 (167); Vrtiak, 1958 (230)
	N	CRE / -	Estudillo, 1972 (63)
Olor cygnus, Whooper Swan	N	- / -	Friend & Trainer, 1970 (70)
Olor buccinator, Trumpeter Swan	N	C / -	Estudillo, 1972 (63)
Olor columbianus, Whistling Swan	N	- / -	Friend & Trainer, 1970 (70)
Cygnus olor, Mute Swan		- / -	Picard, 1952 (169); Telbis, 1986 (212)
Chenopis atrata, Black Swan	N	- / -	Friend & Trainer, 1970 (70)
Sthenelides melanocoryphus, Black-necked Swan	N	- / -	Friend & Trainer, 1970 (70)
Chen caerulescens, Snow Goose	N	CR / -	Estudillo, 1972 (63)
Branta canadensis, Canada Goose	E	N / -	Page, 1958 (157)
	N	- / -	Bradshaw & Trainer, 1966 (31); Palmer, 1969 (160)
	N	- / -	Palmer & Trainer, 1970 (161); Slemons &
	N	- / -	Easterday, 1975 (202); Vickers & Hanson, 1982 a (222)
	N	CR / -	Estudillo, 1972 (63)
	N	- / -	Rosenberger et al., 1975 (184); Hinshaw et al., 1985 (94)
	N E	- / -	Spalatin & Hanson, 1975 (206)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Anatiformes (Anseriformes)			
Anatidae / Anatinae			
Aix sponsa, Wood Duck	N	- / -	Webster et al., 1976 (234); Slemons & Easterday, 1975 (202)
	N	- / -	Deibel et al., 1985 (50); Hinshaw et al., 1985 (94)
Mareca americana, American Widgeon	N	- / -	Quortrup et al., 1957 (177)
Chaudelasmus streperus, Gadwall	N	- / -	Chandra et al., 1973 (41); Vickers & Hanson, 1982 b (223); Tumova et al., 1984 (216)
Nettion crecca, Common Teal	N	- / -	Chandra et al., 1973 (41); Pearson & McCann, 1975 (165)
	N	- / -	Bozorgmehri-Fard & Keyvanfar, 1979 (30)
	N	- / -	Hinshaw et al., 1985 (94)
Nettion gibberifrons, Grey Teal	N	- / -	Hore et al., 1973 (97); Mackenzie et al., 1984 (139)
Dafila acuta tzitzihoa, Pintail	N	- / -	Quortrup et al., 1957 (177); Pearson & McCann, 1975 (165)
	N	- / -	Hinshaw et al., 1985 (94)
Spatula discors, Blue-winged Teal	N	- / -	Deibel et al., 1985 (50)
Anas cyanoptera, Cinnamon Teal	N	- / -	Pearson & McCann, 1975 (165)
Spatula clypeata, Common Shoveler	N	- / -	Chandra et al., 1973 (41); Hinshaw et al., 1985 (94)
Anas superciliosa, Grey Duck	N	- / -	Mackenzie et al., 1984 (139); Alexander et al., 1986 (8)
Anas platyrhynchos, Mallard	N	- / -	Bradshaw & Trainer, 1966 (31)
	N E	- / -	Friend & Trainer, 1970 (70) & 1972 (71)
	N	- / -	Spalatin & Hanson, 1975 (206); Bahl et al., 1977 (18)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Anatiformes (Anseriformes)			
Anatidae / Anatinae			
Anas platyrrhynchos, Mallard	N	- / -	Alexander et al., 1979 (6); Tumova et al., 1984 (216); Alexander et al., 1986 (8)
	N E	- / -	Vickers & Hanson, 1982 a (222)
	N	- / -	Hinshaw et al., 1985 (94); Kelleher et al., 1985 (115)
Anas fulvigula, Black Duck	N	- / -	Deibel et al., 1985 (50); Hinshaw et al., 1985 (94)
Aythya valisineria, Canvasback	N	- / -	Hinshaw et al., 1985 (94)
Aythya americana, Redhead	N	- / -	Hinshaw et al., 1985 (94)
Aythya ferina, European Porchard	N	- / -	Tumova et al., 1984 (216)
Aythya affinis, Lesser Scaup	N	- / -	Pearson & McCann, 1975 (165); Hinshaw et al., 1985 (94)
Bucephala albeola, Buffle Head	N	- / -	Hinshaw et al., 1985 (94)
Oxyura jamaicensis, Ruddy Duck	N	- / -	Hinshaw et al., 1985 (94)
, Feral Ducks/ Waterfowl	N	- / -	Rosenberger et al., 1974 (184)
(without species names)	N	- / -	López, 1976 (132); Kessler et al., 1979 (116)
	N	- / -	Vickers & Hanson, 1982 b (223)
	N	- / -	Turek et al., 1984 (217)
	N	- / -	Alexander et al., 1987 (9)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Phasianiformes (Galliformes)			
Phasianidae / Numidinae			
Numida meleagridis, Helmeted Guinea Fowl	N	- / -	Moine, 1950 (150)
	N	N / -	Adler et al., 1951 (1); Placidi & Santucci 1954 b (172)
	N	GCE/ -	Ballarini, 1964 (19)
Phasianidae / Pavoninae			
Pavo cristatus, Indian Peacock	N	C / -	Jansen & Kunst, 1952 (103)
		- / -	Sheble, 1961 (199)
		- / -	Faddoul, 1974 (65)
	N	GCRE/HCRE	Tsiroyannis et al., 1971 (215)
	N	CRE/ -	Estudillo, 1972 (63)
unspecified Peacock varieties	N	- / -	Grausgruber, 1972 (77)
Phasianidae / Meleagridinae			
Meleagris gallopavo, Common Turkey		- / -	Pomeroy & Fenstermacher, 1954 (175); Vrtiak, 1958 (230)
Phasianidae / Phasianinae			
Chrysolophus pictus, Golden Pheasant		- / -	Alexander et al., 1987 (9); Grausgruber, 1972 (77)
Phasianus colchicus, Common Pheasant		- / -	Beck, 1942 (24); Brandly, 1946 (32)
		- / -	Dobson, 1949 (52), Vrtiak, 1958 (230)
	N	- / H	Baumann, 1942 (21)
	E	- / -	Fenstermacher et al., 1946 (66)
	N	C / -	Levine et al., 1947 (129); Mantovani & Ceretto, 1953 (147)
	N	CR / -	Liebengood, 1949 (130)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Phasianiformes (Galliformes)			
Phasianidae / Phasianinae			
Phasianus colchicus, Common Pheasant	N	- / -	Zuydam, 1949 (246); Skoda & Zuffa, 1956 (201)
	N	N/ -	Placidi & Santucci 1954 b (172)
		- / -	Vrtiak, 1958 (230); Locke, 1960 (131)
		- / -	Reid, 1961 (179); Paschali-Papadopoulou & Panagiotiduo-Mamolouka, 1969 (163)
	N	- / -	Keymer & Dawson, 1971 (120)
	N	C / R H	Borland, 1972 (29)
		RCE / -	Estudillo, 1972 (63)
	N	- / -	Yenilmez, 1974 (243)
		G / -	Smit, 1975 (202)
	N	RE / -	Al-Hilly et al., 1980 (10)
	N	GC(E)/HRE	Higgins, 1982 (93)
	N	- / -	Senne et al., 1983 (195)
Lophura ignita, Crested fireback	N	- / -	Chew & Liow, 1970 (42)
Lophura swinhoii, Swinhoe's pheasant	N	- / -	Grausgruber, 1972 (77)
Lophura nycthemera, Silver pheasant	N	RC / -	Chew & Liow, 1970 (42)
unidentified species	N	- / -	Pearson & McCann, 1975 (165)
unidentified Indian species	N	RC / -	López, 1976 (132)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Phasianiformes (Galliformes)			
Phasianidae / Lophophorinae			
Lophophorus impejanus, Himalayan Monal	N	- / -	Jansen et al., 1949 (102)
Phasianidae / Perdicinae			
Perdix perdix, (Common) Partridge	N	+ / -	Farinas, 1930 (64)
	E	- / -	Farinas, 1930 (64)
		- / -	Crawford, 1931 (48); Hudson, 1937 (98); Kretzer, 1950 (126)
	N	- / -	Dobson, 1949 (52); Ceretto & Maglione, 1953 (40)
	N	- / -	Mantovani & Ceretto, 1953 (147); Torlone, 1954 (214)
	N	- / E	Thompson, 1955 (213)
	N	GC / -	Parnaik & Dixit, 1953 (162)
		- / -	Vrtiak, 1958 (230)
	N	C / -	Borland, 1972 (29)
Indian Partridges	E	- / -	Géral et al., 1976 (73)
	N	GC / -	Parnaik & Dixit, 1953 (162)
non identified species	N	- / -	Pearson & McCann, 1975 (165)
Alectoris chukar/graeca, Chukar Partridge	E	- / -	Fenstermacher et al., 1946 (66)
	E	C / H	Lucas & Laroche, 1958 (133)
	N	- / -	Walker et al., 1973 (233); Pearson & McCann, 1975 (165)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Phasianiformes (Galliformes)			
Phasianidae / Perdicinae			
Alectoris rufa, Red-legged Partridge	N	- / -	Ciurnelli, 1955 (44)
	E	- / -	Géral et al., 1976 (73)
Bambusicola thoracica, Chinese Bamboo Partridge	N	GCRE/ H	Galli & Cessi, 1968 (72)
	N	- / -	Shortridge et al., 1978 (200)
Francolinus pintadeamus, Chinese Francolin	N	GCRE/ H	Galli & Cessi, 1968 (72)
	N	- / -	Shortridge et al., 1978 (200)
Francolinus francolinus, Black Francolin	N	GCRE/ -	Galli & Cessi, 1968 (72)
	N	- / -	Senne et al., 1983 (196)
Pternitis capensis, Cape Francolin	E	C / -	Kaschula, 1950 (111)
Coturnix coturnix (Common) Quail		- / -	Jones, 1963 (105); Dawson, 1973 (49)
	N	CRE/ -	Estudillo, 1972 (63)
	N	- / -	Pearson & McCann, 1975 (165); Hakim et al., 1979 (85)
	N	- / -	Ahmed et al., 1980 (4)
Coturnix japonica, Japanese Quail	N	GCE/ -	Higgins & Wong, 1968 (92)
Phasianidae / Odontophorinae			
Colinus virgianus, Common Bobwhite	E	N / -	Fenstermacher et al., 1946 (66)
Calipepla californica, California Quail	N	- / -	Pomeroy & Fenstermacher, 1954 (175)
Curassaw		CE / -	Estudillo, 1972 (63)
Cuculiformes			
Eudynamidae			
Eudynamys scolopacea, Common Koel		- / -	Shah & Johnson, 1959 (197)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Upupiformes / Upupae			
Upupidae			
Upupa epops, Hoopoe	N	- / -	Sharma & Baxi, 1980 (198)
Upupiformes / Bucerotes			
Bucerotidae			
Anthracoceros coronatus, Indian Pied Hornbill		- / -	Chew & Liow, 1970 (42)
Bucorvidae			
Bucorvus abyssinicus, Abessian Ground-Hornbill	N	- / -	Schoop et al., 1955 (193)
Alcediniformes			
Alcedinidae			
Dacelo novaeguineae, Laughing Kookaburra		C / -	Schoop et al., 1955 (193)
Piciformes / Pici			
Capitonidae, Barbets		- / -	Walker et al., 1973 (233)
Rhamphastidae			
Aulacorhynchus sp., Toucanets		C / -	Estudillo, 1972 (63)
Rhamphastos dicolorus, Red-breasted or Green-billed Toucan	N	N / -	Kauker & Siegert, 1957 (114)
		C / -	Estudillo, 1972 (63)
		GE(CR)/ -	López, 1976 (132)
Passeriformes / Pittae			
Pittidae		- / -	Erickson et al., 1975 (59)
Eucichla guajana, Blue-tailed Pitta	N	- / -	Pearson & McCann, 1975 (165)
Pitta sordida, Hooded Pitta	N	- / -	Pearson & McCann, 1975 (165)
non identified species	N	- / -	Pearson & McCann, 1975 (165)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Passeriformes / Tyrranni			
Cotingidae			
Rupicola rupicola, Guinean Cock-of-the Rock	N	- / -	Senne et al., 1983 (196)
Passeriformes / Passeres			
Menuridae			
Menura novaehollandiae, Superb Lyrebird	N	- / -	Mansjoer, 1961 (146)
Corvidae			
Pica pica, Magpie	N	- / -	Senne et al., 1983 (196); Tumova et al., 1984 (216)
Corvus frugilegus, Rook	E	- / -	Baczynski, 1960 (17)
Corvus corax, Common Raven		- / -	Sahai, 1937 (188); Pearson & McCann, 1975 (165)
Corvus cornix/corone, Hooded Crow	N	- / -	Blaxland, 1951 (27)
Corvus brachyrhynchos, American Crow		- / -	Karstad et al., 1959 (110)
	E	N or -/ -	Spalatin & Karstad, 1959 (205)
	N	- / -	Burridge et al., 1975 a (34)
		- / -	Pearson & McCann, 1975 (165)
Corvus splendens, House Crow	E	- / -	Sulochana et al., 1981 (210)
Coleus monedula, Jackdaw	E	- / -	Baczynski, 1960 (17); Keymer, 1961 (118)
	N	G / -	Keymer, 1961 (118)
Aphelocoma caerulescens, Scrub-Jay	N	- / -	Pearson & McCann, 1975 (165)
Corvus levaillantii japonensis Bonaparte, Japanese Jungle Crow	N	C / -	Hashiguchi & Hayashi, 1969 (89)
unspecified Crow species		- / -	Sahai, 1937 (188); Hanson, 1976 (87)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Passeriformes / Passeres			
Chloropseidae, Leafbirds	N	- / -	Walker et al., 1973 (233)
Irenidae			
Irena puella/cyanea, Asian Fairy Bluebird	N	- / -	Chew & Liow, 1970 (42)
Meliphagidae			
Conopophila rufogularis, Rufous-throated Honeyeater	N	- / -	Alexander et al., 1986 (8)
Nectariniidae / Dicaeinae	N	- / -	Walker et al., 1973 (233); Erickson et al., 1975 (59)
	N	- / -	Pearson & McCann, 1975 (165)
Estrildidae, Exotic Finches	E	GC / -	Winteroll & Grimm, 1974 (238)
Emblema picta, Painted Firetail		- / -	Schönbauer & Kölbl, 1981 (192)
Chloebia gouldiae, Gouldian Finch	N	- / -	Chew & Liow, 1970 (42)
	N	GR / -	Lüthgen, 1972 (135)
	E	- / -	Lüthgen, 1972 (135)
Padda oryzivora, Java Sparrow or Rice Bird	E	(+)/ -	Collier & Dinger, 1950 (46)
Munia castaneothorax, Chestnut-breasted Mannikin	N	- / -	Alexander et al., 1986 (8)
Munia maja, White-headed Mannikin	E	(+)/ -	Collier & Dinger, 1950 (46)
Munia ferruginosa, Java Mannikin	E	(+)/ -	Collier & Dinger, 1950 (46)
Lonchura punctulata, Nutmeg-Mannikin	E	(+)/ -	Collier & Dinger, 1950 (46)
Lonchura leucogastra, White-bellied Mannikin	E	(+)/ -	Collier & Dinger, 1950 (46)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Passeriformes / Passeres			
Estrildidae			
Lonchura molucca, Moluccan Mannikin	E	- / -	Erickson et l., 1975 (59) & 1978 (61)
	E	C / -	Erickson et al., 1977 (60 a)
Ploceidae / Ploceinae, Weavers		- / -	Keymer et al., 1964 (119)
Ploceus manyar, Streaked Weaver	E	(+)/ -	Collier & Dinger, 1950 (46)
Passeridae			
Passer domesticus, Sparrow		- / -	Konno et al., 1929 (123); Donatien & Geyot, 1946 (53)
		- / -	Pomeroy & Fenstermacher, 1948 (174)
		(+)/ -	d'Arces, 1949 (14)
	E	+ / -	Jezierski, 1950 (104)
		- / -	Popovic, 1951 (176)
	E	- / -	Gustafson & Moses, 1952 a (79), b (80) & 1953 a (81)
	N	C / -	Gustafson & Moses, 1953 a (81); Estudillo, 1972 (63)
	E	C / -	Maglione, 1956 (143)
	E	- / -	Hartwigk & Nitsch, 1957 (88); Baczynski, 1960 (17)
	N	- / -	Monda et al., 1960 (151); Winteroll & Grimm, 1974 (238)
		- / -	Burridge et al., 1975 a (34)
	N	- / -	Pearson & McCann, 1975 (165); Alexander et al., 1987 (9)
Passer domesticus nilotus, Nile Sparrow	E	N / N	Magid et al., 1965 (142)

Table 1: Host range of NDV in free-living and pet birds

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Passeriformes / Passeres			
Passeridae			
Passer montanus, (Eurasian) Tree-Sparrow	N	- / -	Telbis, 1986 (212)
Passer melanurus, Cape Sparrow	E	GCE / -	Kaschula, 1950 (111)
Fringillidae, Finches unspecified	N	- / -	Butterfield et al., 1973 (36)
		- / -	Erickson et al., 1975 (59)
	N	N / -	Pearson & McCann, 1975 (165)
	N	- / -	Pearson & McCann, 1975 (165); Pierson & Pfow, 1975 (170)
	N	- / -	Senne et al., 1983 (196)
Fringilla coelebs, Chaffinch		- / -	Pagnini, 1942 (158) & 1943 (159)
Carduelidae			
Chloris chloris, (European) Green Finch		- / -	Pagnini, 1942 (158) & 1943 (159); Bonaduce, 1948 (28)
Carduelis carduelis, (European) Gold Finch		- / -	Pagnini, 1943 (159)
Serinus canaria, Canary		- / -	Bonaduce, 1948 (28)
	N	GE / -	Monda et al., 1960 (151)
	N	R / -	Lüthgen, 1972 (135)
	N	- / -	Erickson et al., 1975 (59); Pearson et al., 1975 (164)
	N	- / -	Pierson & Pfow, 1975 (170)
	E	(+)/ -	Erickson et al., 1977 a, b (60)
	N	- / -	Smit, 1975 (203); Senne et al., 1983 (196)
	E	- / -	Erickson et al., 1978 (61)

Table 1: Host range of NDV in free-living and pet birds

Order / Suborder Family / Subfamily Genus Species	Mode of Infection	Clinical signs / Lesions	References
Passeriformes / Passeres			
Motacillidae			
Motacilla flava, Yellow Wagtail	N	- / -	Hakim et al., 1979 (85)
		- / -	Ahmed et al., 1980 (4)
Motacilla alba, White Wagtail	N	- / -	Tumova et al., 1984 (216)
Emberizidae / Emberizinae			
Junco hyemalis, Dark-eyed Junco	N	- / -	Deibel et al., 1985 (50)
Thraupidae / Geospizinae			
Tiaris canora/collaris, Cuban Grassquit	N	- / -	Schönbauer & Kölbl, 1981 (192)
Sylviidae			
Phylloscopus trochilus, Willow Warbler	N	- / -	Hakim et al., 1979 (85); Ahmed et al., 1980 (4)
Sylvia communis, White-throat	N	- / -	Hakim et al., 1979 (85); Ahmed et al., 1980 (4)
Timaliidae / Pomatorhininae			
Garrulax leucolophus, White-crested Laughing-Trush	N	- / -	Alexander et al., 1982 (7)
Muscicapidae			
Merula merula, (Common) Blackbird	N	- / -	Bonaduce, 1948 (28); Winteroll & Grimm, 1974 (238)
Ficedula sp., Flycatchers	N	- / -	Hakim et al., 1979 (85); Ahmed et al., 1980 (4)
Sturnidae			
Gracula religiosa, Hill Mynah	N	- / -	Erickson et al., 1975 (59)
	E	(C)/ -	Erickson et al., 1977 a, b (60)
	E	- / -	Erickson et al., 1978 (61)

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Passeriformes / Passeres			
Sturnidae			
Acridotheres tristis, Indian Mynah	N	- / -	Pfow, 1972 (166); Butterfield et al., 1973 (36)
	N	- / -	Walker et al., 1973 a (233); Senne et al., 1983 (196)
	N	- / -	Pierson & Pfow, 1975 (170)
	N	- / -	Sulochana et al., 1982 (210)
Sturnus vulgaris, Starling	N	+ / -	Beaudette, 1943 (23)
	N	- / -	Gillespie et al., 1950 (75); Pearson et al., 1975 (164)
	N	- / -	Pearson & McCann, 1975 (165); Pearson & Pfow, 1975 (170)
	N	- / -	Senne et al., 1983 (196); Tumova et al., 1984 (216)
Hirundinidae / Hirundininae			
Hirundo rustica, (Barn) Swallow	N	- / -	Uppal, 1982 (219)
Delichon urbica, European House Martin		- / -	Winmill & Haig, 1961 (237)

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13

GEOGRAPHICAL DISTRIBUTION

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International recording of the presence of Newcastle disease virus or of clinical Newcastle disease is not entirely accurate because of the lack of precise definitions of the virus and the disease. The use in many countries of mesogenic or lentogenic strains of Newcastle disease virus as vaccines is a complicating factor. Although there is probably some under-reporting, it is apparent that the distribution of Newcastle disease virus is not yet world-wide. Freedom from infection is apparently a result of effective quarantine and geographical isolation.

Lentogenic or mesogenic strains of Newcastle disease virus (either naturally circulating virus or vaccine viruses) are present in most of the countries of Asia, Africa, the Americas and Europe, and in the USSR. More than one third of the countries of Asia and about one fifth of the countries of the world acknowledge the presence of velogenic strains of virus. The countries of Oceania are relatively free from Newcastle disease virus. Some larger countries in the area recognise the presence of avirulent strains of virus while many of the island states are apparently free from all pathotypes of Newcastle disease virus. Newcastle disease virus is also absent from some island states of the Americas and from some European countries.

INTRODUCTION

Newcastle disease virus is a very widely distributed virus. The virus is important because some strains cause disease of varying severity and for this reason international monitoring and reporting

is practised. Clinical Newcastle disease is distributed less widely than are strains of Newcastle disease virus and this results in distortions in reporting systems. Some strains of the virus are of low pathogenicity while a few countries recognise the presence of avirulent strains of Newcastle disease virus. Official reporting does not always take note of strains of low pathogenicity and widespread use of vaccines may mask the presence of virulent strains of Newcastle disease virus. Vaccination usually involves the use of live viruses which retain some degree of pathogenicity and Newcastle disease may not be reported unless it is associated with strains of virus that are more pathogenic than the vaccine viruses that are in use (1).

The ease of recognition of Newcastle disease depends on the host population. Commercial poultry, especially if they are not vaccinated, are excellent indicators of pathogenic strains of Newcastle disease virus. The commercial industry comprises dense, focal populations of improved strains of broiler and layers. A traditional poultry industry persists in many tropical and subtropical countries. Scavenging birds of local breeds are kept in small numbers under free-range conditions and they may have contact with feral poultry. Newcastle disease is not readily diagnosed in these populations. Wild birds and poorly susceptible domestic birds (ducks, geese) may also be hosts to strains of Newcastle disease virus. Infections tend to be recognised and recorded when wild birds become victims of the international bird trade, but there has been little investigation of free-living avian populations.

A further complication is the recognition in the last few years of a variant of Newcastle disease virus that is adapted to pigeons (1) and that infects both domestic and feral pigeons. The pigeon-variant of Newcastle disease virus has, under special circumstances, caused disease in domestic poultry. There is need for an international definition of Newcastle disease and Newcastle disease virus to make reporting more useful.

The present review has used the 1985 Animal Health Yearbook of the Food and Agriculture Organization (FAO) of the United Nations

(2) as an official source of Newcastle disease reporting. In this publication the peracute form of Newcastle disease caused by velogenic strains of Newcastle disease virus is reported separately from the less severe forms of disease caused by mesogenic and lentogenic viruses. It is recognised that reporting of the former is probably more reliable than reporting of the latter. Countries that have velogenic strains of virus will almost invariably have the other forms of virus as well, either circulating naturally or being used as vaccines. This publication has been supplemented by literature references that give more detail of the Newcastle disease situation in individual countries.

In the ensuing discussion distinctions will be drawn between the presence of velogenic virus, the presence of mesogenic and lentogenic virus (either as disease agents or vaccines) and the presence of avirulent viruses. Of particular interest are those few countries that do not recognise the presence of any form of Newcastle disease virus. For some this may indicate deficiencies in diagnosis but others, especially island states, may owe their freedom to efficient natural quarantine. One recent source (3) considered that the less virulent strains of Newcastle disease virus have world wide distribution but this may be too pessimistic a view of the situation. Countries are assigned to geographical zones in accordance with FAO practice.

ASIA

Asia was probably the home of Newcastle disease virus, with the first report coming in 1926 from the city in modern Indonesia now known as Jakarta. Most of the other early reports in 1926 or 1927 were also from Asia.

Of the 42 Asian countries listed in the 1985 FAO Animal Health Yearbook (2), only Saudi Arabia and Mongolia made no reports on Newcastle disease. These were not claims that Newcastle disease was absent but failures to comment. Several countries - Cyprus, Israel, Korea D.P.R., Oman, Qatar and Yemen P.D.R. - recorded no disease but used vaccines. Velogenic virus was widely distributed - Afghanistan, Bahrain, Burma, Hong Kong, India, Iran, Japan, Korea Republic,

Kuwait, Lebanon, Malaysia (Peninsular, Sabah and Sarawak), Nepal and Singapore - and caused frequent disease in Bangladesh and Iraq. Thailand was the only country in the zone in which velogenic Newcastle disease had never been recorded, although other sources contradict this (4). Newcastle disease has recently been described as endemic throughout south-east Asia and the most important of the viral diseases of poultry in the region (5). Few would argue with this assessment.

The commercial poultry industries of Asian countries are totally reliant on vaccines to control Newcastle disease. There are sometimes problems with vaccine quality and the provision of cold chains for the transport of vaccines (6). However the traditional village flocks in rural areas rarely receive conventional vaccination and the losses caused by Newcastle disease are poorly appreciated outside the area. Entire flocks are frequently lost, for example in the Philippines (7) while in Nepal it was reported that most of the rural poultry population (95% of the poultry population of the country) is destroyed annually (8).

Wild birds are known to carry strains of Newcastle disease virus (9) but their role in the epidemiology of endemic disease is not understood. In Asia non-avian hosts may further complicate the epidemiology, for Newcastle disease virus has been associated with experimental mortalities in rice field crabs (10).

AFRICA

Newcastle disease is also a serious problem in Africa. The 1985 FAO Animal Health Yearbook (2) contained reports from 54 African countries and Newcastle disease was present almost throughout the continent. Information on Newcastle disease was not available from three countries (Equitorial Guinea, Sao Tome and Principe, Western Sahara), six were free of the disease and used no vaccine (Djibouti, Libya, Mauritania, Rwanda, Senegal and Somali) and in Niger the disease was suspected but the diagnosis was not confirmed. Only eight countries recognised the presence of velogenic virus. These included Kenya where the virus was enzootic and Mozambique, Sudan, Toto and Uganda where the velogenic disease occurred at high frequency.

Traditional poultry raising is still practised in many African countries. In some of these countries Newcastle disease is recognised as a serious problem and the difficulties seem to be similar to those in Asia. An effective vaccine cover is difficult to attain. Christensen (11) regarded Newcastle disease in village chickens as one of the major problems in all developing African countries and described the control of the disease that was attainable with conventional vaccines in commercial poultry in Malawi. Conventional vaccination had not been successful with village chickens in Malawi, although an earlier study with a naturally spreading Newcastle disease vaccine had been successful (12). A report from Zambia (13) emphasises the African problem of outbreaks of Newcastle disease in village poultry whose traditional owners fail to vaccinate.

THE AMERICAS

Thirty seven countries were included in this section of the 1985 FAO Animal Health Yearbook (2) and Newcastle disease was reported from most states of the mainland. Freedom from Newcastle disease was confined to island states (Antigua and Barbuda, Bahamas, Barbados, Bermuda, Dominica, Falklands, Grenada, St. Lucia, St. Vincent Grenadin) and to Uruguay. Canada and Chile were free of disease but used vaccines. Velogenic virus was recorded in Argentina, Bolivia, Dominican Republic, Ecuador, Guatamala, the United States and Venezuela.

EUROPE

Thirty European countries made official reports in 1985 (2). Most were then free of Newcastle disease and none except Belgium and Greece reported outbreaks attributable to velogenic virus. Outbreaks of Newcastle disease were recorded that year in Albania, Belgium, German Democratic Republic, the Federal Republic of Germany, Greece, Italy, Romania, Switzerland and Yugoslavia. Vaccine was used in several countries in the absence of disease. Two European countries, Luxembourg and Norway, have never recorded outbreaks of Newcastle disease.

The picture in Europe has been complicated by the earlier spread of the pigeon-adapted variant of Newcastle disease virus. This virus, apparently originating in Iraq in 1978 (14), spread from there to Mediterranean countries and through Europe reaching Britain and Scandinavia in 1984 (1). The outbreak of Newcastle disease in European pigeons has been controlled by vaccination and the spread to domestic poultry that occurred in Britain has been controlled by protecting feed stores from contamination with pigeon droppings (1).

OCEANIA

The eleven countries of Oceania were mostly free of Newcastle disease in 1985 (2). Only in East Timor was the disease prevalent. The islands of the region were free of Newcastle disease and three (French Polynesia, New Caledonia, Vanuatu) had never recorded Newcastle disease. Australia (15), New Zealand (16) and Papua New Guinea (17) have avirulent strains of Newcastle disease virus present in their poultry populations. These strains produce antibodies but no disease and Newcastle disease vaccination is not practised in these countries. Similar avirulent strains of Newcastle disease virus have been isolated from wild birds in Australia (18).

USSR

Newcastle disease was present in certain parts of the USSR in 1985 but velogenic strains were not recorded. Vaccine was used.

COMMENT

The distribution of the various pathotypes of Newcastle disease virus is summarised in table 1. The various countries are assigned to groups depending on the presence of strains of Newcastle disease including velogenic strains, the presence of mesogenic/lentogenic strains or the presence of avirulent strains. The presence of mesogenic/lentogenic strains is recorded if vaccines are used, and not all countries in this category would have experienced clinical disease when the information was collected in 1985 (2).

The table emphasises the problem of Newcastle disease in Asia.

Table 1. The geographical prevalence of various pathotypes of Newcastle disease virus

Geographical zone	Number of countries reporting					
	Velogenic and other strains	Mesogenic/ lentogenic strains	Avirulent strains	No virus	Not reporting	Total
Asia	17	23	0	0	2	42
Africa	8	36	0	7	3	54
The Americas	7	19	0	10	1	37
Europe	2	14	0	14	0	30
Oceania	0	1	3	6	1	11
USSR	0	1	0	0	0	1
Totals	34	94	3	37	7	175

All 40 countries that reported recognised the disease or were obliged to use vaccines. Seventeen of the Asian countries (about 40%) reported problems with velogenic virus. This is almost certainly an under-estimate. Some countries indicated that information was lacking and several countries that did not then report velogenic disease would now acknowledge its presence. Poultry production is practised by many large Asian rural populations and in some areas poultry products are a source of animal protein second only to fish. There is a desperate need for methods for controlling Newcastle disease in village poultry populations in Asia and in other tropical areas.

By contrast, the countries of Oceania suffer little from Newcastle disease. This is probably a result of isolation as quarantine procedures are more easily enforced in island environments. Small poultry populations may also be a factor. It is not known how large a population of domestic chickens is needed to maintain Newcastle disease virus. In the absence of reservoirs in wild birds, it may be large. Where strains of Newcastle disease virus are recognised in this zone, they are mostly avirulent viruses. A similar avirulent virus was also recovered in Ulster at a time when other forms of Newcastle disease virus were not present (19).

It is possible that such strains are widely distributed but that they remain undetected in countries where virulent virus is present or where vaccines are used.

The geographical distribution of Newcastle disease virus is not static. The virus when introduced into new areas can spread rapidly. The international trade in aviary birds is often responsible for this spread. This trade is now well established and where it can not be prevented it should be strictly monitored. Successful eradication programmes also alter the distribution of Newcastle disease virus. The usual targets are outbreaks caused by velogenic viruses and eradication, usually slow and expensive, can be achieved. Eradication of other strains is probably more difficult but it should be feasible in countries where domestic poultry are the only hosts.

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14

NEWCASTLE DISEASE : METHODS OF SPREAD

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INTRODUCTION

Over the sixty years that have passed since the recognition of Newcastle disease (ND) one of the most consistent and alarming characteristics has been the ability of the disease to appear suddenly in a poultry population and spread with singular rapidity. This property is so marked that many studies that have been undertaken during the history of ND have included evaluation of this aspect of the epizootiology of the disease even if not specifically aimed at achieving an understanding of the problem. As a consequence, most of the chapters in this book have touched on the epizootiology of ND. The nature of the disease, the pathogenicity and other properties of the virus, the hosts and their environment, the use and misuse of vaccines and the control policies imposed all have important bearings on the ability of NDV to establish infection and cause disease and the reader is referred to the relevant chapters for details. The object of the present chapter is to cover the transmission and spread of the disease and virus rather than every parameter associated with the epizootiology of ND.

TRANSMISSION

ND virus (NDV) isolates show marked variation in the organs they affect (1) as well as in their pathogenicity (2, 3) and this

will have considerable bearing on the mode of spread between individual birds. Basically, it can be considered that natural infection of hatched birds occurs by the respiratory or the intestinal routes following either inhalation or ingestion of infectious virus.

Infection by inhalation

Inhalation of infectious virus may occur as the result of the presence of either larger droplets or fine aerosols containing virus. The former may occur in the birds' environment due to the presence of infected hosts in which the virus is replicating in the respiratory tract or as a result of contaminated drinking water, e.g. as with vaccination by this medium. In addition, respiratory infection may product fine aerosols containing virus which may cover much greater distances before infecting susceptible hosts by inhalation. Faecal excretion may also result in the production of both large and small particles containing infectious virus, the latter most probably resulting from dried faeces.

Transmission by inhalation has not proven easy to establish by experiments using conditions intending to reflect natural poultry environments. However, the ease with which live vaccines may be distributed to poultry by sprays and aerosols has been considered strong evidence of this mode of spread (4). Estola et al (5) were able to show transmission from caged infected chickens to caged susceptible chickens placed in the same air space and the prevention of such infection by the use of a constant negative corona discharge to increase air ionisation. Hugh-Jones et al (6) related deaths in contact (but not separated) suceptible birds to the amount of virus in air samples taken from a room containing birds inoculated with virulent NDV.

Infection by ingestion

Because of the obvious possibility of spread of inhalation

when diseased birds are showing respiratory signs and the dominance of respiratory distress as the primary disease sign for most forms of pathogenic ND, infection by ingestion has been often overlooked as a mode of transmission of NDV strains. Beard and Hanson (4) suggested, with some justification, that vaccines presented to poultry in drinking water may well establish infection by inhalation rather than ingestion and this argument may be applied in most cases where ingestion may be implicated as a possible route.

Nevertheless, in some infections of poultry, such as those with enteric avirulent viruses (4), there is no evidence of respiratory involvement and the primary site of replication in the intestine would indicate ingestion of faeces or contaminated material as the chief mode of transmission. The variant virus responsible for the panzootic in pigeons during the 1980s also failed to induce respiratory signs in either uncomplicated infections of pigeons (7, 8) or in chickens (9). Spread of the disease to chickens in Great Britain was shown to be due to food contaminated with faeces and carcasses of infected feral pigeons (9) and Alexander et al (10) confirmed that virus presented in this way would result in infection of susceptible hens. A noticeable property of the disease seen in laying birds in Great Britain during the 1984 outbreaks was the extreme slowness of spread through a house. This was attributed by Alexander et al (9) to the barrier battery cages would present to a virus dependent on faecal/oral transmission.

Vertical transmission

The concept of vertical transmission in birds implies that the virus is passed directly from the parent to the progeny via the embryonated egg and excludes infection which may occur after the egg has been laid. The facts that virus may penetrate the shell of the egg after laying (11) and that faecal contamination of eggs or the environment has frequently resulted in infection and disease early in the life of chicks hatched from infected parents have

often confused the assessment of true vertical transmission. Experimental assessment has also been greatly hindered by the cessation of egg laying which is usually associated with infection by pathogenic strains of NDV. There are many instances of infected embryos in eggs obtained from hens undergoing field infection (4, 12) but this generally results in death of the embryo long before hatching. In addition, the presence of infected eggs may further complicate assessment of vertical transmission in the field as cracked or broken infected eggs at the hatchery represents another source of virus that may result in early infection in chicks and apparent vertical spread. In contrast to infection of embryos with virulent viruses, lentogenic or vaccinal viruses may not cause death of the embryo and infected chicks may hatch from such eggs (13, 14). Again, it is not clear at what point such eggs become infected although La Sota vaccine has been shown to be present in the ovaries, oviduct and uterus after vaccination (15).

The spread of ND during epizootics has frequently been associated with hatcheries and the movement of eggs or day-old chicks (12) but contaminated trucks or packaging materials such as egg flats are as likely to be the source of infection as the eggs or chicks (16). During the epizootic in Great Britain in 1984 broilers hatched from infected hens developed disease due to the same variant virus (9). However, Alexander et al considered it most likely, from the evidence available, that spread was mechanical and due either to faecal contamination of unfumigated eggs, resulting in infection of hatched progeny, or by direct contact between infected broiler breeder and broiler farms.

METHODS OF SPREAD

Some authors reviewing the methods of spread of avian viruses have considered that different mechanisms may be involved for primary introduction into a country or area than for secondary spread within that area. While this may have been true in the past the present international nature of trade in poultry and other birds

and the ease with which birds, products and personnel may be transported around the world make such differentiation redundant and in the present review no distinction will be made.

Movement of live birds

a) Feral Birds

NDV is capable of infecting an enormous range of birds and has frequently been isolated from feral birds (17). Lancaster and Alexander (12) reviewed the reports of spread from feral to domestic birds and concluded that most instances occur during epizootics in poultry. There seems little doubt that in such circumstances the virus passes from poultry to feral birds and back to poultry (18). Nevertheless, it is clearly indicative of the contact that occurs between domestic and feral birds and the potential mechanism of spread.

NDV strains have often been isolated from waterfowl and other aquatic birds (19, 20) but generally these viruses have been of very low pathogenicity for domestic poultry. Russell and Alexander (21) were able to place such viruses into several distinct antigenic groups using mouse monoclonal antibodies. The isolation of viruses from domestic poultry which show identical binding to these (21, 22) probably represents direct spread from feral to domestic birds.

The migratory nature of waterfowl and the reported isolation of pathogenic NDV from migratory passerines during an epizootic in chickens (19) indicates the potential spread of NDV over large distances as a result of infection of feral birds.

b) Pet/exotic birds

Caged or aviary birds, kept as pets or for show purposes, are generally trapped in countries where they occur as feral birds and are then transported to countries where they are exotic. The volume of trade in such birds can be enormous. For example, between 150-250,000 exotic birds have been imported into Great Britain each

year since 1977 (23) while in the USA nearly 3 million birds were offered for importation between 1973-1981 (24).

The primary introduction of viscerotropic velogenic NDV (VVNDV) resulting in the epizootic in California in 1971-1973 was directly linked to the movement of psittacines into the area (25, 26). While Francis (27), in a detailed review, associated the transport of psittacines with many of the outbreaks occurring throughout the world during 1970-1971. As a result of these apparent relationships between ND and captive bird movement, many countries imposed quarantine restrictions on imported birds. Such quarantine measures have usually included assessment of the ND status of the birds by attempted virus isolation. In the USA this resulted in isolation of virus from birds in 7.6% of the 2,274 lots of birds submitted for importation during 1974-1981, 147 of the viruses proving to be virulent for chickens (24). Similar results have been obtained in other countries. There is no doubt that in countries imposing quarantine smuggling inevitably occurs and in the USA several outbreaks of ND in domestic pet birds have been associated with contact with smuggled birds or birds removed illegally from quarantine (24).

As discussed by Kaleta and Baldauf (17) it is not clear how exotic birds become infected but there can be no doubt that they do pose a serious threat in terms of the introduction of ND into a country. Modern transport methods mean that such birds can be moved rapidly over large distances while incubating virus infections which may not always result in overt disease. Quarantine restrictions appear to be successful, but avoidance of such regulations by unscrupulous dealers and the evidence that some psittacine species may become persistently infected with intermittent excretion of virus over several years (28) indicate areas where such control may break down.

c) Game birds

Modern methods employed in game bird production mean these

birds undergo a considerable change in environment during their lives as they are frequently raised initially in intensive units with subsequent release and dispersal in the wild.

Lancaster (12, 29) reviewed reports of outbreaks of ND in pheasants (Phasianus colchicus), partridges (Perdix spp), Guinea fowl (Numida meleagridis) and peacocks (Pavo spp). Such outbreaks tended to occur only at times of epizootics in domestic poultry, suggesting game birds were not necessarily primary introducers of NDV. Nevertheless, since the disease in such birds appears to be similar in course to that in poultry (30, 31), once infected they are just as likely to transmit the disease and more likely to disseminate the disease if released for shooting. Shortridge et al (32) reported the isolation of virulent NDV from Chinese francolins (Francolinus pintadeanus) and bamboo chickens (Bambusicola thoracica) imported into Hong Kong from the People's Republic of China. While these birds were presumably imported for food, in some European countries game birds are imported and released for sport without undergoing the strict control measures imposed on domestic poultry.

d) Racing pigeons

The panzootic disease occurring in racing, show and food pigeons caused by a variant NDV has been dealt with in detail in two chapters of this book (3, 7). The extremely rapid spread of the disease across Europe and the rest of the world probably resulted from contact associated with races, shows, trade and the gregarious nature of the sport. The practice of large transporters collecting birds in a wide area to take to the release point for races presents an excellent environment for the spread of NDV and was considered a prominent method of spread in Great Britain (33). In addition racing of diseased birds offers opportunities for the wider dissemination of the disease as birds are expected to cover long distances and frequently fly widely off course (especially if sick). Such birds may be taken into other pigeon lofts to recuperate or

mix with feral birds. In Great Britain pigeon races from continental Europe were banned in an effort to prevent introduction of the virus but it probably reached that country by stray racing pigeons from Portugal being taken into a loft to recover (34).

In Great Britain, where there was no vaccination of domestic poultry at that time, the virus passed from racing pigeons to feral pigeons which resulted in the infection of domestic hens via foodstores infested with infected feral pigeons (9).

e) Commercial poultry

During the first ND panzootic (3), movement of commercial poultry was probably by far the most important method of spread of the virus. At that time the poultry industry throughout the world was based on small farm units and that, plus the general lack of availability of current methods of freezing and storage, meant that it was the practice to move live birds to and from markets for trade purposes. Intensivism has meant that for many developed areas of the world this has become a relatively unusual method of trade. But there can be no doubt that for a major part of the world such bird markets are still an extremely important part of poultry trade, even in the USA recent investigations have revealed that live bird markets still flourish in certain areas and the spread of influenza virus to commercial poultry have been associated with these (35). The problems associated with live bird markets are not only that diseased birds may be taken and placed in close proximity with susceptible birds but that many species may be confined together, even in the same cage, including birds such as waterfowl which are refractory to NDV but may nevertheless carry the virus (36). Implicit in the holding of live bird markets is the movement into a central area, concentration of poultry for a short time followed by widespread dissemination - ideal conditions for the spread of disease.

Despite evolution of the industry away from small farms and the ability to preserve poultry products for long periods, movement

of live birds was still considered the major method of spread in developed countries during the panzootic of the early 1970s (16, 37). While this mainly represented local spread, even today, despite further increases in intensive rearing, the international nature of the poultry industry results in the frequent movement of birds, both nationally and internationally, from breeding to rearing farms or as genetic stock. Modern transportation means that distance is no real barrier to such trade and that birds may be moved rapidly while incubating disease (38) or, more likely, may be infected in transit due to contact with other birds, including pet birds, either in cargo holds or holding depots.

Movement of people and equipment

Man may transfer NDV either mechanically on his person or equipment, or as a result of infection, which is usually manifest as conjunctivitis (12, 29). The risk from the latter source, in a country free from disease, such as Australia, was brought home in 1987 when a technician employed at a high security laboratory, was shown to be suffering from conjunctivitis caused by a very virulent strain of NDV as a result of an accident at the laboratory, and who had been in close proximity to poultry between accident and diagnosis (39). Although no infection of the birds took place in this case, it highlighted the potential of humans to spread the disease. The technician could easily have travelled to any country in the world during the time she was excreting virus. Personnel working with poultry, especially those dealing with disease in areas where NDV is endemic, should be fully aware of the potential of carrying NDV in this way, or on contaminated clothing and equipment.

Utterback (16) considered the movement of personnel and equipment to be the most important method of secondary spread of disease during the 1971-1973 epizootic of ND in California. Other authors have similarly stressed this method of spread during epizootics (26, 37). In the 15 years since these outbreaks the commercialisation of the poultry industry has increased in most developed

countries, resulting in greater centralisation of such processes as rendering, removal of spent hens etc. which have proved more economical when carried out by contractors dealing with a large number of farms. Even processes involving handling birds such as vaccination may be dealt with by contractors travelling from farm to farm. The potential effects of such movement between farms were demonstrated during the 1983-1985 highly pathogenic influenza outbreaks in the USA. The occurrence of this disease, which may be considered similar to ND in its methods of spread, in a intensive, highly integrated area of high density poultry rearing resulted in extremely rapid spread which was largely attributed to movement of man and his equipment. King (40) listed eleven types of personnel who, with their equipment, may move from one farm to another:- egg collection contractors, food delivery contractors, refuse collectors, rendering contractors, health service personnel (including veterinary surgeons and poultry health advisors), blood sampling contractors, machinery service personnel, spent hen contractors, catching crews, farm helpers and neighbours and friends. NDV, like influenza virus, is excreted from infected birds in high concentrations in faeces which represents an extremely stable medium for the survival of the virus (29) as well as being of a consistency that readily contaminates man and his fomites facilitating transfer of disease from one farm to the next. On most poultry farms in most countries biosecurity measures are either non-existent or merely perfunctory and render the flocks open to infection by this method.

Movement of poultry products

Birds slaughtered for meat during disease episodes may represent an important source of virus. Most organs and tissues have been shown to carry infectious virus at some time during infection with virulent NDV, although the affected organs may be more localised in infections of less pathogenic strains. The presence and stability of viruses in infected carcasses were described in early reports of ND and have been reviewed in detail by Lancaster (29).

Infectious virus may persist in bone marrow after several days storage at 30°C (41). In developed countries there is a greater potential for preservation of virus in infected carcasses due to the practice of freezing most poultry meat at slaughter. Meat preservation results in equally good virus preservation and infected meat has been shown to retain viable virus for over 250 days at -14 to -20°C (42) and infectivity has been retained in bone marrow and skin after 300 days at -4°C (43).

Although dissemination by frozen meat appears to hae been an extremely common method of spread up to the 1970s (review 29) control policies, including restrictions on the movement and importation of products that were imposed at that time, appear to have greatly reduced the instances of spread by contaminated meat. It should be emphasised that at a time when virus is likely to be widely distributed throughout the organs and tissues of an infected turkey or chicken the bird will be overtly sick and should not be slaughtered or should be detected at meat inspection which is required in most countries with developed poultry industries. However, problems of contaminated frozen meat should not be minimised as apparently healthy birds may be excreting virus in their faeces and this could result in contamination of the meat or wrappings during processing and prior to freezing.

Transfer of virus from infected meat to susceptible birds may occur by direct human contact, but, in the past, it has been commonly the result of feeding offal or poultry scraps untreated to susceptible birds. In the initial report of Doyle on the outbreaks in 1926 (44) he records the suspicion that the primary outbreak was the result of feeding offal obtained from the seaport of Newcastle upon Tyne. Gordon et al (45) considered 33% of 542 outbreaks of ND occurring in England and Wales in 1947 were attributable to feeding infectious poultry waste.

Eggs represent the other major poultry product and the presence of virus in or on eggs laid by infected hens has been covered above under "vertical transmission".

Poultry manure may also be regarded as a commercial product as

in some countries faeces from poultry is incorporated, either untreated or merely dried, into crop fertilisers. Such material may even be exported from one country to another. As reviewed by Lancaster (29), NDV may be extremely stable in poultry faeces. Spread of ND has been associated with the use of faeces from infected chickens as fertiliser (46).

Airborne spread

Airborne spread of ND from one poultry house or farm to another has received considerable attention due mainly to observations and reports in Great Britain during the 1970-1972 epizootic of severe respiratory disease and unusual patterns of spread (47). Dawson (37) considered windborne spread to be of major significance during the early outbreaks of that epizootic.

In a detailed study of the survival of airborne NDV under various conditions Hugh-Jones et al (6) stressed the importance of factors such as high relative humidity on the survival of airborne virus. These authors were able to demonstrate detectable levels of viable virus 64 metres, but not 165 metres, down wind of infected premises. Gloster (48) reviewing the prerequisites determined for the airborne spread of other viruses and the ability of NDV to fulfil these, concluded that such spread was feasible but difficult to predict with certainty due to lack of information on several important parameters.

It should be noted that during the epizootic in California in 1971-1973, with ostensibly the same virus as in Great Britain (21), airborne spread of disease was considered to be of little importance (25) and there have been few reports of significant spread by this method from other countries.

Contaminated poultry food

Although, as discussed above, it is well known that feeding poultry meat or offal from infected birds has been a common method

of spread, this practice has been largely associated with backyard farming methods. The industrialisation of poultry farming in many countries, resulting in commercialised food production has greatly reduced this method of spread and of more concern has been the potential dissemination of disease by food delivery trucks travelling from farm to farm. However, the events occurring in 1984 in Great Britain highlighted the potential of centralised poultry food production and distribution in the spread of ND. At that time a non-vaccination policy had been in force in Great Britain for some years (34) and the national poultry flock could be considered fully susceptible to NDV. The pigeon variant NDV had reached Great Britain in June 1983 and had spread rapidly in racing pigeons and to feral birds (33, 49). In 1984, 23 outbreaks of ND were confirmed in chickens and 20 of these were shown, unequivocally, to be due to the variant virus responsible for the disease in pigeons (33). Epizootiological tracing confirmed 14 of these outbreaks to be linked directly to the feeding of untreated food originating from stores held at Merseyside docks which were known to be infested with diseased feral pigeons (34).

Several important epizootiological points emerged from this episode. The tracing of the source of virus was greatly facilitated by the ability to identify the virus responsible and distinguish it from other NDV strains with confidence; this being possible using a monoclonal antibody fingerprint technique (50). Direct infection occurred only in birds which had received untreated food, in particular the pelleting process to which most poultry food is subjected and which involves heating to 80°C for about 30 seconds appeared to be sufficient to remove infectious virus (34). NDV was able to survive, presumably in faeces, for long periods under the drying conditions usually associated with poultry food. This further emphasises the importance of storing food and food constituents, both on a large commercial scale and at farm yard level, under bird-proofed conditions.

Spread by water

One of the successful methods used for the mass application of live NDV vaccines is to present the virus in the drinking water. This is indicative of both the stability of the virus in water and the potential for spread by this medium. Generally, apart from occasional reports (51), little consideration appears to have been given to this method of spread during epizootics of disease despite the high likelihood, in many parts of the world, of contamination of the water supplies by seepage of liquid from chicken manure or carcasses.

Wild birds, especially waterfowl, that carry NDV may be responsible for the contamination of water supplies. Disease in captive kestrels (Falco tinnunculus) caused by the variant NDV strain associated with the panzootic in pigeons (49) was thought to be due to using rain water gathered from a source frequented by feral pigeons.

The isolation of NDV from pond water on commercial duck farms in Hong Kong has been reported (52).

Spread by non-avian species

Any animal, including flying insects, that travels between infected and susceptible birds must represent a potential for the spread of disease by mechanical transfer of virus. In addition, a large number of species have been shown to be suitable hosts for the replication of NDV (reviewed by Lancaster, 29) and, like humans (see above), may offer a means by which virus can be spread over long distances. The extent to which this happens under natural conditions is unclear.

Spread by vaccines

Vaccines may be responsible for the spread of NDV by several mechanisms, all of which require mishandling, laboratory errors or

control failures in the manufacturing process. They are: a) Contamination of live vaccines (NDV or other pathogens). For example, Beard et al (53) reported the presence of vaccinal NDV in vaccines aimed at several other poultry diseases. b) Contamination of inactivated vaccines after vaccination. c) Incomplete inactivation. d) Mislabelling of vaccines or mixing seed strains, i.e. resulting in administration of mesogenic viruses as lentogenic vaccines.

Control measures have been drawn up by international agencies specifically to deal with such problems and are the subject of a chapter of this book (54).

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15

NEWCASTLE DISEASE IN TROPICAL AND DEVELOPING COUNTRIES

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INTRODUCTION

In tropical and developing countries (TDCs) poultry and eggs are among the most easily produced forms of animal protein for the human diet. While Newcastle disease (ND) occurs globally (1,2), its impact in terms of morbidity and mortality among chickens, the economic cost to the community, and the resulting reduction in available animal protein for human consumption, is greatest in certain TDCs.

The occurrence of ND in several Asian countries has been described elsewhere (3,4). In preparing this chapter the authors have also considered current reports from numerous countries. It has become apparent that the high incidence of ND in TDCs is attributable to epidemiological and husbandry factors rather than to unique properties of the ND virus (NDV) in these countries. Here, we deal with these contributory factors rather than with national incidence in individual TDCs. In order to convey the complex interactions involved we describe in detail the circumstances prevailing in Hong Kong, with reference to other TDCs where comparison or contrast is informative. Indeed, the New Territories of Hong Kong is a striking example for a case study, exhibiting almost all the factors potentially allowing importation, persistence and spread of ND, and suffering numerous constraints on the effective control of this disease.

The New Territories Microcosm

The New Territories is an area of about 1000 square km, north of the Kowloon peninsular (Fig. 1). Along with 235 out-lying islands it was leased to Britain by China for 99 years under the Convention of Peking, 1898. Traditionally, the New Territories has been rural and agricultural, with the exception of mountainous and rather remote areas. The production emphasis has been to provide fresh vegetables, live poultry, and some pigs to the growing urban

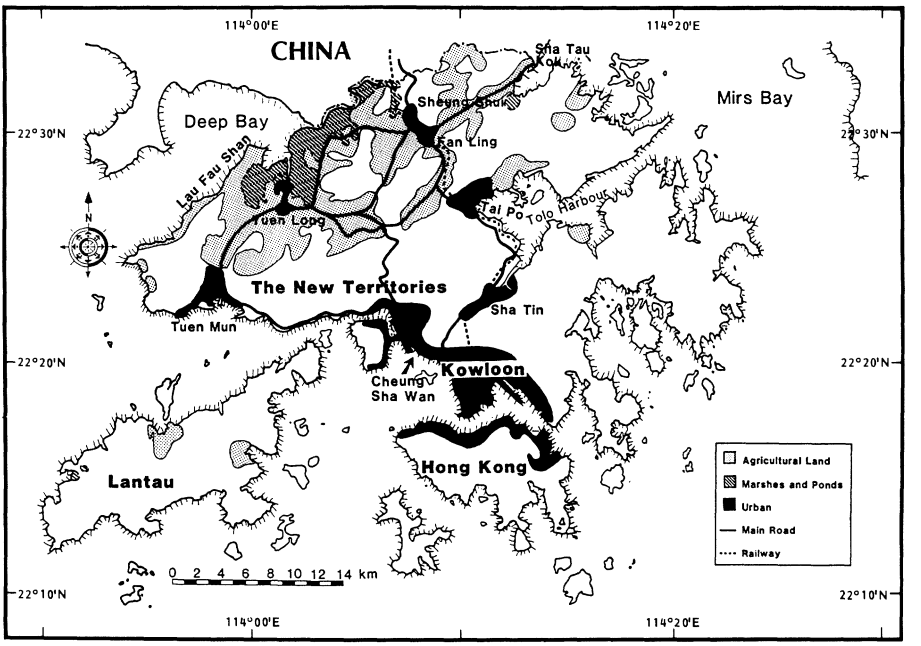

Fig. 1. Map of the territory of Hong Kong showing the New Territories located between Kowloon and China. The main agricultural, urban and marshland areas are indicated. Duck farms are most common in the coastal area of Deep Bay around Lau Fau Shan and in the marshes and ponds of the north-western New Territories. Chicken farms are distributed throughout the agricultural areas, but are most common in the vicinity of Yuen Long. Common resting areas for migratory birds are the marshlands of Deep Bay and the shallow bays of Lau Fau Shan. The main road and rail routes for transportation and importation of poultry are shown; poultry imported for consumption go predominantly to a wholesale market in Cheung Sha Wan, while fertile eggs and day old chicks are hatched or sold in Yuen Long.

areas of Hong Kong and Kowloon. The New Territories has attracted many people with farming heritage from China, such as the Hakka and Chiu Chow people, resulting in a different ethnic mixture from the predominantly Cantonese and Shanghainese business areas of Hong Kong.

In recent years the agricultural emphasis of the New Territories has diminished in the face of industrialisation, northerly spreading of the Kowloon urban area, the development of "new towns" and enlargement of the existing towns of Yuen Long, Fan Ling and Sheung Shui. Land availability is rapidly decreasing, and the previously tranquil, and rather unique, farming areas of the New Territories are disappearing. This in no way reduces the availability of high quality vegetables, poultry and other meat in Hong Kong, for agriculture has expanded in southern China and road, sea and rail transport are efficient.

According to Government statistics (5) in 1985 there were about 1504 chicken, 223 duck, 70 quail and 652 pigeon farms in the New Territories, holding a total of 9.2 million birds. Annual production is estimated at about 14 million chickens worth almost HK$300 million (US$1 = HK$7.8) (Table 1). However, in recent years the poultry population and production, particularly of chickens, has fallen steadily (Table 1) reflecting the current emphasis on

Table 1. Hong Kong poultry population and production figures, 1981 and 1985*.

Species	Population		Production (number in '000s/value in HK$'000+)	
	1981	1985	1981	1985
Chickens	7037500	5487000	17388/$344248	14017/$299880
Ducks	817370	872800	4446/$97972	4413/$113709
Geese	19480	15130	232/$8218	192/$6854
Quail	525600	651000	12019/$17069	12435/$18043
Pigeons (pairs)	209400	577200	732/$20825	1589/$60016

* Source: refs 5 and 22. + HK$7.80 = US$1.00

industrial land use. Considerable seasonal fluctuation in stocking rate occurs, with greatest increases prior to the Chinese New Year festival, which is in January or February. In addition to poultry meat the industry produces annually about 30 million chicken eggs, 1.6 million duck eggs and 150 million quail eggs with a total value close to HK$150 million. The industry can be very dynamic, quickly responding to demands for new products; e.g. farmers are prepared to initiate quail or pigeon farming if the market suggests it would be profitable. A few farms are quite large (~80,000 birds) but irrespective of size most are of rather low husbandry standards (Figs. 2 and 3). Some poultry farms can be found isolated in remote areas, but most are clustered together in small villages served by adequate roads and convenient transport (Fig. 4). Duck farms are most common in the marsh and fish pond areas of the western New Territories.

Traditional Chinese cuisine demands a large, brown-skinned chicken with much subcutaneous fat; brown-shelled eggs are also preferred. The Cantonese breed is ideal for these requirements; unfortunately it grows slowly, converts food inefficiently, and lays rather few eggs albeit these are brown-shelled. Cross-breeding with imported birds is aimed at retaining the desirable culinary qualities, while improving productivity. Table birds are usually purchased live (Fig. 5). Prepacked, deep-frozen chicken is becoming accepted for its domestic convenience, but would usually not be used in restaurants or for special occasions.

HOST AND VIRUS

Incidence of ND in Hong Kong

ND is regarded as the most important disease of chickens in Hong Kong, mortalities in field outbreaks varying from 5-95%. However, the exact occurrence and economic impact are difficult to assess. Between 1956 and 1968 the number of recorded outbreaks rose from 12 to 238; these figures were based on field reports most of which were not backed up by laboratory investigation. In recent years statistics have been based on laboratory confirmation of notifiable diseases, and in 1985 this amounted to 21 outbreaks of ND affecting flocks totalling 23748 birds. However, the troublesome restrictions accompanying the notifiable status of a disease make farmers reluctant to report outbreaks. Mortalities

Fig. 2. A typical New Territories chicken farm. Note the proximity of the buildings, also the fold-down shutters and roll-down blinds to adjust ventilation.

Fig. 3. A small flock of the Cantonese breed of chicken in typical accommodation. The birds are overcrowded and there are insufficient drinkers. One wall of the building is boarded precluding good ventilation.

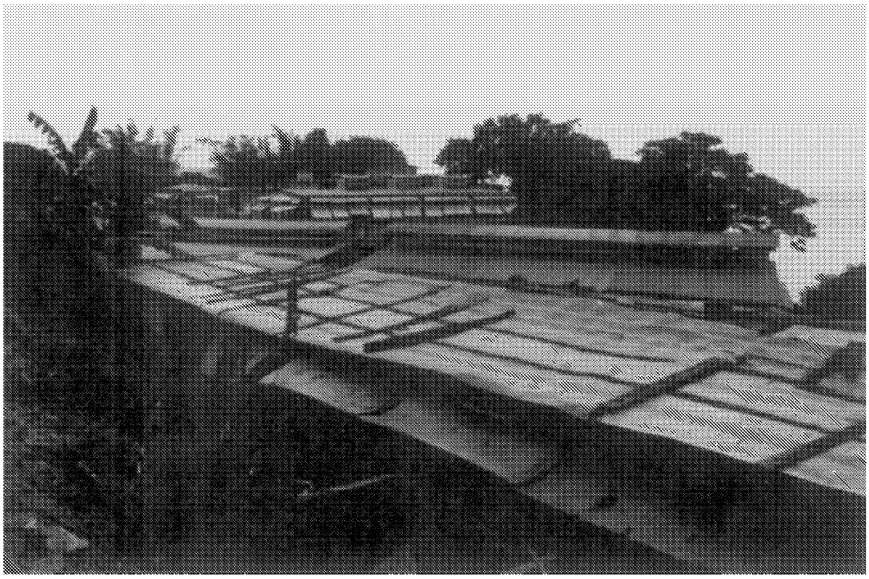

Fig. 4. A cluster of chicken farms in the western coastal area of the New Territories. The buildings are situated against the slope at the side of a main road; this location restricts ventilation. The proximity of houses also reduces air flow and facilitates spread of virus from flock to flock.

Fig. 5. A shop selling live poultry for domestic consumption. The birds are delivered in and sold from the cane baskets and then held in cages overnight if necessary. The cane baskets are usually used once only before being discarded.

among affected birds have remained fairly constant, being 26.8% in 1968-69, and 33.2% in 1984-85. In the period 1968-70 most outbreaks occurred between September and February, and exactly half the outbreaks were in birds 10-39 days of age (6). Disease is associated with a variety of neurologic, enteric, respiratory and systemic signs. Field viruses are mesogenic and velogenic, but whether variation in virus virulence accounts for variation in clinical signs is not known. Concurrent infections are common (vide infra), with less than 20% of cases yielding NDV as the only agent. Vaccination is practiced intensively, and between this and possible additional natural exposure, serum antibody titres are generally remarkably high. The predominant question remaining is why birds with high levels of antibody are so consistently affected by disease.

Are local breeds more susceptible?

Rural societies with a history as long as the Chinese have selected their domesticated animals for desirable features. Disease resistance has been selected for both intentionally and by the natural outcome of pandemics. For these reasons local breeds of animals and poultry are often more resistant to indigenous diseases than imported breeds. There is, however, no evidence to suggest that the popular local breeds (Cantonese, Wai Chow) are more susceptible to ND than imported breeds. Experimental evidence has not been sought. Field evidence would be unreliable; a farmer importing exotic breeds would be likely to employ more modern husbandry techniques and to spend more money on feed, housing and other equipment than his neighbour who employs traditional methods to rear local chickens.

Do field strains of NDV occurring in TDCs differ from viruses occurring in temperate countries?

In so far as virulence is concerned the direct answer to this question is: not invariably. Based on clinical signs and lesions, the velogenic viscerotropic (VVND) and velogenic neurotropic forms of NDV occur in Hong Kong. However, such classification criteria are inappropriate since signs and lesions are often modified by intercurrent infectious diseases or non-infectious stress factors. Considerable circumstantial evidence points to the involvement of mesogenic vaccine viruses as disease agents in stressed birds.

A simplistic explanation of the failure of these antibodies to

protect against ND would be antigenic variation. However, serological techniques provide no evidence that NDV of TDCs express unique antigens, or undergo antigenic variation. Whether the virulent NDV isolated in TDCs will belong to a unique group remains to be seen but this, and the relationship between NDV common in TDCs and the sporadic appearance of VVND in temperate countries, should be priority areas for investigation (vide infra).

Reservoirs of NDV

The world literature confirms that while ND is of greatest economic importance among chicken flocks a variety of wild birds, as well as turkeys, pigeons and quails, are susceptible (1,2,7). Ducks and geese are considered more resistant, though disease has been reported. Among these species vaccination is sometimes performed, but generally it is not. The reasons for not vaccinating are two-fold: i. since disease occurs infrequently in these species vaccination does not have high priority; ii. some of these species (notably ducks) develop little or no measurable antibody (8,9) suggesting that vaccination would be a waste of effort and money.

An important area of ignorance is whether any of these species can become permanent asymptomatic carriers of NDV virulent for chickens. The main candidate is the duck. Over 3% of oral and cloacal swabs taken from apparently healthy ducks at a dressing plant in Hong Kong yielded NDV, with most isolates being from the cloaca (10,11). This might be a parallel to the better-understood situation of the duck as a reservoir of influenza A viruses. The few reports of clinical ND in, and prolonged excretion of virus by, ducks (9,12,13,14) must be set against wide experience showing these birds to be refractory to experimental infection (8,13,15) and capable of excreting virus for only 4-5 days (13).

The rural ecology of southern China and Hong Kong is believed to be conducive to the prolonged survival of influenza A viruses in ducks and the dissemination of these viruses to humans (16). A similar situation might exist with regard to NDV persisting without causing clinical signs within a host species other than the chicken. In Hong Kong and southern China the duck would again be the prime candidate for reservoir status.

Ducks, geese, pigeons and quails are farmed in the New Territories of Hong Kong. Some species occur predominantly in

certain areas, others are distributed widely. Either way, they often exist in close contact with chicken flocks, on the same or neighbouring farms, and spread of NDV from reservoirs to susceptible birds would occur easily.

Wild birds and the global dissemination of ND

Many species of wild birds have been found infected with and sometimes affected by NDV (2,7,17). Their global importance in the spread of ND is not known for certain, though some outbreaks have been attributed to contact between domesticated and wild birds, and the introduction of VVND into USA has sometimes been associated with the importation of infected psittacine cage birds (17,18,19).

Two wildlife reservoirs of NDV were identified by Hanson (20): a chicken-avirulent virus among migratory waterfowl of temperate zones and a chicken-virulent virus among tropical jungle birds. It might therefore be relevant that many TDCs lie on the great north-south migratory routes and see a tremendous traffic of transient visitors twice a year. Migratory birds visiting Hong Kong and other areas of South-East Asia are flying between Siberia and Australasia; birds visiting central Africa are migrating between northern Europe and southern Africa or Antarctica.

The farming areas occupied by chicken farms are less likely places for migratory birds to stop over than the marshlands and shallow bays of the north-western New Territories. While direct contact with chickens probably does not occur, there is reasonable chance of contact with domesticated waterfowl which could play an important part as intermediate hosts.

BORDER CONTROL

Many TDCs have borders which are ill-defined or, for a variety of reasons, difficult to control. Apart from possible spread by wild birds NDV can cross borders in at least three ways: by spread from affected chickens in neighbouring farms across the border (Fig. 6), in transported birds, or on fomite vectors. In addition, the possibility exists that man and other mammals, e.g. mice (21), can be infected transiently and might transport the virus.

Problems arise in Hong Kong because of the volume of traffic (human, vehicular, goods, animals) crossing the border between the New Territories and China. The exact number of poultry imported to Hong Kong is not known. However, most (probably about two-thirds)

of the poultry imported for consumption are sold at the Cheung Sha Wan (Fig. 1) poultry market where the daily average sales in April 1987 were 48900 chickens, 12300 ducks, 4400 geese and 7500 pigeons (22); of these it is estimated that about 30000 chickens are imported daily by road from China, the remainder entering by train or ship. These birds are for immediate consumption and either go first to a holding station (where they should not come into contact with local farm birds) or direct to market. They are sold live (Fig. 5), and birds left unsold at the end of a day's trading are then held overnight in cages at the market. Some birds might be diverted to farms in the New Territories, but they will be few if any. Thus, imported live birds infected with NDV are unlikely to act as a source of infection for local farm birds. Indirect spread of virus might, however, occur. The cages and trucks used for transportation are used intensively, travelling from China to the New Territories, and within the New Territories from farm to market to farm, with no disinfection in between.

The other possible source of imported virus lies in illegally imported livestock. Usually this means individual birds carried by families for immediate domestic consumption. The border is well-defined, with clear crossing posts, but the numbers of people crossing daily makes thorough inspection impossible. The origins and health status of such poultry are unknown, but this avenue for importation of NDV does not offer much chance for establishment of infection in farm stock.

Elsewhere, notably in some large African countries, borders can be difficult to control because of their length. Spread of ND across borders is recognized by some countries as the most important source of new outbreaks. This is particularly the case where neighbouring countries have rather different infrastructures - population density, disease control regulations etc. - and where a country with few poultry and hence no observable ND is adjacent to a more densely populated one in which ND is endemic. Important factors in eradication of these "imported outbreaks" are early diagnosis, restriction of movement from the infected focus, ring vaccination and, if the infection persists or appears to be spreading, slaughter of affected and in-contact stock.

VACCINATION

Availability and use

Vaccines are freely available from agricultural supply companies (Fig. 7) in the market towns of Yuen Long and Sheung Shui. There is no restriction on importation, sale or purchase, and no legislation controlling methods of storage and administration. Vaccines are imported from numerous suppliers in several countries; most are the products of reputable manufacturers, but some are of dubious standard. Although they are invariably kept in refrigerators in the shops and on the farms, there is no assurance that the cold chain has been adequate during shipment, customs clearance or warehouse storage.

ND vaccination is practiced intensively in Hong Kong. Live vaccines in common use are the lentogenic F, B1 and La Sota and mesogenic Ranikhet, Mukteswar and Roakin strains; it is noteworthy that the mesogenic vaccines used in Hong Kong are among the more virulent of the available mesogenic isolates. The basic programme is to administer lentogenic vaccine by the nasal or ocular route within one week of hatching, followed by intramuscular injection of mesogenic vaccine at one and three months of age. Layers should be revaccinated at six months of age, at point of lay and thereafter every six months. Most farmers are, unfortunately, far from rational and tend to hyper-vaccinate, sometimes injecting mesogenic virus monthly. In addition, vaccine will be administered if birds become sick, often irrespective of the nature of the disease signs, or if farmers suspect that outbreaks of ND are occurring on neighbouring farms. The vaccination techniques in use are intraocular, intranasal and intramuscular, and are time-consuming and labour-intensive.

To a simple farmer the true content and mode of action of vaccines are incomprehensible: agents, live or dead, which require ice-cold temperatures for the persistence of their efficacy and which act by stimulating complex immune systems. Rather, vaccine is seen either as an immediate injection of protection, or as a panacea. Survival requirements of the vaccine virus, its interaction with host cells and with other infectious agents, and its possible neutralization by residual immunity in the recipient chicken are cumbersome concepts to convey to rural people.

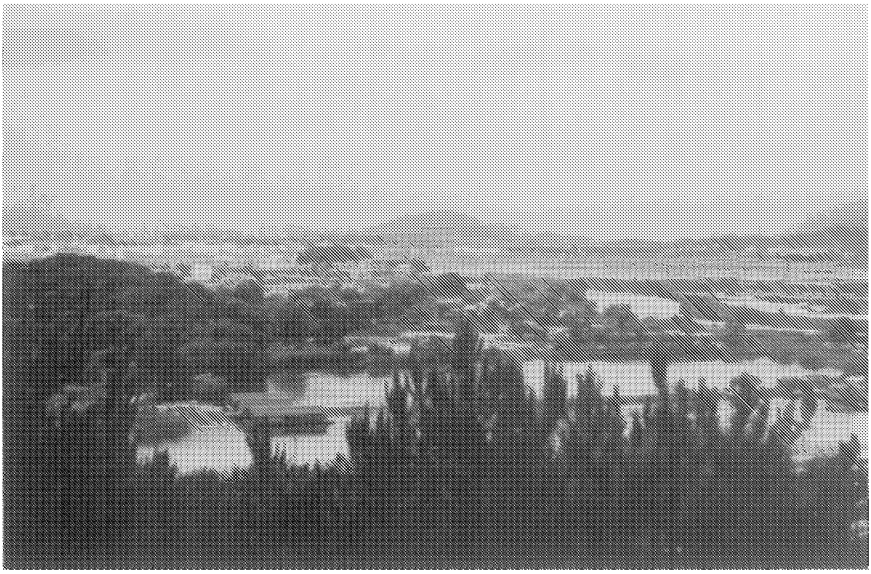

Fig. 6. A view of part of the border area between Hong Kong and China. The Shum Chun river constitutes the border at this point and the buildings visible in the distance are part of the Special Economic Zone of Shen Zen (formerly Shum Chun). Ponds are plentiful on either side of the river, and a New Territories duck farm can be seen about 200 metres from the river. The potential for trans-border spread is apparent.

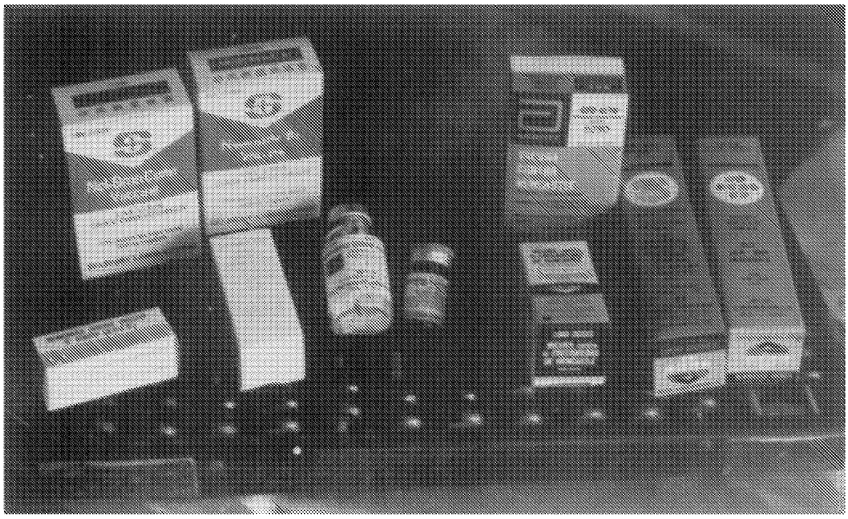

Fig. 7. The selection of ND vaccines found available for purchase in one of the many agricultural supply shops in Yuen Long.

Consequences of vaccine overuse

Throughout TDCs vaccines for commercially raised poultry are used empirically and excessively. These excesses are universally aimed at reducing disease in broilers attributable to inadequate active immunity arising from neonatal vaccination in the face of maternally-derived passive immunity.

The intense vaccination programmes in use have several important outcomes. In general extremely high levels of serum antibody are stimulated. Since flocks are often in close contact, vaccine virus spreads rapidly. Sometimes young birds with minimal protective immunity are thus exposed to mesogenic virus. Laboratory assessment of NDV isolated from cases of clinical disease often indicates that they might be mesogenic vaccine strains. Mesogenic vaccine strains are also frequently implicated in clinical disease, sometimes with neurologic signs, where birds sick from causes other than ND are nevertheless vaccinated or are in close contact with vaccinated birds.

Comparison of various TDCs reveals a remarkable range of vaccination practices. Day of first vaccination, route of administration and strain of vaccine are variable parameters. Surprisingly, in some countries vaccination at day-old is still favoured, albeit there is now a general concensus that the only way to induce immunity reliably is to wait 2-3 weeks while passive immunity wanes. The most unusual practice - and one which admirably illustrates the lack of understanding of vaccines - occurs in a country where the recommendation is for first inoculation by the nasal route at 14 days; here the farmers attempt a compromise with a half dose at 7 days. In all TDCs, the subsequent vaccination programme involves rather heavy use of live mesogenic vaccines.

A high proportion of TDCs report the isolation of lentogenic and mesogenic viruses from diseased birds; direct attribution of disease to these strains is rarely so unequivocal. However, there is general awareness of their potential as disease agents and an important observation in South Africa is the participation of lentogenic NDV in a multifactorial aetiology of colisepticaemia (23).

An additional point to stress is that a vaccine should not be used unless the relevant pathogen has been identified in the

country or region in question. It is pointless to use vaccines against diseases that do not exist. Unfortunately this situation arises for a variety of reasons including: inadequate information on the occurrence of disease, aggressive sales tactics of vaccine manufacturers, and the failure of uneducated farmers to understand that the protection offered by a vaccine is agent-specific and not a panacea. Indiscriminate use of vaccines undoubtedly occurs world-wide but is extreme in TDCs. It is an additional stress factor to the chickens, an unnecessary burden of expense and labour to the farmer, and can generate an undesirable pool of circulating avirulent virus within farm stock. The residual virus and the antibodies resulting from vaccination and from adventitious exposure to vaccine virus make interpretation of results arising from surveillance difficult.

HATCHERY MANAGEMENT

Structure and practice

Poor hatchery hygiene and the mixing of fertile eggs from different sources are believed to contribute to the problem of ND in Hong Kong.

Some years ago, employees of local hatcheries could be seen cycling round the countryside collecting small batches of eggs from numerous farms, eventually bringing 12-20 trays of 30 eggs apiece to the hatchery on the back of the bicycle. Nowadays, only few of the chicken flocks in Hong Kong are kept for egg production, and some of these farms run their own hatcheries. Most eggs for consumption and for hatching of commercial broilers are imported from China; for breed improvement purposes, fertile eggs are imported from all over the world.

Commercial hatcheries are mostly found in the town of Yuen Long (Fig. 1). The premises are converted shops, each fitted with 2-5 small incubators, allowing a total capacity of up to 10,000 eggs (Fig. 8). The local practice is to allow the last day of incubation and actual hatching to proceed outside the incubator, so one end of the hatchery is usually fitted with suitable wooden bunk-like shelves covered loosely with hessian. The standard of these hatcheries varies from quite good to very poor. Most are dark, dirty and dusty, eggs are not cleaned prior to setting, and newly hatched birds are placed into straw baskets lined with

newspaper covered in the droppings of several previous hatches.

Laying hens shed NDV for several days or weeks after infection or vaccination (24,25,26). However, it would seem unlikely that the embryo would survive and hatch unless the NDV was extremely avirulent. Nevertheless, cracked and broken eggs can contaminate the hatchery. Also, the virus is sufficiently thermostable to survive for the incubation period on eggs coated with contaminated droppings (27,28) from where virus might penetrate through the shell (29). Contaminated egg flats have been recognized as a major source of infection (30).

Fertile eggs are imported at various stages of incubation, and a single set can be a mixture of the products of different flocks (Fig. 8). Little is known of the disease or vaccination status of the parent flocks but ND is endemic in the areas of southern China neighbouring Hong Kong and vaccination is believed to be as intensive there as it is in the New Territories.

Vaccination dilemma

There seems to be no doubt that the failings of ND vaccination in Hong Kong begin with the wide range of antibody titres encountered in newly-hatched chicks. The effects of passive immunity on the immunogenic potential of vaccine virus have been well documented (31-35). Should a farmer vaccinate within one or two days of hatch and risk neutralization of the virus by high levels of maternally-derived antibodies? Or should he wait one or two weeks until high antibody titres in some birds have fallen, during which time birds inheriting low levels of maternal immunity will be susceptible to field infection? This dilemma is common in TDCs and has stimulated much research into methods of vaccinating chicks against ND (36-38).

Whatever action the farmer takes, some birds will develop protective levels of immunity, to varying degrees, but in others vaccination will be ineffective. It seems probable that a combination of circumstances including immunological immaturity, inadequate passive immunity, virulence of vaccine virus, level of exposure, and infectious and environmental stress factors can result in susceptibility to NDV which would not normally be pathogenic. These problems will only be overcome when hatcheries set eggs in batches from single flocks in order to produce day-old chicks of more uniform immunological status.

PREDISPOSING FACTORS

A variety of factors are known to make birds more susceptible to ND. Most of these occur in Hong Kong.

Intercurrent disease

Infection with other viruses (infectious bronchitis, infectious laryngotracheitis, infectious bursal disease, Marek's disease, adenoviruses, reoviruses), bacteria (Pasteurella multocida, Salmonella spp., Escherichia coli, Haemophilus gallinarum), Mycoplasma spp., gastrointestinal nematodes and coccidia are common. Cases of chicken disease referred for laboratory diagnosis usually reveal multiple infection with a variety of potential pathogens. It is reasonable to believe that these agents play an important role in predisposing chickens to ND.

All TDCs report a high incidence of background disease, and in almost all cases these are considered important predisposing factors in ND. In some countries, as in Hong Kong, a variety of diseases is reported; in other countries, certain agents are singled out as predominant in their association with ND, e.g. in the Yemen Arab Republic, M. gallisepticum and M. synoviae are of singular importance. It is noteworthy that M. gallisepticum can provoke disease signs when mixed infection with lentogenic NDV occurs (39). Almost all the pathogens associated with increased susceptibility to ND - apart from those causing marked systemic debility - are respiratory pathogens. This confirms the importance of the respiratory route of infection in ND, and the need for a healthy respiratory tract to ensure resistance. This, in turn, requires not only a disease-free environment but also one which is well-ventilated and dust-free.

Housing and related husbandry practices

Farm buildings are usually make-shift, built of wooden frame, wire-mesh sides and corrugated iron roof (Fig. 2). Most have fold-down tin flaps to protect against the cold winds of winter; nevertheless, for 2 to 3 months of the year heaters are necessary. In summer the air is hot and humid, so large pedestal fans are used to improve air flow (Fig. 9). In spite of the contingencies, most poultry houses are too cold in winter and too hot in summer. Since houses stand close together and are often located where there is little breeze (Fig. 4) the fans will not draw sufficient air and

Fig. 8. A typical hatchery. This one has a mixed batch of eggs incubating and another mixed batch ready to hatch in the lower compartment. Egg trays, cardboard boxes and unset or discarded eggs clutter the hatchery, suggesting that general hygiene standards are low.

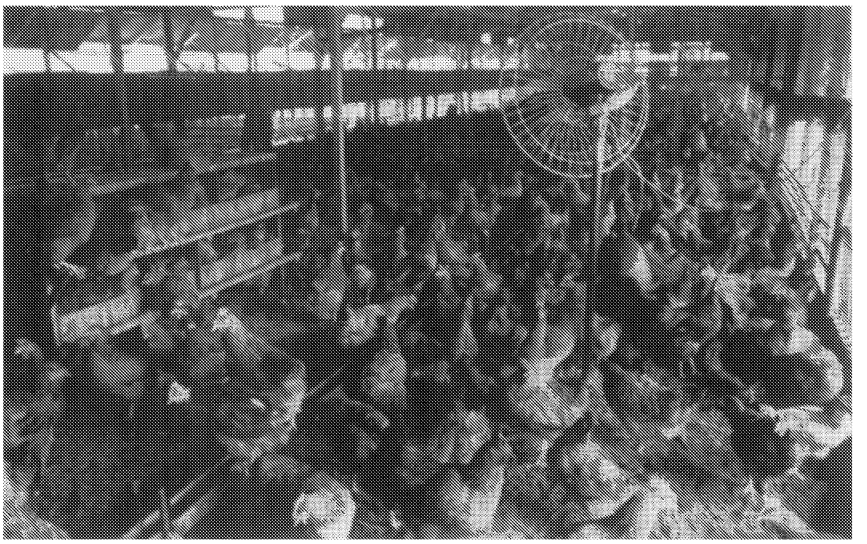

Fig. 9. An overcrowded, dusty chicken house, stocked with breeding hens. Note the pedestal fan to improve ventilation, but which will also raise dust into the air, and the inadequate number of make-shift laying boxes.

ventilation remains inadequate. The buildings are cleaned rarely and dust particles and cobwebs accumulate. During winter, when ventilation is restricted, the air becomes stale and dusty. In the hot humid summer, the air becomes ammoniacal, and birds can often be seen gasping. Under these conditions, it is not surprising that birds are highly susceptible to respiratory diseases and ND.

Most farmers in TDCs cannot afford substantial farm buildings, elaborate apparatus for heating, ventilation or automatic feeding, or fuel bills for heating costs. It is unrealistic of Western conglomerates to expect farmers in TDCs to import the latest equipment and technology. Too often modern equipment is donated within the structure of development assistance programmes to TDCs which do not possess adequate support facilities or on-going funding. The result is either redundant unused equipment or an added burden of expense on the farmer who maintains it. Hence the most useful contribution Western agricultural technologists could make would be to investigate ways to improve farm buildings in TDCs using readily-available materials and minimum funding.

A major conceptual difference between farmers in relatively affluent temperate countries and those in TDCs is their attitude to the needs of their farm stock. Persuading poor rural people that their chickens require ventilation, dust-free atmosphere, constant supply of clean water, quality-controlled feed free from mycotoxins and containing important (and expensive) additives such as vitamins is indeed a formidable task. It is nevertheless a task which is essential to improve the humanitarian aspects of farming, the profitability of the farm, and the quality of life in TDCs.

A related, and often overlooked, problem is the inability of farmers to acknowledge the importance of environmental factors outside the chicken house. Not uncommonly Asian farms are cluttered with disused equipment, discarded vehicles, various forms of rubbish, and overgrown plant life. The minimum distance required between buildings to allow good ventilation and dissemination of dust and microorganisms rather than transmission is open to some debate; 30 metres is a figure often stated in the West. Compared to this, the 1-2 metres often seen between farm buildings in Hong Kong (Fig. 2) and South-East Asia is clearly insufficient, and the insufficiency is compounded by the disorganized piles of rubbish found in this area.

It is arguable that the pressures of inadequate space in densely populated countries force farmers to overstock their premises. But while these pressures exist in east Asian countries, they do not generally exist to the same extent in, say, the Middle East and Africa yet farmers there often overstock their houses and allow insufficient space between buildings. This is attributable to the inability of uneducated farmers to view their stock and their husbandry in a holistic way. They cannot see the relationship between overstocking, dirty conditions and disease. Nor can they comprehend an epidemiological link between disease occurring on two adjacent farms.

Feed

The best feeds are imported to Hong Kong or milled locally from imported ingredients under strict quality control; they are correspondingly expensive. Cheaper products are generally more popular but are of inferior quality particularly in terms of protein content and vitamin additives. Some farmers, notably those with just a few free-range chickens, feed loose corn, rice and other grains.

In recent years aflatoxicosis has been identified in many TDCs as a major problem. Chronic low-level exposure to aflatoxins results in mild liver pathology. Such birds are probably immunosuppressed (40), resulting in reduced responses to vaccines and increased susceptibility to field viruses.

Handling

Farming in the New Territories of Hong Kong and in most TDCs is labour-intensive. As labour is cheap, this is not a major drain on the rural economy. However, chickens are handled with remarkable frequency: moving from hatchery to farm; from pen to pen; debeaking; injection of hormone pellets (now officially illegal) and for numerous vaccinations. The effects of handling stress, seen in the reduced productivity of egg-laying birds, is probably also translated into increased susceptibility to ND.

Climate

In TDCs generally, ND seems to occur throughout the year, although incidence may be higher during inclement weather or in periods of intense heat or extreme cold. In Hong Kong the main seasonal incidence of ND is in January and February, when maximum daily temperature in the New Territories can be as low as 4°C and

the overnight minimum might be below 0°C. In the Philippines ND is more frequent in the summer when typhoons and heavy rains occur. In South Korea, a country with harsh winters, the period of peak occurrence is April to July, i.e. late spring, early summer (41).

However, assessing the direct significance of climate in the occurrence of ND is difficult. The peak occurrence in Hong Kong corresponds with the period just prior to the Chinese New Year when farm stock is increased. The extra density of the chicken population will facilitate spread and the valuable stock will receive mesogenic vaccine with possible clinical side-effects. Similarly, in South Korea the April to July period of increased outbreaks of ND coincides with sharply increased production of broiler chickens commencing about April in expectation of important festivals.

These contrasting situations suggest that while adverse climatic conditions and inferior housing might exacerbate susceptibility to indigenous NDV in TDCs, widespread movement of poultry and increases in stocking rates might be more important factors contributing to the number and severity of ND outbreaks.

PERSISTENCE AND SPREAD

Clustering of farms

As already noted, farms in the New Territories occur in clusters (Fig. 4). Premises within a cluster are usually separated only by wire mesh fences. Birds on neighbouring premises are effectively in contact and houses holding different flocks within one farm are separated only by a few feet (Fig. 10). Sometimes flocks of different ages are penned inside a single house (Fig. 11). ND is highly contagious and, under such advantageous conditions, will spread rapidly from flock to flock and from farm to farm. The only barriers to its progress would be greater separation of premises or confrontation with birds of uniformly-high immune status, neither of which is likely.

The proximity of flocks of different age, and hence of different vaccination status, within individual farms and within farm clusters not only facilitates spread; the possibility of persistence of virus by perpetual rotation through a cluster of farms becomes very real. Zander (42) has emphasized the ability of diseases to remain endemic within such farm clusters ("megafarms").

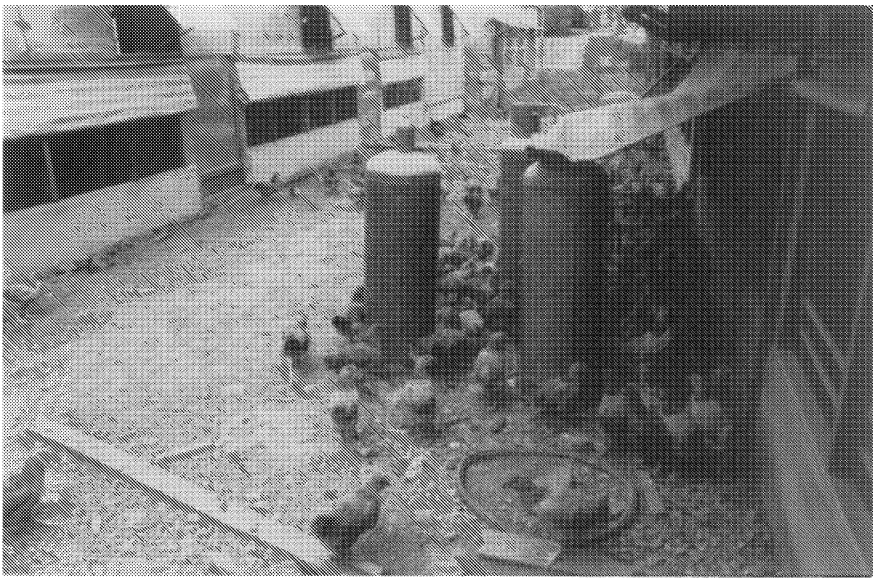

Fig. 10. The space between these chicken houses is about 4 metres. It is cluttered with gas tanks for brooders and some rubbish, yet is used as a free-range area. The free-range chickens will easily transfer infectious agents and vaccine virus between birds held in the adjacent houses.

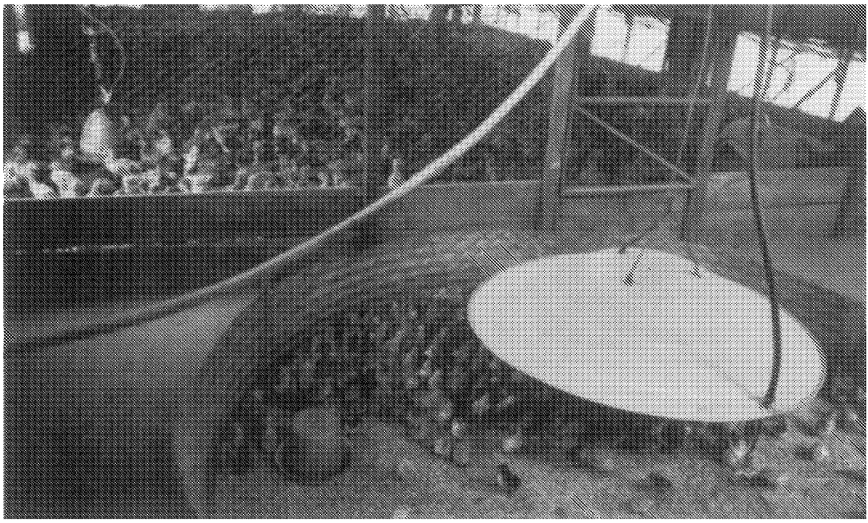

Fig. 11. Brooding chicks adjacent to a flock about 21 days old. The older birds are already somewhat overcrowded, and will soon be receiving mesogenic ND vaccine which will easily spread to the younger flock.

It is essential that stocking, vaccination, treatment and eradication programmes be coordinated within all units of a farm cluster.

Some other factors will, from time to time, assist spread of ND in the New Territories. These include wind-borne spread, spread by wild mammalian and avian vectors, fomite spread by human vectors, fomite spread by vehicles or in infected litter and feed, and spread by transportation of infected stock. However the evidence suggests that the most important single factor in persistence and spread is the physical relationship of farm premises. Alterations and improvements will require a total restructuring of the rural, social infrastructure of southern China.

Undoubtedly the proximity of farms and the overall density of the chicken population are major factors in the persistence and spread of NDV within TDCs. This is emphasized by the structure of the poultry industry in those TDCs where ND is not a problem. These are usually large countries with relatively small human populations living in isolated villages; isolation can be by virtue of distance, poor transportation facilities, or physical barriers such as hills. Papua New Guinea is an excellent example of the value of physical barriers. Here, remote villages rear sufficient chickens for local needs; since the villages are isolated in inaccessible valleys ND does not occur and should there be an outbreak it would not spread. The effectiveness of the sea as a physical barrier is exemplified by the Fiji islands where ND does not occur.

Countries in which ND does not occur by virtue of physical isolation usually do not practice vaccination. Should NDV gain access to those susceptible poultry it will cause widespread mortality. The veterinary services must remain alert to this possibility, and must have the resources to impose efficient control measures, including compulsory slaughter of affected flocks and ring vaccination, to contain outbreaks.

THE VILLAGE CHICKEN

As the rural element of the New Territories disappears, traditional villages are becoming fewer. It is now rare to see chickens scavenging around villages and, as such, these chickens

can have little effect on the epidemiology of ND. In some TDCs, including Indonesia and many African countries, the village chicken is economically very important. Indeed, in Indonesia, it is estimated that village chickens constitute a national flock of about 90 million birds (43). Serological evidence suggests that there is widespread exposure to NDV (44). The birds act as susceptible hosts, with facility to spread the virus widely as they wander the village premises. Vaccination is practiced either not at all or on a primitive basis - vaccine virus viability is not assured and administration is uncontrolled, and it is invariably impossible to round up all birds for vaccination. Current research is centred on attempts to develop a stable, non-pathogenic vaccine strain with capacity for efficient lateral spread.

In some TDCs the free-range village chicken is believed to be a reservoir of NDV. Elsewhere infection has been attributed to contact with infected wild birds. In many primitive countries free-range chickens are given as gifts when villagers travel to visit friends and relatives and this might be a mechanism for dissemination of ND.

It is apparent that the potential contribution of the village chicken to the epidemiology of ND is great and that the social and veterinary problems in reducing this role will be formidable.

CONTROL OF OUTBREAKS

In the field

ND is a notifiable disease in Hong Kong as it is in most TDCs. Since enforcement would impose hardship on owners of affected farms and would require considerable resources, the notifiable status of ND is essentially ignored. This is probably a major difference between Hong Kong and other TDCs in many of which legislation aimed at controlling animal disease is vigorously enforced.

It is important to realize that in traditional Asian rural communities considerable stigma is attached to having diseased farm stock. It suggests bad fortune, a result of wrong spiritual ambience of the owner or his premises. Bad management could also be implied. A farmer will go to great lengths to conceal his bad fortune or any possible reflections of his poor husbandry.

A common sequence of reaction to occurrence of disease is: i. treat with antibiotics and sometimes with "traditional" medicines;

ii. vaccinate; iii. send the birds to market. Failing recovery, and assuming the farmer does not dispose of the birds through the market (he might want to wait for an impending festival when prices will be higher) he might seek advice. An extension officer of the Agriculture and Fisheries Department will visit, make a field diagnosis and submit samples to the veterinary laboratory. There is no charge for these services when applied to a notifiable disease. Advice will include: the need to isolate affected flocks from unaffected flocks - usually impossible within one farm or cluster of farms; a request not to move birds to other farms or markets and pointers on how to avoid fomite spread - often ignored; a recommendation to slaughter affected flocks in cases of severe disease - almost invariably ignored; suggestions to vaccinate neighbouring unaffected flocks; and suggestions for future improvement of vaccination programmes. As this advice is regarded by many farmers as too much to achieve and is requested too late to be effective, its impact is small.

Extension services

The extension officer is the link between the farmer and both the diagnostic and regulatory arms of the veterinary services. It is he who is responsible for initial field diagnosis, referring samples to the diagnostic laboratory, monitoring the disease situation and on-site implementation of control measures. The combined efforts of the extension services and the veterinary diagnostic laboratories are the key to disease surveillance in TDCs.

It is of paramount importance that extension officers are adequately trained in disease recognition, vaccine storage and administration, hygiene and proper record keeping. It is also vital that the extension officers develop relationships of trust and respect with the farmers and through this impress the need for correct use of vaccines, improvement in hygiene and early diagnosis should disease occur.

Laboratory diagnosis

Hong Kong has a well-developed agricultural extension service and a well-equipped veterinary diagnostic laboratory. As the Territory is small, advice and investigation services can be obtained within a few hours of reporting disease. Diagnosis is based initially on flock history and clinical signs, later on the results of veterinary laboratory tests including autopsy, serology

(HI tests) and isolation of virus in fertile eggs.

Interpretation of serology and virus isolation presents problems not encountered in temperate countries. In the face of hypervaccination, many birds develop very high serum antibody titres. Thus, it is impossible to differentiate between vaccine-induced and infection-induced antibodies.

The significance of isolating embryo-lethal NDV is clouded by the widespread excessive use of mesogenic vaccines. Skilled questioning to obtain detailed case histories often reveals that farmers have vaccinated birds when signs of disease were first apparent seeking advice several days later when the situation became uncontrollable. Reagents enabling rapid distinction between field and vaccine viruses, and of strains of field virus are urgently required.

Hong Kong and Singapore are probably the only TDCs wherein the entire territory can be served by a single veterinary laboratory. However, many TDCs boast a well-structured network of regional laboratories capable of autopsy, serology, bacteriology and parasitology and some doing virus isolation in fertile eggs. Cases requiring more detailed or sophisticated procedures will be sent to a central reference laboratory which is usually located in the capital or in the area of most intensive agricultural development, or sometimes to university veterinary colleges. This structure of veterinary diagnostic facilities and its integration into larger extension services is a vital aspect of animal disease control and an important source of disease statistics in TDCs.

RECOMMENDATIONS

Research directions

With the advent of more precise genetic and antigenic analytical techniques, it is now possible to obtain far greater information about the virus and its immunogenic sites. Techniques applicable to NDV include oligonucleotide mapping (45), one dimensional peptide mapping (46) and the use of monoclonal antibodies (47). Monoclonal antibodies were successfully employed in tracing an outbreak of ND in chickens in the United Kingdom in 1984 to a virus derived from racing pigeons (48).

Thus, it may now be possible to answer important questions relevant to the control of ND in TDCs (and elsewhere). These

questions include -

1. Are there relevant, epidemiologically important differences between the NDV of TDCs and temperate regions?
2. What is the relevance of apparently avirulent field isolates?
3. What is the relevance of highly virulent (under laboratory conditions) strains isolated from asymptomatically infected poultry?
4. Are the vaccine strains currently used in TDCs appropriate? Can they cause clinical ND?
5. What is the effect of laboratory passage on the antigenic, immunogenic and pathological properties of vaccine and other strains?

Central to these questions is the need for a better understanding of the relationship between mesogenic and velogenic strains and whether "true" mesogenic strains (as opposed to naturally attenuated velogenic strains) exist at all. Current research indicates that passage of mesogenic strains through chicks leads to an increase in virulence of a small proportion of the virus population (49). This supports our contention that in Hong Kong constant passage through neighbouring flocks, plus interacting stress factors, might favour the emergence of virulent virus. Experimental observations in the laboratory will be difficult to translate to the field situation where variable levels of maternally-derived or vaccine-induced antibodies will undoubtedly affect the ecology of NDV and here the use of monoclonal antibodies as indicators of virulence has considerable potential.

The problem remaining is that of transfer to TDCs of appropriate recent technology. New techniques of molecular biology or methods using monoclonal antibodies can require sophisticated equipment and appropriately trained technicians. Such technology is beyond the reach of local diagnostic laboratories. A programme of technical development aimed at establishing new methodology in central or regional reference laboratories would have a considerable impact on the evaluation of virus isolates in an epidemiological context.

Recommendations for control

We have attributed the high incidence of ND in Hong Kong and other TDCs to the inadequacy of measures to rationalize husbandry and marketing measures related to ND and to control outbreaks. ND

would be reduced by introducing or enforcing the following:

1. Rational vaccination by monitoring and controlling the importation, sale, possession and use of vaccines. This would include: licencing for sale only vaccines of known potency within strains believed to be suitable for local use; licencing premises importing and selling ND vaccines, qualification for licences including provision of adequate cold storage subject to regular inspection. Limiting commercial availability often means having to offer an alternative "official" product free or heavily subsidized, but this in turn is a lever to controlling abuse through professional vaccinators and extension officers who administer the subsidized vaccines.

2. Restriction orders should be placed on infected premises to prevent movement of affected and in-contact stock, and contact with potential fomite vectors. In the case of outbreaks occurring on farms situated within larger clusters or village units, then the village or cluster of farms, rather than the individual farm itself, would have to be treated as the infected premises. Penalties for failing to inform of the occurrence of new outbreaks or to observe the restrictions imposed should be severe. In reality a balance must be struck between relaxed enforcement, aimed at helping individual farmers, and stringent control aimed at preventing spread and protecting the national flock.

3. In outbreaks of severe disease with potential for rapid and economically damaging spread, compulsory slaughter should be undertaken.

4. Upgrading of hatcheries is essential and should be aimed at improved hygiene together with control over mixing of hatches in order to minimize differences in passive immunity within flocks.

5. Better husbandry should be encouraged in terms of improved buildings with better ventilation and cleaner environment, greater isolation of flocks and farms, and higher quality of feed. While vaccination is effective in temperate countries where low flock densities and well segregated farms ensure that exposure to NDV is low, it will fail where poultry movements and high farm density may give rise to endemic infection. Reducing contact between host and virus is a more effective way of preventing and eradicating vaccine-induced immunity of unpredictable strength and time-span. Thus, the improvement of husbandry methods and the development of

effective zoo-sanitary routine is as important, or even more important, than the perfection of vaccination techniques. This can be achieved by farmer education through extension services and farmer associations, and by offering subsidy incentives to farmers, millers and hatchery men.

6. The use of extension services and veterinary diagnostic facilities for early diagnosis, advice on treatment, eradication and prevention, surveillance and eradication of intercurrent diseases, and possibly for serological monitoring of vaccine efficacy must be encouraged. In most cases this means that such services must be free. However, the cost to enforcement agencies is small compared with the body of information obtained and the potential for effective control of ND.

These suggestions, though made with Hong Kong in mind, hold for most TDCs. In addition, some general points can be made. It is important that animal disease control regulations are tailored to the country concerned. There is no point in attempting to apply legislation used in, say, the United Kingdom to other countries. Such attempts have failed in the past and will continue to fail because they are generally impractical, unacceptable to many, and often irrelevant. However, provided the approach is appropriate and is well organized there is no reason why ND should be as devastating as it is in many TDCs. Given sufficient funds, legal backing, logistical support and staff motivation ND can be controlled and eliminated.

ACKNOWLEDGEMENTS

The authors are grateful to the following who have provided information on the situation in various countries: N.H. Christensen, S.B. Dhanapala, D.W. Francis, M.H. Hakim, N.H. Heng, D. Hoffman, R.F. Horner, F.W. Huchzermeyer, I. McIntyre, G. Mukiibi-Muka, P.I. Orafu, S.Z. Ou, R. Rattanarajchatikul, P. Saville, N.B. Singh, J.W. Thompson, M.C. Wigglesworth, A.J. Wilson and P. Young. R.B. Bousfield, K.S. Lo, Y.C. Wong, K.L. Tse and C.C. Chan are thanked for providing information concerning Hong Kong. The map was drawn by S. Lam and the chapter was typed by Miss W. Fok.

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16

NEWCASTLE DISEASE : CONTROL POLICIES

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INTRODUCTION

Control policies for Newcastle disease (ND), depend on the specific requirements of the poultry industry in different countries. In most of them, ND and Avian Influenza, are the main diseases which are considered as very dangerous for poultry and hence are submitted to regulations which aim to prevent their introduction, to limit spread and to lead to their eradication.

Most of these control measures originated at a time when the disease was recognised only as a severe form usually termed plagues or pests. But since the first description of ND, knowledge has progressed and control methods implemented have been adapted to the evolution of the poultry industry and greater understanding of the virus. Each new outbreak of disease has helped to define the most suitable methods of control according to the epizootiological context and poultry production characteristics of each country.

Sanitary concepts were, for many years, limited to hens and chickens but over the past years have been extended to other avian species due to development of their production -for example the great increase in turkey production, or due to their sensitivity to certain Newcastle disease viruses- pigeons for example.

Because the development of the international trade in live and dead poultry increases the potential risks of introduction of Newcastle disease virus (NDV), certain countries have used

this argument to impose arbitrary restrictive measures in order to limit live and/or dead poultry importations. Consequently, poultry exporting countries particularly fear the occurence of ND in their own country as this could lead to indirect economic losses, due to the embargo measures that can be taken, which may be more important than direct losses caused by the virus in the flocks themselves.

A few other countries, generally where poultry production is not of prime importance, base their entire ND prophylaxis on sanitary measures, prohibiting vaccination, and are thus more vulnerable to risks of occurence of the disease, the origin of which can be domestic, pet or wild birds.

Many factors such as the diversity of poultry production structures in each country, the volume of live or dead birds imported and/or exported, the geographical situation, the sanitary status of poultry flocks, must be considered by State Veterinary Authorities to define the best control policies for ND the country involved.

CONTROL OF THE DISEASE AT INTERNATIONAL LEVEL

Definition of the disease

In view of the variations in disease caused by different strains in different hosts, assessment of the pathogenicity of PMV-1 virus isolates should be based on laboratory findings.

Once it was evident that different strains of NDV showed differences in virulence for chickens and other susceptible hosts, both in natural and experimental infections, it became important that reproducible tests were developed to enable distinction between isolates on the basis of their virulence (3, 4, 11, 26).

Ideally, a quick reproducible in vitro test would be most useful but, so far, none has been developed, in vivo tests are, therefore, usually employed (16, 24, 27, 35). As a result embryo mean death time and intracerebral and intravenous pathogenicity indices are still employed as the international reference methods, although new diagnostic reagents such as monoclonal antibodies

indicate promising potential which eventually may allow more accurate and rapid results (1).

The intracerebral pathogenicity index (ICPI) test, provided that it is carried out strictly according the method oultlined by National Academy of Science (27), gives the most reliable results. In this method 10 one-day-old chicks are inoculated intracerebrally with 0.05 ml of a 1/10 dilution of fresh infectious allantoic fluid, at a recommended haemagglutinin (HA) titre greater than $\log_2 4$, and are observed for disease and death over an eight day period.

In 1985 the European Pharmacopeia recommended inoculation of $10^{5.7}$ 50 % infectious doses (EID_{50}) in 0.05 ml for screening vaccines strains (2). However, as NDV may grow to titres as high as 10^9 EID_{50} in 0.05 ml, this dilution may lead to the elimination of any minority virulent subpopulation that may be present in the initial material.

To define, on an international basis, an ICPI value above which an NDV strain must be considered as virulent is very difficult : a strain classified on the basis of ICPI value as virulent in one country may be used as vaccine in an other country where the epizootiological situation needs the use of more virulent strains.

Nevertheless, in most countries where there is a developed poultry industry, the mesogenic strains are considered as virulent and their use as vaccines is banned. For this reason, in countries where vaccination with inactived or live lentogenic vaccines only is allowed, the ICPI value of 0.7 is considered the upper limit for a non-virulent strain.

Some countries, where vaccination is forbidden, consider that this limit must be lower, using an ICPI value of 0.3, and require special measures for the importation of live or dead poultry from countries using lentogenic vaccines of ICPI values up to 0.7. However on an international basis, it would appear impracticable to take into account the potential threat of strains of ICPI value between 0.4 and 0.7.

Consequently, whether or not there are signs of disease, if a NDV is isolated from birds and if it has an ICPI greater than 0.7, these birds should be considered as infected with a virulent NDV and the disease should be reported.

Reportable disease

In all developed countries Newcastle disease must be a reportable disease (21,39). It is only by open and full declaration of outbreaks that this disease will be brought under international control. Countries should imperatively participate fully in membership of agencies, such as the Office International of Epizooties (OIE) whose objectives include the recording of outbreaks of diseases, such as ND, on an international basis.

As the declaration of the disease needs virus isolation, identification and assessment of virulence, each country must have diagnosis facilities under supervision of State Veterinary Services and acceptable monitoring and reporting programs.

Outbreaks of NDV to be reported to the international agency, OIE, should be considered to be every flock from which a PMV-1 virus is isolated which has an ICPI greater than 0.7.

Conversely, a country or a defined region, depending of the size of the country, is considered free of ND if no virus of an ICPI greater than 0.7 has been isolated from domestic poultry for at least one year, or six months if a stamping out policy is practised.

If ND appears in an country free of the disease, following importation of birds, the responsibility of the exporting country, must be involved in case of lack of declaration of ND outbreak by the Veterinary Services of this country.

Import and export restrictions on live birds, poultry manure, vaccines, poultry products and on movement of personnel

Trade and movement of live birds

Due to the efficiency of modern transport it is possible for infected poultry to be moved over long distances in a very short time.

The risk of infection is maximum during the incubating stage

of disease when no clinical signs are evident. Infection in transit or by mixing of birds of different sources in the same transport vehicle (especially aircraft) must also be considered (25,30).

The trade of live domestic birds must be limited except, for valuable genetic stocks, to one day old birds or, preferably, fertile eggs as these constitute the minimum risk.

If the exporting country is not free of ND according to the previous definition, the State Veterinary Services should certify that the flock(s) of origin have been inspected and found free of ND and that the premises are located in area of a minimum radius of 20 km where no outbreak of ND occured during the previous 2 months.

Additionally, serological samples with negative results should be required from the flock of origin in the case of a non-vaccination policy in the exporting country. If vaccination is practised, only lentogenic live or inactived vaccines must be allowed and the flock of origin vaccinated more than two weeks before collecting eggs.

Live domestic birds must be moved from a single source, as one cargo with a single destination and should be quarantined at destination.

Non-domestic live birds may be infected with NDV and certainly have the ability to carry and excrete virus with little or no evidence of disease (14). Game birds, such as pheasants and partridges imported for the shooting season in some countries, represent a very considerable risk (7, 13, 41).

In spite of the fact that the isolation of NDV from wild birds have been usually restricted to lentogenic viruses, except at times of epizootics in domestic poultry, the potential spread from feral bird is sufficiently high to recommend taking considerable measures to prevent contact between domestic and wild birds (5, 11, 30, 36, 37, 38).

Among wild birds it must to be pointed out that psittacines, either colonies of free living or pet psittacines originating from tropical forests, are considered as a major source of very

virulent NDV strains (15, 17, 22, 43).

Racing pigeons, in view of the large distances travelled during races and the international nature of pigeon racing and trading, also represent a high risk for the introduction of NDV into country or area (6, 34).

Game birds and pet birds should be quarantined at arrival in the importing country, but quarantine need not be applied to show and racing pigeons and doves, provided these birds, before attending shows or races beginning or terminating outside the country of origin, are fully vaccinated, using inactivated vaccines according to the manufacturers'recommendations.

Poultry manure

In many countries untreated or merely dried poultry faeces may be used as a constituent of crop fertilizer. There are some instances of export of such material which represents a risk of ND virus spread, this trade should be not recommended (9, 30).

Vaccines

Vaccine production is a large international industry and, although standards of production are very high, accidental contamination is a possibility for live vaccine and partial inactivation of viral particles is a possibility for inactived vaccines prepared with virulent ND strains (8, 10, 40). A control policy and a State Laboratory control of vaccines must exist in each country for both vaccines manufactured in that country or imported vaccines and as far as possible, inactived vaccines should be prepared with non-virulent strains.

Trade in dead birds and poultry products.

Regulatory control at international level for poultry products is necessary if trade exists between countries. But the objective should be to allow trade without restriction between countries provided this does not result in the spread or potential spread of contagious disease.

As overtly sick birds are, theoretically, not slaughtered, the potential risk mainly concerns carcases contaminated by faeces or viraemic birds slaughtered during the incubation period,

reaching susceptible poultry by feeding offal or uncooked scraps to backyard flocks (18, 23, 32).

In fact, the potential risk of exporting poultry products from the first flocks to be infected by NDV, in a country free of ND up to that time, which were slaughtered during the incubation period is pratically nil. For this reason there should be freedom of movement of poultry products from all countries free of ND as defined above, to all other countries.

If a country is not free of disease, all exportations of all poultry products originated from flocks located in the infected area(s) must be prohibited.

Some countries where ND vaccination is banned, taking in account the great susceptibility of their poultry flocks, have specific requirements in order to prove that the poultry products imported are free of all NDV infection, despite the fact that the contamination of poultry products by lentogenic strains which may include vaccines, is of low incidence, because the low titres to which these viruses replicate in birds and the relatively high temperature used during the processing.

Nevertheless, these countries also consider that there is a risk of transitory inapparent infection of vaccinated flocks with a virulent NDV and consequently require that poultry meat must be imported only if originating from unvaccinated flocks showing neither signs of disease nor positive HI titres within 7 days of export, or from vaccinated birds showing no disease and from which virus isolation attempts are negative.

For table eggs, as the disease is not considered to be egg transmitted, no restriction are imposed by these countries.

Control of movement of personnel. The major risk concerns consultants visiting poultry farms in countries where ND is enzootic subsequently visiting susceptible birds in a different country (42).

As controls of movement of personnel on an international basis appears very difficult to manage, specific measures must be taken at a national level.

CONTROL OF THE DISEASE AT NATIONAL LEVEL

Where no ND exists in a country or area, control should be aimed at prevention of introduction of disease which may or may not be linked to a vaccination policy.

Quarantine

Quarantine premises for imported poultry, game birds or commercial exotic birds preferably should be run by the national government, if private they should be under strict government supervision. The quarantine period should be at least 35 days. Specific-pathogen-free chickens should be kept in the same air and examined for seroconversion after 28 days if any ND vaccination is practised in the quarantine.

All non domestic birds should have at least one faeces or cloacal swab tested for the presence of virus except for very small birds or large numbers of birds for which it would be sufficient to remove fresh faeces from the cage. Faeces samples or cloacal swabs from birds in the same cage may be pooled for virus isolation (27). All birds dying in quarantine should be tested for virus isolation. Samples from dead birds from the same cage may be pooled.

The quarantine premises should be secure, bird and vermin proof, capable of beeing easily cleaned and disinfected, all air outlets should be fitted with a dust filter, personnel should shower and change clothes, vehicles must be disinfected.

A buffer zone around the quarantine delimited by a wire fence, should be employed to prohibit access to the bird houses by foreign personnel or vehicles.

If pathogenic NDV of ICPI greater than 0.7 is detected all birds on the premises should be slaughtered and disposed of, but an infected area need not be declared.

Monitoring and reporting system

As mentioned above, a good organisation of veterinary services including an efficient monitoring and reporting system is essential to prevent the spread of ND from the affected premises, by quick diagnosis and application of a stamping out policy.

Each state must have diagnositic facilities related to the importance of the poultry industry and at least one or more national reference laboratory at which facilities and expert personnel should be maintained to allow full antigenic and biological typing of avian paramyxoviruses (20, 29, 31, 33, 43).

While waiting caracterisation of the virulence of the ND virus isolate, all measures must be taken if ND infection is suspected, to avoid spread of the virus from the farm and to prepare for the slaughter of the birds.

In order to be sure that farmers rapidly inform the State Veterinary Services of the suspicion of ND, they must be fully and quickly compensated by the State for the eventual slaughter of their birds.

Slaughter policy

All countries should adopt a slaughter policy for all birds on site and properly dispose of the birds and all products (e.g. eggs, food, manure).

To kill the birds the most efficient and humane process must be employed, involving the minimum amount of movement of the birds while they are alive. Different gasses have been employed when poultry houses are suitable for their use e.g. cyanide, methylbromide, carbone dioxide, however the gas used may depend on the laws of the country (43).

Once birds are killed minimum movement of carcases is desirable. Burial on site in deep-pits to which calcium oxide is added has proved effective, but some countries have used burning on site, while others have available specially designed air-tight trucks to transport carcases to local incinerators. Disposal of contaminated faeces or litter is also a considerable problem especially in deep-pit houses. Burial with calcium oxide or sterilization by heat or chemical treatment are possible alternative methods.

Cleaning and disinfection is important on all premises where NDV has been demonstrated ; following depopulation either by slaughter or normal marketing, the premises must be thoroughly

cleaned and disinfected before restocking takes place.

These cleaning and disinfection procedures must be supervised by state employed inspectors and the disinfectants used must be tested and approved for use against NDV (44).

Infected area

Definition of the infected area.

The infected area should be between ten to twenty kilometres radius around the infected premises. The recommendation for setting up an infected area should not be interpreted too dogmatically and a 10 to 20 kilometre radius should be regarded as a minimum requirement. Where local conditions such as topography of the land or prevailing winds or nearness of poultry houses make it necessary, larger and non-circular areas should be designated infected areas. Similarly the extension of an infected area to include feed mills, hatchery or slaughter plant can increase the efficiency of the eradication of the disease.

Measures applied in the infected area.

Within the area all movement of birds and poultry products must be controlled by state authorities under the supervision of Veterinary Services.

Meat birds declared healthy at a clinical inspection must be processed at the nearest slaughterhouse, if possible, and at the end of the day before the disinfection process of the slaughterhouse.

As NDV is not usually considered to be egg transmitted, table eggs could be sold without special restriction. Fertile eggs from healthy breeders must be incubated and hatched separately and birds originating from these eggs should be submitted to inspection by the State Veterinary Services to make sure that these birds show no evidence of NDV infection.

In infected areas local poultry markets must be prohibited.

Restrictions on infected areas should be removed when at least 21 days have elapsed since a stamping out policy and disinfection of on the infected premises have been completed.

Ring vaccination.

Where there is evidence of spread of pathogenic NDV to more than one farm, ring vaccination with live lentogenic viruses should be considered and if decided on administered compulsory to all commercial poultry stocks within the infected areas around the farm.

In countries not normally using vaccination, applying ring vaccination, a further buffer zone determined by a 15 km radius from the affected farms and treated as an infected area will be set up.

Vaccination policy

Vaccination policies depend on an appreciation of the risk of introduction of the NDV and of the effect of direct and indirect potential economic losses due to the disease on the poultry industry of the country.

The majority of countries have a vaccination policy, either to vaccinate all poultry flocks or vaccination of breeders and layers only, excluding meat birds which are reared only for a few weeks.

In these countries inactived and live lentogenic vaccines are used, in some countries a low ICPI of 0.3 or less, is required for lentogenic vaccines (8, 12, 19).

CONTROL OF THE DISEASE AT FARM LEVEL

Economic considerations have led to the development of poultry industries in particular areas or regions of different countries, which increase the risk of spread of infectious diseases.

Measures taken in order to prevent the disease are to a large extent governed by the method by which the organism responsible is spread. NDV strains show variations not only in virulence and tissue tropism but also in the major routes of spread during different epizootics (30).

Transport of virus particles by wind has been reported to be extremely important during epizootics of ND (28). The spread is correlated with local factors as direction and spead of wind,

temperature, relative humidity and topography of the land. Affected birds or contaminated dust, dried faeces are involved in the spread of the virus. It is pratically impossible to prevent airborne spread of viruses except with controlled environment housing possessing filtration of air and positive pressure. Such equipment and the running costs involved are so expensive, that they are usually limited to valuable genetic stocks, grandparents and parents stocks.

The other potential risks of infection can be more easily prevented by classical measures aiming to limit the movement of personnel and vehicles. Changing of clothes and disinfection of vehicles should be routinely practised even in the absence of disease.

Man may carry infectious NDV, both as an infection and as a passive vector with contaminated clothing or shoes. The personnel concerned may be technicians who move from farm to farm or staff of companies or experts and specialists who move from country to country. Of these perhaps the most potentially important group are those who are involved in giving expert advice at the farm level in countries where NDV is endemic.

Contaminated food and water are also a potential source of infection. The spread of NDV to poultry by food contaminated by feral pigeons was clearly demonstrated recently in Great Britain. Some ingredients used in poultry food originate in countries where ND is endemic in domestic and wild animals may represent a potential risk of infection.

It would seem wise to subject all poultry food to heat treatment at least equivalent to that experienced during the pelleting of food and to ensure that at all times the food is stored and transported under bird-proofed condition.

Poultry houses must also be, as far as possible, under wild bird proofed conditions and easy to clean and disinfect.

One-day-old birds for re-stocking farms must have originated from hatcheries and breeders under the supervision of State Veterinary Services. One species and one age of birds should be

reared on a single farm to avoid contamination by trucks passing to and from the slaughterhouse. In case of layers flocks egg trays must be disinfected.

CONCLUSION

As ND is endemic in many countries and occurs sporadically in others, this disease constitutes a permanent threat for domestic birds.

For this reason the knowledge of the sanitary situation in each country and the control policies concerning the prevention of infection must be considered of outstanding importance for the poultry industry.

The active cooperation between poultrymen, technicians, veterinarians, the diagnostic laboratories under supervision of a reference laboratory and the State Veterinary Services, is essential to succeed in the prevention of ND and in its eradication.

ACKNOWLEDGMENTS

Although the opinions and views expressed in this chapter are entirely his own in the preparation of this chapter the author has drawn heavily on the discussions and recommendations of the European Economic Community Scientific Group of private experts on Contagious Diseases of Poultry. The Group consisted of : Prof. Dr. J.B. McFerran, Dr. D.J. Alexander, Dr. G. Bennejean, Prof. Dr. E.F. Kaleta, Dr. B. Kouwenhoven, Dr. G. Meulemans, Dr. T.M. Murphy, Dr. M. Guven, Prof. Dr. R. Rott and Dr. F. Contardo. The report and recommendations of this Group to the EEC Scientific Veterinary Commitee will be published in due course.

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CONTROL BY VACCINATION

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INTRODUCTION

The control of Newcastle disease (ND) is based on complementary hygienic and medical measures. Full protection can only be assured if vaccination programmes are combined with commonsense hygienic precautions.

Immunity against ND is due primarily to antibody or activity directed against the two viral glycoproteins; the haemagglutinin-neuraminidase (HN) and the fusion (F) proteins (66). Resistance to infection is usually associated with the presence of moderate to high titre of neutralizing and haemagglutination inhibiting (HI) antibodies. The haemagglutination-inhibition test (HI) is a reliable and practical serologic indicator of immunity (12).

Antibodies appear in the serum and, in case of vaccination with live virus, in local secretions, 6 to 10 days after vaccination and persist for various periods of time. Early protection following vaccination can be demonstrated in the presence of low levels of antibody or in their absence (9, 47). This protection can be explained by the rapid onset of a cell-mediated response. Cell-mediated immunity is indeed the first immunological response and can be demonstrated as early as 2 days after vaccination (40, 92).

Infectious diseases that reduce the immune response of chickens can have a marked influence on success of immunization against ND. Infection of day-old chicks with infectious bursal disease virus depress the ability of the chicks to respond to inactivated or to live ND vaccines administered three weeks later (5, 36, 37). This immunodepressive effect of infectious bursal disease virus on ND vaccination can be avoided by vaccinating the chicks against ND at day-old or by using attenuated strains of infectious bursal disease virus in all commercial vaccines (65). Genetic differences between chicken lines (78) and social stress (70) can also be responsible for differences in immune response to ND vaccination.

Vaccination against ND can be performed using either live or inactivated vaccines.

VACCINATION USING LIVE VACCINES

Vaccinal strains

Live virus ND vaccines have been used by the poultry industry for more than 30 years. Whereas mesogenic strains such as the Roakin and Van Roekel strains were employed in the early years, the less virulent lentogenic strains now predominate (50). Their effectiveness and safety when used under field conditions were responsible for this transition. Different lentogenic strains have been used during this time (102); however Hitchner B1 and La Sota strains are the strains of choice and have proven to be highly efficient on a world wide base.

As Hitchner (53) explained, the exact origin of the B1 strain is not known. Beaudette had identified this virus strain as an infectious bronchitis virus and sent it to Hitchner who later demonstrated it was a non pathogenic ND virus capable of inducing immunity against ND in susceptible chickens (54). The La Sota strain was isolated in 1946 by Beaudette in New Jersey. This virus was shown by Beaudette (13) and by veterinarians working at the Vineland Poultry Laboratories as being a good vaccine when administered by intramuscular, intranasal or drinking water routes (46).

The efficiency of a live virus vaccine depends on its invasiveness and its power to multiply sufficiently within the chicken to set up an adequate immune response (3). In general, La Sota vaccines give better protection than B1 vaccines but variations were found between products prepared from the same strain depending on their source (17, 32, 91, 103). The La Sota strain has also a greater tendency than the B1 strain to spread from bird to bird within a house (84).

Although vaccination against ND, using B1 and La Sota vaccines, proved to be succesful in most parts of the world, Kaschula and co-workers in Iraq were the first to report difficulty in controlling a virulent strain of strongly pneumotropic ND virus using these vaccines. In order to control the disease, Kaschula and co-workers selected a lentogenic virus designated AG 68L which was able to immunize chickens without causing visible signs of disease or distress when given in the drinking water. This strain was shown to be much more effecitive than the B1 and La Sota strains especially in the presence of maternally derived homologous antibodies (17). A cloned virus derived from the AG 68L strain was found to be significantly more immunogenic than the La Sota virus when administered in the drinking water to either fully susceptible or maternally immune chicks; it was shown to be significantly less

pathogenic than the parent strain and slightly more pathogenic than the La Sota strain when given as an aerosol but without detectable pathogenicity when given in the drinking water (18).

Recently, a thermostable lentogenic Australian virus, isolate V4, was selected and successfully used as vaccine in Malawi where La Sota and Komarov vaccines were often inefficient in controlling ND (83).

Drinking water vaccination using either AG 68L or V4 lentogenic viruses could be highly valuable for developing countries where ND remains a major cause of economic loss; it could replace individual vaccination with live mesogenic vaccines.

Live mesogenic vaccines such as the Roakin, Komarov, Herts and Mukteswar strains are still widely used throughout Africa, the Middle East and S.E. Asia. These strains are pathogenic for chickens under 8 weeks of age, nor are they recommended for adult birds not previously immunized using lentogenic vaccine. The use of live mesogenic vaccines should therefore be discouraged, they could undoubtedly cause serious disease problems in fully susceptible poultry.

Live freeze-dried vaccines can be stored at 4°C for a year without significant loss of titre. They should never be held above 8°C for more than an hour or two immediately before use, taking care that, during transport of bulk supplies to local storage facilities, the temperature does not exceed this limit (11).

Combined commercial vaccines are available which are used for simultaneous vaccination of chickens against ND and infectious bronchitis (21, 104) or ND and Marek's disease (111). Using these vaccines, no evidence of interference in the inducement of immunity to ND was observed (63, 104, 111).

Administration of live vaccines

Live vaccines can be administered using individual or mass vaccination methods. Individual methods of application produce more consistent protection in more birds than mass vaccination methods. However, individual methods are not economically feasible in broilers and are therefore limited to the early vaccination of replacement layers and breeders if necessary.

Individual methods. Intranasal/Ocular. The vacine is reconstituted with distilled water at room temperature as the use of very cold water may cause severe conjunctivitis (29). A volume of 30 - 35 ml per 1000 vaccine doses usually provide enough to give one drop each per 1000 birds. The drop should be applied from a distance of approximately one centimetre to the open eye or nostril (11).

Beak dipping. One vial of 1000 doses of vaccine is reconstituted in approximately 150 ml water and placed in a shallow dish. The beak of each chick is

dipped, submerging the nostrils for a fraction of seconds. Excessive wetting of the head of the chicks and inhalation of the vaccine should be avoided (11).

The efficiency of the intranasal drop and conjunctival sac instillations were demonstrated by Doll et al. (28). Birds vaccinated intraocularly with the B1 strain are resistant to intraocular challenge with virulent NDV from two to four days after vaccination (47, 98). Day-old vaccination by beak dipping gave protection for at least 8 weeks (96).

Mass methods. Drinking water administration. Drinking water administration is certainly the most common and simplest method of ND vaccine administration. The objective of this procedure is to get as many birds drinking vaccine water within the shortest period of time. Therefore, the birds should be deprived of water for one or two hours before vaccination and adequate trough space should be provided by temporarily installing supplementary drinkers. This is particularly important as many apparent failures of vaccination have been traced to faulty watering systems and, more commonly, inadequate trough space (10).

To avoid losses of virus infectivity, the drinking water containing vaccine should be drunk as soon as possible and the quantity used should take account of the age of the chickens, the local conditions and weather. During the first week of age, 2 to 5 litres of water should be used to vaccinate 1000 chickens. This quantity should be increased afterwards to reach 9 to 11 litres from the 2nd to the 4th week of age, 14 to 18 litres from the 5th to the 7th week and 20 to 23 litres if the chickens are older than 7 weeks. The vaccine should be applied with plastic watering cans directly into drinker and supplementary troughs. The vaccine dose should be split in order to make two to three rounds of the drinkers in the house.

Under normal conditions, vaccinal virus is sufficiently stable in header tank systems for this method to be used for drinking water application of vaccine. Most mains water supplies do not inactivate the vaccine virus, but as the stability of diluted vaccine is dependent on the nature of the water supply, it should not be assumed that virus will be stable for more than four hours under more adverse conditions (6).

Impurities in drinking water have a significant influence on the effectiveness of the vaccine, viral inactivation can be prevented by the addition of one teaspoon dry skim milk powder (DSMP) per 5 litres water (68). Algae and organic matter adsorb vaccine virus, therefore the drinking water system should be cleaned scrupulously without using disinfectants or detergents. Water from copper pipes does not usually contain more than 0.2 ppm copper so water supplies through copper piping are unlikely to be deleterious to ND vaccination by the drinking water route (4). In laboratory trials, different water pipes (black, polyvinyl chloride pipe; Hart cups,

green polyvinyl chloride pipes; Swish cups and old, rusty, galvanized pipes) normally used in cage operations were demonstrated to adversely affect vaccinal virus. DSMP was essential in stabilizing vaccine virus and maintaining an adequate vaccine virus concentration in the three types of pipes (106).

Water vaccination elicits a minimum respiratory reaction as compared to some severe reactions observed after vaccination by aerosol when mild to moderate respiratory signs are observed 4 to 5 days post-vaccination (31). Winterfield and Seadale (100, 101) established under laboratory conditions that the serological response to high virus intake ($>10^6$ EID_{50} per bird) is consistently better than at a lower dosage level; it is therefore desirable to provide at least 10^6 EID_{50} of B1 per bird. The same authors did not observe adverse post vaccination reaction after administration of as much as $2x\ 10^8$ EID_{50} per chicken. Respiratory signs were slight or absent, irrespective of the age of the chickens (100).

Parental immunity against ND can interfere with drinking water vaccination. Chicks 4 days to 2 weeks of age derived from ND vaccinated parent stock and carrying high level of passive immunity give poor immune response to vaccination through the drinking water using the B1 strain (100, 101). Comparing the immune response of broiler chickens vaccinated with B1 and La Sota strains, Woernle and Scholtyssek (105) observed a better protection against challenge with a velogenic ND virus strain in broilers vaccinated with La Sota than in chickens vaccinated with B1. The La Sota strain posses also a slightly greater spreading potential than the B1 strain (31). As a consequence, the La Sota strain can engender immunity in susceptible contact chickens but the immunity so obtained wanes quickly in contrast to immunity due to direct vaccination which persists for weeks (74).

Although the La Sota strain may have a greater spreading potential than the B1, it may be more advantageous to use B1 for primary vaccination and La Sota for subsequent vaccinations because of potentially more severe reactions from the La Sota strain after the primary vaccination. Severe respiratory reactions are undesirable especially in broiler chickens (31).

Spray and aerosol administration. Spray and aerosol vaccinations are presently widely used for primary vaccination and revaccination of chickens against viral respiratory diseases. Approximately 80 % of the commercial broiler companies in the USA vaccinate chicks at day 1 with a cabinet that drops a coarse spray over the birds simulating eye and\or nose drop infection (44).

The main advantages of aerosol and spray vaccinations are that mass application makes it possible to vaccinate a maximum number of chickens in a minimum period of time. The major limitations of these techniques are the difficulties

for standardization and the risk of severe vaccinal reactions, particularly if mycoplasma positive chickens are vaccinated by aerosol (64, 95).

An aerosol may be defined as any system of liquid droplets or solid particles dispersed in air, of fine enough particle size, and consequent low settling velocity, to possess considerable stability as an aerial suspension. The upper size limit of aerosols for practical purposes is well below 10 µm (51). A spray consists of larger particles which sediment with considerable velocity.

The site of deposition of inhaled particles in the respiratory tract of chickens is a function of particle size (52). Particles of 3.7 to 7 µm are captured in the nose turbinates and anterior trachea, smaller particles are deposited uniformly throughout the rest of the respiratory tract.

Between 40 and 90 % of the dry particles generated by aerosol during ND vaccination are 0.5 to 5 µm (73, 93, 109) causing a deep penetration of vaccinal virus and the possible occurrence of severe vaccinal reactions (93). This stress effect is not influenced by the aerosol particle size on which the virus is carried over a mean particle size range of 1.0 to 4.0 µm (8). Therefore, aerosol vaccination is mainly restricted to revaccination of mycoplasma free chickens. A standard practice is to give young chickens a coarse spray of vaccine early in life and to follow this with a fine aerosol when a basic level of immunity has developped.

It is preferable to spray day-old chicks either in the hatchery or on site in boxes or on the floor. The spray should be neither an aerosol nor a watering can as either deep penetration from aerosol-sized droplets or chick soaking will cause problems. It is generally thought that chicks obtain their dose of vaccine from the backs of other chicks, not as a result of direct droplet inspiration; the virus being introduced to the birds by the nasal or ocular route. Chicks should be allowed 10 to 15 minutes to dry off away from direct heat (11).

Several spray and aerosol generators are commercially available. Coarse spray generators used to vaccinate day-old chickens are for example the Spra-vac®, Select. Inc., Gainsville, Georgia, USA; the ASL® Associated Sprayers Ltd., Birmingham, England and the Beak-O-Vac® (Beak-O-Vac® Inc., Gainsville, Georgia, USA) which is a machine that simultaneously debeaks and sprays vaccine in the mouth simulating tracheal instillation. Coarse spray generators used for revaccinating chickens are the ULVA Fan® (Micro West, Houston, Texas, USA) and the Spray Master® (Intervet Inc., Millsboro, Delaware, USA). Aerosol equipments are the Root-Lowell Atomist type 1021 (Lowell, Michigan, USA), the SACI Turbair (Braintree, Essex, England), the Birchmeier Flox 10 (Künten Aargau,

Switzerland) and the Medap Collison type atomiser (Bad Hamburg, German Federal Republic).

Several factors can influence successful vaccination by aerosol, e.g. : particle size distribution, airborne concentration of active virus and stability of the virus in aerosol. Particle size distribution depends both on the aerosol generator and on the diluent used. Machines which produce the finer particles induce a faster serological response (48). Measuring the size and distribution of wet particles produced by different aerosol and spray equipments, Yadin and Orthel (110) showed that the Collison atomiser, which is a typical laboratory instrument, delivers predominantly small droplets of which 94.7 % are below 10 µm. The Atomist, Turbair and Flox 10 produce a median droplet size of 20 - 39 µm and this size class makes up 51, 43 and 30 % of the respective distributions. The Atomist is useful for large operations where mean dry particles of 2 to 5 µm in diameter are needed; this aerosol equipment was proved to be most practical in the field for revaccination purposes (81). The Turbair is less safe and less practicable because the operator is contaminated. The Flox 10 and ASL generators are suitable if medium sized or coarse particles respectively are required (110).

Comparing different diluents, Gough and Allan (48) demonstrated that distilled water produces fine droplets which are more immunogenic than the nuclei derived from vaccines containing tap water or gelatin as diluents. The use of distilled water is therefore recommended; moreover, it has the advantage to obviate the presence of chlorines or other viricidal agents which are sometimes present in tap water and may completely inactivate the virus during the evaporation phase of the aerosol. The stability of ND vaccine in aerosol under practical conditions can only be controlled by the selection of suitable generators and vaccine diluents since other factors such as temperature and relative humidity are difficult to control.

Vaccines are most stable in aerosols generated in distilled water (48, 94, 110), the airborne concentration of infectious virus remaining constant provided that the ventilation is shut off and all air inlets and outlets are closed during vaccination and for 30 minutes afterwards (110). In practice, in the field, 50 % or more of the particles smaller than 5 µm in diameter remain in suspension after 30 minutes if losses through ventilation are prevented (110). In serial experiments in semi-field conditions where virus was nebulised in an amount of 4.4 ± 0.4 $\log_{10}$ EID_{50} per litre of air, the average amount of inhaled virus during a 30 minutes exposure was 3.6 ± 0.56 $\log_{10}$ EID_{50} per chicken (109). This value is largely in excess of minimum values of 3 EID_{50} of La Sota virus, 48 EID_{50} of Hitchner B1 (16) and 100 EID_{50} of

lentogenic ND virus strains reported by Kohn (57) and Monreal et al. (71) to be required to immunise chickens by inhalation.

In practice, the amount of water used to dilute the vaccine is such that it takes about 3 minutes to spray 1000 doses. The generator is moved evenly about the house and pointed in all directions while held horizontally, 50 cm above the chickens. Technicians need to wear approved gas mask and gloves while spraying.

Maternal immunity can interfere with aerosol and spray vaccinations; this interference depends both on the generator and on the vaccinal strain used. Maternal immunity interfers greatly with day-old vaccination by Beak-O-Vac (30, 42) but to a lesser extent with coarse spray (43, 44). Hitchner B1 vaccines were demonstrated by Borland and Allan (17) to be less effective, in the presence of maternal antibodies than vaccines made from the La Sota strain, although the latter are recognized as being capable of inducing a significant degree of respiratory damage when administered to susceptible young chicks.

Vaccinal reaction also depends on the vaccinal virus used. Allan and Borland (7) compared 14 different lentogenic strains using a standardized pathogenicity test which measures the effect of vaccinal virus on the respiratory tract. Significant differences in values between the mildest and the most damaging strain were demonstrated and the finding of Lancaster (59) that the La Sota strain was more immunogenic than the B1 strain was confirmed. When the stress indices and HI responses for both types of vaccines were subjected to analysis by the Student's test, it was found that the mean stress indices for the La Sota group were significantly higher than the mean stress indices for the B1 group. The mean HI response for the La Sota group was higher than that of the B1 group, the difference being highly significant ($p < 0.001$) which suggested that although the La Sota strains are in general more damaging than the B1 strains, they produce a better immune response (7). The administration of La Sota virus by aerosol to day-old chicks is however highly dangerous and should be discouraged. Indeed, mortality percentages reaching 20 % and 34 % were observed respectively in chicks with maternal antibodies or without such antibodies after vaccination at day-old with La Sota virus given in aerosol (17).

VACCINATION USING INACTIVATED VACCINES

During the last twenty years, oil-based, inactivated ND vaccines have been developed and demonstrated to be largely more immunogenic than the previous aluminium hydroxide inactivated vaccines (27, 38, 55, 61, 75, 77, 82, 112).

The efficacy of oil-emulsion ND vaccines depends on their formulation : emulsifier contents, aqueous-to-oil ratios and antigen concentrations (22, 23, 85, 86, 87).

Because of the cost of the vaccine and the cost of individual vaccination, oil based inactivated ND vaccines are used mainly for revaccinating laying and breeding stocks at or near point of lay. Chickens vaccinated with a live ND vaccine and subsequently revaccinated with an inactivated oil emulsion ND vaccine have higher and more persistent haemagglutination inhibition (HI) antibody titres and lay more eggs than birds vaccinated using only live ND vaccines (33, 34). Revaccination at or near point of lay using oil based inactivated ND vaccine affords protection for the whole production period.

Day-old chicks can be simultaneously vaccinated using live B1 and oil based vaccines, such vaccination protects the chickens until the age of 11 weeks (15, 79, 97) and could be very helpful in developing countries where ND is still endemic.

If vaccination is performed by a vaccinating team, all hygienic measures should be taken to avoid contamination of the flock. Therefore, each member of the team needs to disinfect thoroughly his equipment, overall clothing, head gear, footwear and hands and wash his face before beginning vaccination and again before leaving the premises. To limit the possible spread by vaccination of any other infection within the flock, there should be frequent changes of needles, using sterilized replacements. The birds should be handled carefully and quietly during vaccination. Vaccination with dead vaccine does not produce stress or losses but these may result from rough handling.

The bottle of vaccine should be shaken thoroughly before and during use. The vaccine should be injected either under the skin or into muscle. The most satisfactory place is the thick breast muscle which extends from the point of the keel bone to the shoulder joint. The needle should be directed forwards and slightly outwards. An alternative site for older birds and turkeys is the leg muscle, and the needle should be inserted just above the hock, where the feathers begin, and parallel to the bone, taking care not to insert it too deeply.

Oil emulsion vaccines retain their potency for at least one year provided they are stored in the refrigerator at 4° to 8° C (25). Commercial vaccines available today are mainly polyvalent vaccines containing different antigens such as ND, infectious bursal disease, infectious bronchitis, egg drop syndrome virus and reoviruses (49, 61, 62, 72, 80, 88, 89, 90, 107).

VACCINATION PROGRAMMES

In chickens

Many important factors affect the success of vaccination. These include the level and persistance of maternal immunity, the time intervals between two successive vaccinations and the health status of the chickens, particularly in terms of Mycoplasma contamination. As a rule, broilers are generally vaccinated once during the two first weeks of age either by spray, aerosol or drinking water methods using the Hitchner B1 or the La Sota strains.

Laying and replacement chickens are generally vaccinated before five weeks of age using the B1 strain administered through the drinking water or in aerosol. Revaccination occurs around the 10th week of age using the La Sota strain and at point of lay or just before transfer to the production unit using either the La Sota strain or oil adjuvanted inactivated vaccine.

In other poultry

Young turkeys are best vaccinated using the La Sota strain administered either by intraocular/intranasal instillation, spray or aerosol (39, 40, 108). Aerosol vaccination with La Sota virus induces tracheal histological lesions consisting mainly of cell proliferation. These lesions are particularly evident four to six days after vaccination, but from day 8, there is regression of the proliferative lesion and by day 14 the tracheal mucosa regains its normal histological appearance (1, 2). Primary vaccination with La Sota virus is usually followed by revaccination using oil-emulsion inactivated vaccine (19, 20). Maternally immune turkey poults can be successfully immunized at day-old by using concurrently La Sota live vaccine and emulsion killed vaccine (20).

One-day-old guinea fowl can be successfully immunized using La Sota virus given either by the oculo-nasal route or by intramuscular administration of oily adjuvant inactivated vaccines (14).

Willemart and Schricke (99) found that the B1 strain is slightly pathogenic for the gray partridge (Perdix perdix) and highly pathogenic for the red partridge (Alectoris rufa) when administered by eye-drop inoculation while the La Sota strain similarly administered is non pathogenic for either breed.

Partridges can sucessfully be immunized using the La Sota strain given by intranasal, intraocular or aerosol application (58).

FUTURE DEVELOPMENTS OF VACCINES

The advantages expected from the use of vaccines obtained by genetic engineering over the currently available vaccines are lack of possible reversion,

purity, lack of side effects following administration and possibility of differentiation between vaccinal response and seroconversion due to infection.

The development of recombinant DNA technology now makes it possible to clone the genes coding for the immunizing proteins of ND virus. Expression of these genes into various vectors such as bacteria (E.coli), yeasts (Saccharomyces cerevisiae), viruses (double-stranded DNA viruses) could lead to the production and use of new vaccines. Insertion and correct expression seems unlikely into bacteria but could be successful in yeasts and viruses as proteins synthesized in these vectors are glycosylated.

Using the method of Paoletti et al. (76), success in inserting and expressing the genes coding for the F and HN proteins into vaccinia virus has recently been obtained (35, Wemers et al., in preparation).

Vaccination against ND using a recombinant virus expressing the F protein only would be of great interest as it would allow differentiation between immunological response induced by vaccination or infection. The vaccinia recombinant virus expressing the F protein of ND virus multiplies in the cerebrum of newborn chickens and affords protection against challenge performed 4 weeks later with a velogenic strain. However, as vaccinia virus is not able to infect older chickens, this recombinant cannot be used as a vaccine in poultry unless the virus is adapted to avian species (67). Insertion of the F gene into fowl pox virus could solve this problem, but the use of turkey herpesvirus (HVT) which is presently the most common virus utilized as vaccine against Marek's disease, as vector virus, would present unique advantages. HVT causes a persistant viraemia and genes cloned into this virus may be continuously expressed, providing a solid, lifelong immunity (24).

The sequence of the HN and F proteins of different ND viruses are now known (26, 35, 45, 56, 69) and specific immunogenic epitopes can be predicted by computer analysis and short peptides synthesized. Since these polypeptides are generally too small to be antigenic, they would have to be conjugated with a larger protein or incorporated into liposomes. Such synthetic vaccines could replace the inactivated vaccines used currently and obviate all risks linked to the inactivation of the virus such as denaturation of the immunogenic proteins, or persistance of infectious virus despite inactivation.

Vaccinations procedures against ND could be fully modified in the near future but it will need much more research and evaluation before large scale field application of new vaccines issued from genetic engineering could occur.

CONCLUSION

The control of Newcastle disease on a worldwide basis is essential for preventing important economic losses to the poultry industry. Efficient control measures involve accurate vaccination programmes, monitoring and reporting systems including serotyping and pathogenicity testing of viral isolates.

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NEWCASTLE DISEASE - VACCINE PRODUCTION

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INTRODUCTION

Newcastle disease (ND) is highly contagious, and attempts to control it by slaughter, sanitary measures and quarantine are often unsuccessful. In those instances when the disease either exceeds the capacity of an eradication authority to contain it, or when it becomes endemic, vaccination of flocks at risk is a highly effective method of control.

The production and testing of veterinary vaccines is controlled by regulatory authorities, and standards and requirements have increased markedly over the past 30 years. Although neither may acknowledge it, there is a common purpose between a vaccine manufacturer and a regulatory authority - to ensure that the user gets a safe product that is potent and efficacious at the time of use. The manufacturer needs to comply with requirements but at the same time make a profit.

However, there are several factors which combine to make profit margins slender. Vaccine development requires demanding and complex production and quality control protocols. High salaries need to be paid to properly qualified and motivated personnel. The low return on research and capital investment is difficult to justify economically, especially when capital equipment is underutilised for much of the year. The time taken to prepare a seed and produce and prove a test batch of vaccine can be as long as 18 months. In order to reduce another time factor, namely, the period regulatory authorities take to review a licence application, many manufacturers now liaise with Government from the early stages of vaccine

development. This also avoids the not uncommon problem of Government reviewers giving unfavourable assessments of licensing data. Furthermore, the time between commencing a subsequent production batch and its sale in final container lots can be from 6 to 12 months, thus creating storage, inventory and cash flow problems. Although there are large numbers of birds in the poultry industry which need to be protected by vaccination, the profit margins for vaccine manufacturers are still low, since vaccines must be cheap and easy to administer, preferably via mass vaccination techniques to minimise labour costs. This is why the demand for live ND vaccines is much greater than for inactivated vaccines which tend to be used only in flocks of high genetic merit. Patent protection is not as effective for vaccines as it is for other pharmaceuticals, and a competitor could purchase a final container of live vaccine and use it to produce a vaccine. In the case of ND, the virus may not mutate after being multiplied on to produce a vaccine, the final container lot acting as a "seed".

Conventional ND vaccines are of two types, those involving replicating antigens (lentogenic and mesogenic) and those involving non-replicating antigens (killed or inactivated). The former have been selected for spreadability and immunogenicity and consist of infectious viral particles which range in pathogenicity according to the strain. The most common vaccinal strains are V4, Ulster 2C, F, B1 and La Sota (all lentogenic) and Komarov, Mukteswar, and Roakin (all mesogenic). Inactivated vaccines consist of whole virus particles which have been treated to destroy their ability to replicate, but retain their immunogenicity. Inactivated vaccines are expensive to produce, and must be administered to individual birds. They can, however, fail to induce mucosal immunity in non- or inadequately-primed birds. The use of adjuvants to increase the immune response and pre-immunisation with live vaccine or natural exposure is necessary to obtain protective immunity.

FACILITIES

Following recommendations by the World Health Organisation, several countries have developed codes for regulating the quality of

vaccines intended for both local and international trade (1,2,3, 4,5,6).

Vaccine production facilities should be separate from research or diagnostic areas, and the respective staffs should each have their own amenities. The building should have filtered air and be designed to allow effective cleaning and disinfection. Drainage should be such as to prevent wash water and product residues from accumulating. The simultaneous production of more than one vaccine must be avoided.

All equipment should be designed to facilitate thorough cleaning and disinfection, and constructed of materials which are impervious and do not react with or absorb materials and product. All personnel should be capable of assuming responsibility and be trained regularly in the principles of good manufacturing practice. Records should be kept of all procedures relating to production. Type-written master instructions for each product should be prepared and endorsed by a designated person.

A specific pathogen-free (SPF) chicken flock should be maintained by the manufacturer in secure facilities and tested at regular intervals for antibodies to specified pathogens.

LIVE VACCINES

Source Materials

a. Seed Virus

Considerable time and expense is involved in producing and validating a seed, and a manufacturer is unlikely to make a seed virus available to others, especially a competitor. Consequently, a potential manufacturer will have to obtain a strain that is more than likely polyclonal.

Prior to cloning the strain, it should be centrifuged at 10,000 g for 30 minutes to remove clumped virus, after which a sample is removed from the supernatant, this sample then being subjected to ultrasonic vibration to break up any minute clumps. The ND virus may then be genetically purified, using either a plaque isolation method or a limit dilution technique.

The resulting virus suspensions are assayed for virus content and passaged to produce the primary seed, usually in SPF chicken

eggs. The primary seed must be tested for identity, freedom from designated extraneous agents, be genetically stable and homogeneous and have acceptable characteristics with respect to relative infectivity for the target animal. These characteristics will also include immunogenicity, transmissibility and residual virulence.

The primary seed is used as a stable source material to produce vaccine batches. The test batch of vaccine is the first batch produced from the seed, and is used to obtain all validation data for licensing purposes. It is produced at the highest passage level achievable under optimal growth conditions. The multiplication limit is then determined as the ratio of infective virus yield of the virus culture unit to the total infectious inoculum, used to infect the culture unit. Subsequent batches must not exceed this limit, so that they can be related to the test batch on antigen content alone, provided the same production protocol is used.

As long as the manufacturer stores seed virus properly and practices conservation of seed virus, there should be sufficient for 10-20 years' production. Seed is used at a much higher rate for the production of inactivated vaccines.

b. Substrate

The majority of ND vaccines are produced in SPF chicken eggs, although some seeds and mesogenic vaccines are produced in SPF chicken cell monolayers. When SPF eggs are used, a history of the source flock, its testing protocol and results of testing should be provided to regulatory authorities. Chicken cell monolayers used in vaccine production should be derived from such flocks.

c. Media

The composition of media, including the method of preparation and the concentration of antibiotics in the product, should be stated. Animal sera and trypsin must be tested for freedom from extraneous agents. Mammalian or chicken sera are potential sources of contaminant viruses and mycoplasmas. Chicken serum should be obtained from an SPF flock and heat treated at 56^{o}C for 30 minutes before use. Trypsin is a potential source of contaminant viruses, bacteria, fungi and mycoplasmas and should be treated to remove such

contaminants during preparation. Some antibiotics contain bacteria and must be shown to be sterile.

Production Methods

A manufacturer uses a validated seed virus to produce a test batch on which all registration or licensing data are obtained. Subsequent batches produced from the same seed may be related to the test batch on antigen content alone, as long as the same production protocol is used. Should the production protocol be varied, regulatory authorities will require new validation data.

It is obviously important that the sole inoculum used for the production of batches be derived from the Primary Seed Lot. It is in the interest of the manufacturer to practice conservation of seed by adhering to optimal growth in substrate, inoculation doses, incubation temperatures and harvest times.

Manufacturing methods should be provided to regulatory authorities and will include descriptions of the:

a. substrate and the method of its preparation;
b. dilution and inoculation of seed virus, incubation of substrate, and harvesting of virus;
c. the methods of clarification, purification, addition of stabilisers and preservatives, filling of final containers and the containers used, and lyophilisation;
d. the diluent, if one is used, including information on its sterilisation, safety in the target animal, and innocuity to vaccine virus.

Embryonated SPF chicken eggs are potential sources of bacterial contamination. Eggs should be chilled as soon as possible after incubation, harvest materials should be rapidly chilled and the size of harvest pools should be limited. Harvest pools should be tested for designated adventitious agents and further pooling restricted to uncontaminated pools only. Preservative should be added to pools as soon as possible and pools should be stored frozen at $-20^{\circ}C$ while being tested prior to lyophilisation.

Freeze Drying

The method of freeze drying of each live virus vaccine must be determined by the manufacturer. As a general rule, the thickness of

the pellet should not exceed 5 mm, to allow drying to an acceptable residual moisture level. If cooling is too rapid, small ice crystals form in the pellet, resulting in a longer drying time. Slow cooling results in the formation of large ice crystals and a more rapid drying time. Since rapid cooling will preserve virus viability, the manufacturer should aim to cool and dry the pellet at a rate which gives acceptable viability. The container may be sealed under vacuum, or sealed after an inert gas such as nitrogen is used to displace the atmosphere above the pellet. In the latter case, there is less chance of contamination should the seal be faulty.

Storage and Transportation

Final container lots of vaccine should be stored under conditions which ensure maximum survivability of vaccine virus. Each batch should be assayed for live virus prior to release, and should be transported and stored under conditions recommended by the manufacturer and approved by the licensing authority.

Testing Methods

The vaccine is tested for identity and freedom from bacterial and mycotic contaminants, including mycoplasmas. The test batch should be tested as follows:

a. Virulence. The seed virus will not be used in the production of vaccine if the test batch fails a test for virulence. The test batch must not demonstrate reversion or enhancement of virulence, and this must not occur after a designated number of chicken-to-chicken passages.

b. Safety. The vaccine should not contain designated extraneous pathogens and should not induce untoward reactions when administered to poultry by all the recommended routes and age groups.

c. Stability. *In vitro* stability tests are carried out to demonstrate that the vaccine has acceptable potency under the conditions of storage as recommended by the manufacturer. These data are obtained by using the recommended conditions for the whole of the proposed expiry period, or by evidence obtained under conditions of accelerated degradation, or by a combination of the two methods. Data should be derived from at least three representative batches and

should be in a form that can be evaluated statistically, for example, by linear regression analysis.

d. Efficacy. For live ND vaccines 'potency' is the term used to denote virus titre. 'Efficacy' indicates a measure of the performance of the vaccine virus in inoculated birds, such as the degree of protection afforded against a standard challenge, or the specific antibody response following vaccination, that antibody response having been related to protection afforded against a standard challenge in comparison to that afforded to control animals. It is extremely important that experimental vaccine used in field trials is obtained from a properly validated seed. In the design of field trials, Government regulatory authorities should be consulted, and care should be taken to include control groups and group sizes that provide statistically valid data. For ND vaccines, the test vaccine should be compared with a standard vaccine, and the effect of maternal antibodies should be determined. The vaccine must give the protection claimed for it by the manufacturer. This must be demonstrated by natural and/or artificial challenge, and evidence of duration of protection must be given.

A clone of strain V4 has been selected for thermostability and immunogenicity, and is coated on to pelleted feed for administrationion to village chickens in Asia. This vaccine must survive storage in the hot Asian environment.

(e) Consistency of manufacture. Evidence of consistency of manufacture must be provided for a designated number of consecutive batches.

(f) Dose response trials should be conducted to validate the recommended dose for each recommended route.

(g) Multiple antigen vaccines. If two or more antigens are combined in one vaccine, data must show that the combined vaccine is no less potent, efficacious, stable or safe, than the respective components.

INACTIVATED VACCINES

The production of inactivated ND vaccines differs from that of live vaccines in that the product is inactivated and adjuvanted. Greater antigenic mass is required, resulting in a higher use rate of

seed. It is advantageous to use strains with high EID_{50} growth potentials, such as Ulster 2C and V4. Strains F, B1 and La Sota may also be used, as may more virulent strains, but since the antigen yield is lower the antigen may need to be concentrated. If virulent strains are used, one must be very confident that the inactivation process is effective. It is important that the test batch is produced under optimal conditions so that subsequent batches produced from the same seed and under the same protocol may be related to the test batch on antigen content alone.

Multiplication Factor

The multiplication factor is the ratio of infective virus yield of a virus culture unit to the total infectious inoculum used to infect the culture unit. This must be known in order to place a multiplication limit on subsequent batches so they can be related to the test batch on antigen content alone.

Inactivation Kinetics

The inactivation kinetics must be determined for each of three replicate batches made from the seed. Prior to inactivation, it must be ensured that the vaccine is a homogeneous suspension free of clumps that may not be penetrated by the inactivating agent. Care should be taken to ensure that excessive agitation does not occur, leading to splashing and coating of areas of the container with live virus which may not come into contact with the inactivating agent, but be incorporated into the inactivated product subsequent to the inactivation process. Inactivating agents include formaldehyde, beta propiolactone or other alkylating agents, cis-diamino-dichloro platinum II, and various imines. The inactivation kinetics are determined by treating bulk vaccine containing the highest level of virus likely to be present. Samples are taken for assay of residual infectivity at intervals during the inactivation period, and deviations from linearity investigated. If a subsequent batch is produced from the seed, and it has a higher titre than the test batch, the vaccine batch falls outside the definition of the test batch and the kinetics of inactivation no longer apply. Every batch must be shown to be inactivated. Any residual free formaldehyde should be neutralised. The United States of America Department of

Agriculture requires that the residual free formaldehyde content does not exceed 740 parts per million. If the inactivating agent is beta-propiolactone, then this determination is not required.

Non-SPF Substrate

A manufacturer may choose to use non-SPF substrate. In this case, experimental evidence should be provided to show that the inactivation method will inactivate the largest potential amount of the most resistant viral contaminant (for example, avian reovirus or infectious bursal disease virus) likely to be present in the incubated substrate. This can be demonstrated by inactivating such a contaminant virus after it has been added to a sample of virus harvest fluids. If production using non-SPF substrate is performed in an area of the facility which is normally used for the production of SPF live vaccines, the area must be effectively cleaned and decontaminated prior to being used for the production of SPF live vaccines.

Retention Samples

Manufacturers should retain samples of the viral harvest prior to and after inactivation, and after addition of adjuvant, as it is extremely difficult to extract antigen from emulsions in a consistent manner from batch to batch. Tests on inactivated ND vaccines are described in the Chapter on Quality Control of Vaccines.

Adjuvanting

Inactivated vaccines require immunopotentiation, and must be combined with safe adjuvants to induce protective immunity. Adjuvants also reduce the amount of antigen required for protection, thus making vaccine production more economic.

The adjuvants commonly used in veterinary practice are aluminium hydroxide, aluminium salts or oil emulsions, the latter based on either a mineral oil or a vegetable oil. Vegetable oil adjuvants, such as peanut oil, were developed to overcome the problem of long-term persistence of mineral oil in human tissues. Various formulations are used, one containing peanut oil, aluminium monostearate as a stabiliser and Arlacel A as the emulsifier. Another has peanut oil with glycerol and lecithin. The emulsion may be oil-in-water or water-in-oil; the antigen must be trapped in the

aqueous phase of the emulsion for maximum immunopotentiation. Double emulsions (water-in-oil-in-water) are made by re-emulsifying a single water-in-oil emulsion in Tween 80. Double emulsions are as antigenic, but are much less viscous, easier to administer and are more stable. Peanut oil must be tested for aflatoxin content. Other adjuvants used include liposomes and various saponins, including Quil A. All oils, stabilisers and emulsifiers must be sterilised prior to mixing with the vaccine. The World Health Organisation recommends that adjuvants must not be carcinogenic, must not promote tumour formation as a co-carcinogen, as does Arlacel A, and should be biodegradable and safe to use either intramuscularly or subcutaneously (7).

Stability of emulsions should be determined by storing vaccine in final container lots under recommended conditions for the whole of the proposed expiry period, or by data obtained under accelerated degradation, usually at three temperatures, 4°C, 20-30°C and 55-65°C. Regulatory authorities may allow an expiry period of at least 12 months, this being increased or decreased as data become available. Stability is determined by lack of separation or cracks in the emulsion, and the formation of discrete droplets when the emulsion is dropped on to the surface of water. It is important that the protocol for adjuvanting is not varied, because this might affect the immunogenicity of the batch, and its antigen content might not relate to the protection proven by the test batch.

Sterility testing of final product is by the method of membrane filtration, using a suitable solvent. The solvent should not have antimicrobial activity under the conditions of the test or change the pore diameter of the filter membrane. The method of direct transfer may be used if a suitable solvent is not available. Before addition to media, the test sample should be emulsified with an agent which will improve contact between the sample and the medium (e.g., polysorbate 80 or light mineral oil). The volume of material under test should not be greater than 20% of the volume of the medium. The sterility test is valid only if it is shown that the mixture supports the growth of specified microorganisms at the beginning and end of the period of incubation.

Leaflets should specify that accidental injection of adjuvanted vaccine in man can cause serious localised reactions, and should this occur, medical attention should be urgently sought and the attending doctor advised that the vaccine is an oily emulsion.

SUBUNIT VACCINES

One of the main thrusts of molecular biology is the development of sub-unit vaccines, or vaccines which consist of viral proteins or peptides free of genomic nucleic acids. These protective antigens may be produced in bulk quantities by transferring and cloning the corresponding genes into a suitable host, or by chemical synthesis if the amino acid sequences are known. With chemical synthesis, the chains may not be properly conformed, and so will fail to stimulate protective immunity. Cloning may be performed in prokaryocytes, but bacteria do not glycosylate the product, resulting in a form which is incorrectly shaped for the induction of protective immunity. Better results have been obtained with eukaryotic systems, but this method of production is very expensive.

Genetic manipulation may be used to produce antigen-replicating vaccines. The construction of mutant viruses from which a pathogenic gene or genes have been removed, without affecting the ability of the virus to replicate and induce protective immunity, is being investigated. Such a mutation cannot be corrected by future spontaneous mutations, and the virus is permanently attenuated. An attenuated virus strain may be used to express foreign protective immunogens. Vaccinia, fowl pox, adeno and herpes viruses all have large genomes which would make them suitable as hosts. Herpes virus of turkeys would be especially suitable because vaccinated poultry become viraemic for life and thus would be continually vaccinated. Foreign DNA could be inserted into a non-essential gene of the vector virus. This technique could be used to prepare recombinant viruses which could then express the F and HN proteins of ND virus. It is extremely important that the vector multiplies in the target animal.

It is difficult for regulatory authorities to define specific requirements for biologicals resulting from recombinant DNA (rDNA) technology. Each product will need to be considered on an individual

basis to assess its purity, safety, potency, stability and efficacy. Requirements for conventional vaccines have developed over the years, and this will apply to recombinant vaccines. Intending manufacturers should follow guidelines formulated by the National Institutes of Health Guidelines for Research into Recombinant DNA Molecules, the World Health Organisation, and the National Institute for Biological Standards and Control in the United Kingdom. The Commonwealth Department of Health in Australia has issued guidelines for the manufacture and quality control of biologicals produced by rDNA technology (8). These are based on the guidelines issued by the Department of Health and Human Services of the Food and Drug Administration of the United States of America.

Source Materials

Regulatory authorities will expect a description of the method used to prepare the specific segment, in particular a nucleotide sequence analysis and restriction enzyme digestion map of the cloned segment and a description of any additional sequences (introns and /or flanking sequences) present in the coding segment. The construction of the vector with the specific coding segment, including the source and component parts of the vector, should also be described. A restriction enzyme digestion map of the complete constructed vector, as well as the source, phenotype and genotype of the host cell should be provided. The mechanics of transfer of the expression vector into the host cell, and whether the vector is integrated or episomal as well as the source and composition of all culture media should be provided.

Production Methods

In general, production methods will comply with current requirements. All precautions should be taken to prevent cross-contamination with adventitious agents. The seed lot system will also apply to biologicals produced by rDNA technology. The manufacturer should clone the host cell containing the expression vector in order to establish a master cell bank, and this should be stored in a manner which ensures genetic stability. All production

batches are derived from the seed, and limitations on the number of passages permitted during manufacture should be established. The host cell and expression vector should be genetically stable, and the fidelity of the nucleotide sequence of the specific coding segment with the amino acid sequence of the desired product should be verified for the master cell bank. In addition, the master cell bank should be tested for adventitious agents. If mammalian host cells are used, they should be examined by electron miocroscopy for virus-like structures, and tested for tumourigenicity and karyology. The cells in each production run should be characterised by analysis of phenotype and genotype, and tested for adventitious agents prior to termination of culture. The manufacturer should establish criteria for rejection of culture lots.

The method of harvesting, extraction and purification of the rDNA should be described. The identity and purity of the rDNA product should be compared with the equivalent natural vaccine or a reference standard and analysed using polyacrylamide gel electrophoresis or high performance liquid chromatography. The product should have proper conformational structure, and this should be compared with that of the natural product.

Final Product

The final product should undergo physicochemical tests for identity and purity, and should be biologically characterised by comparing its potency and immunogenicity with those of the equivalent natural vaccine or a reference standard. Final container vaccine should be sterile, and tested for safety, stability and pyrogenicity.

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QUALITY CONTROL OF VACCINES

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INTRODUCTION

Adequate quality control of vaccines is essential to avoid economic losses due to the application of unsafe or ineffective products and to ensure the health and welfare of the recipients of the vaccines. It is particularly important in the poultry industry where there is a large population with a rapid turnover and vaccines are in almost universal use: an ineffective product may lead to widespread outbreaks of disease; a contaminated product can introduce new disease to the poultry industry which may take many years to eradicate or control.

There are several areas to consider in relation to quality control. Adequate safeguards must be present in the manufacturing premises to ensure the vaccine cannot be exposed to contamination. Care is needed with ingredients in the final product: stabilisers and emulsifiers must be sterile; substances of animal origin should be free from contaminants. The unnecessary use of antibiotics is to be discouraged, though inclusion in the seed virus inoculum is usually considered acceptable in order to prevent undue loss during manufacture. However, they should not be used to disguise poor aseptic techniques because the latter will allow ingress of mycoplasmal and viral contaminants.

Newcastle disease (ND) vaccines are not usually grown in cell lines. It is therefore rarely possible to use a verified cell seed for production, so the substrate represents a new threat to each production run. Specific pathogen free (SPF) eggs should be used, the donor flock of which is tested regularly to ensure freedom from disease agents. However, new and newly recognised diseases keep

occurring in poultry and, therefore, SPF eggs will always represent some risk.

It is also essential to ensure that the seed virus is pure, safe and capable of producing consistently effective batches of vaccine (1). Live vaccines produced from well known strains of ND such as Hitchner B1 and La Sota may contain non-homogeneous populations of virus (2) and often show different properties, either because passaging over the years at different laboratories has led to the selection of distinct populations or due to deliberate efforts to produce highly attenuated or especially immunogenic vaccines (3). It is therefore necessary to examine safety and immunogenicity whether the vaccine is to be prepared from an established or a novel strain. It may be considered desirable to clone purify the vaccine seed before verifying its properties in order to reduce the chance of future changes in the population of virus (4).

The vaccine should be produced by a consistent method, and consistency should be checked by in process tests such as the virus and antigen content of the harvest.

Finally, it is necessary to test samples of each batch of vaccine to ensure the product is safe, effective and of suitable quality (5).

LIVE VACCINES

Characterisation and identification

Various tests are available to characterise and to distinguish between ND vaccine strains. These tests can be used on the seed virus as an aid in identifying it, checking its purity, and to use as a reference against which future seed lots can be assessed. They can also be used to compare vaccines from different sources and may be of value in epizootiological tracing when vaccine safety or efficacy is being questioned, as a means of distinguishing the vaccine from a field strain.

The mean death time in embryos (6) can be used to distinguish lentogenic, mesogenic and velogenic strains, lentogenic strains having values in excess of 90 hours, velogenic strains being below 60 hours. Virus strains differ in their ability to haemagglutinate red blood cells of various species (7). For instance, it is

usually found that La Sota and F strains haemagglutinate horse red blood cells whereas B1, Ulster and V4 do not. The rate of elution from chickens red blood cells can also be used to characterise strains (7). B1 and F strains are rapid eluters, whereas La Sota, Ulster and V4 are slow. There are also differences in the thermostability of the haemagglutinins (6). Those of B1, F and La Sota are unstable (destroyed within about 5 minutes), whereas Ulster and V4 are stable for 2 hours. Peptide mapping (8) and oligonucleotide fingerprinting (9) can also be used to characterise vaccine strains, as can reactions with a suitable panel of monoclonal antibodies (10,11).

Examination of plaque size and morphology can be used to detect non homogeneous populations (12). In cell cultures, ND virus may cause total destruction of the cells, partial destruction resulting in hazy plaques, or it may just alter the cells' permeability to dyes, resulting in coloured plaques. The size of the plaques depends on the incubation conditions, so standard strains should be cultivated in parallel. Uncloned stocks are likely to give rise to a mixture of plaque sizes and types. Lentogenic virus produces small plaques, clear or hazy, but only in the presence of DEAE dextran and magnesium ions or of trypsin (13). Mesogenic and velogenic viruses produce plaques without these additives. Presence of mesogenic or velogenic sub-populations in lentogenic vaccine can be detected by culturing high concentrations of virus in the absence of these additives.

It may also be possible to use monoclonal antibodies with neutralising activity to detect variant populations that are not neutralised by the particular antibody being used (14).

It is suggested that these characterisation tests are carried out on the vaccine seed virus. Provided a seed lot system is in use for vaccine production, where the number of passages between master seed and final product is strictly limited, and provided that adequate checks are made to ensure the correct batch of seed is used, there should be no need to characterise each batch of final product in this way. However, a test should be carried out to demonstrate that the final product is in fact ND virus, such as failure to haemagglutinate, or failure to infect susceptible

embryos, after mixing with a monospecific antiserum.

Safety

Several in vivo tests are of use in assessing the safety of the vaccine strain and in characterising it further. These tests are the intracerebral pathogenicity index (ICPI) (7), intravenous pathogenicity index (IVPI) (4) and the aerosol stress index (15). In the ICPI test, the lentogenic strains have low values, but there is disagreement about the exact method of conducting the test and about the pass level, some authorities considering these strains should have ICPI values of less than 0.3, other expecting values of up to 0.7. At the lower range of this test, small differences in results can be crucial and the test is perhaps inappropriate for characterising lentogenic strains. An agreed test procedure is required, which standardises factors such as the exact age of the bird. Standard preparations should be tested in parallel with the vaccine strain in order to control the validity of the test. The IVPI values for lentogenic strains should be zero. Mesogenic vaccine strains have intermediate ICPI values and low IVPI values.

The aerosol stress index is of particular value in assessing the safety of vaccines that are likely to be applied by aerosol. It involves the use of special apparatus to generate a controlled aerosol, and this test also should involve the use of standard preparations. The results of the test product must be judged in accordance with the claim for innocuity and the recommendations for method of application in the field.

Interference with diagnosis can be considered as an additional criterion for acceptability when considering the use of vaccines in conjunction with eradication schemes (16). In this case, it is desirable to have well characterised strains which do not spread.

These tests should not be necessary as routine batch tests, for which observation of vaccinated chicks for 2 to 3 weeks for respiratory and enteric signs and deaths should suffice.

All safety tests should be done using material as close to the passage level of the master seed virus as possible, normally using at least the maximum virus content likely to be encountered in batches of vaccine.

As a final consideration of safety, Newcastle disease virus can infect man, causing conjunctivitis, so care should be taken when handling vaccine and other strains, and appropriate warnings should be given to vaccine producers and users.

Reversion to virulence

It is necessary to establish that enhancement of virulence does not take place when the vaccine is passaged from bird to bird (17). This may happen through selection of a more virulent component of a mixed population, and such virulent components have been demonstrated in vaccines (18). However, acquisition of virulence could also occur because of genetic changes to individual virions in an initially homogeneous population. With a readily-spreading agent like ND virus, it may be considered desirable to carry out up to ten chick-to-chick passages. It is essential to do direct passaging without intervening embryo passages which would reverse the direction of selection. The vaccine strain is unsuitable if there is any enhancement of virulence.

Protection

Laboratory protection tests are straightforward with ND vaccines so it seems unnecessary to carry out field trials. A single point vaccine and challenge assay is often recommended. However, a more discriminating test is achieved by using a multipoint assay where graded doses of challenge are given (19). This test can be used to distinguish between (20), or to characterise (3), vaccine strains, for instance to select a particularly immunogenic clone. It can also be used to measure the effect of additional components such as infectious bronchitis vaccine (21). The use of a standard vaccine is recommended in this test and a standard challenge strain is helpful (22). The standard vaccine should have been shown to give protection equivalent to that provided by the International Reference Preparation for Newcastle Disease Vaccine (Live). For vaccines prepared from the B1 strain, the potency of the vaccine should not be less than that of the standard vaccine. However, for vaccines selected either to be particularly mild or especially immunogenic, different pass levels might be appropriate (20).

The protective response elicited by vaccine in chicks with maternal antibody is of great importance in the field situation and a laboratory test in such chicks can also be used for characterisation (3).

A batch of vaccine prepared from the master seed virus in the typical manner but at or beyond the maximum permitted passage level should be tested. This should be used at or below the minimum permitted virus content. Provided a seed lot system is used for production, there should be no need for production batches to be tested for protective potency.

Virus content

The specification for minimum batch titre should be based on that amount shown to be effective in the immunogenicity test. It is also possible, after many years of use of ND vaccines in the field, to recommend an overall minimum titre; the USDA recommend not less than $10^{5.5}$ EID_{50} per dose (23), the standard in the UK is 10^{6} (24) and $10^{6.5}$ has been suggested as a minimum (25). There is no upper limit put on the virus content, but it should be ensured that the vaccine is still safe to use when applied at the maximum likely dose.

Replicate assays should be used to determine the virus content (26) in conjunction with a standard vaccine that has been calibrated against the International Reference Preparation for Newcastle Disease Vaccine (Live). In laboratories experienced in titrating ND vaccines it has been shown that there is little value in expressing the results of the test vaccine in terms of the standard, possibly because determination of infection is straightforward with ND (27). However, the standard preparation is of value in determining the validity of the assay. It may also be of value in reducing laboratory-to-laboratory variation in cases of dispute.

The specified minimum virus content should be present throughout the shelf life of the product. Products shown to be stable at 37° for 7 days are likely to be stable at 4°, but some products are stable at 4° but not at 37° (28). However, stability at 37° also indicates a product's ability to withstand a certain amount of exposure to adverse effects.

Extraneous viruses

Both the seed virus and the final product should be subjected to extensive tests to detect extraneous viruses because of the serious harm that can occur through use of contaminated vaccines. Even though the seed virus may have been purity tested, the extent of testing of the final product cannot be reduced because there are other sources of contamination: the growth substrate and the manufacturing environment.

Extraneous bacteria, fungi and mycoplasma

In all cases it is essential to verify the growth supporting properties of the media in the presence of the product under test. It is sometimes considered permissible for the vaccine to contain up to 1 non-pathogenic fungal or bacterial organism per dose because the vaccine is applied in the field in a non sterile manner. No Mycoplasma organisms are permitted because their presence usually indicates something other than a casual breakdown in aseptic technique in a single batch of vaccine.

Chemical and physical factors

Tests for vacuum and moisture can be usefully carried out to detect faults in manufacture that might result in loss of stability.

Tests for residual antibiotics can also be done (29).

Diluent

If a diluent is supplied for reconstitution this should be tested for sterility, safety and lack of viricidal effect.

INACTIVATED VACCINES

Characterisation and identification

Tests similar to those described for live vaccines can be done, though the range need not be so extensive. There is little evidence that the properties of the strain influence the protective capacity of an inactivated vaccine. It may be desirable to select an innocuous strain as it will cause fewer problems if it is accidentally released into the rest of the manufacturing plant or the field.

The final product can be identified by its serological and protective responses.

Safety

The final product formulation should be tested extensively. Although the domestic fowl occasionally produces local reactions in response to oil emulsion vaccines (30), turkeys and especially pigeons are less tolerant (31, 32). It may not be possible to detect potential problems in laboratory-scale trials, so the vaccine's use in the field should be carefully monitored.

A safety test should be conducted on each batch of final product.

An additional safety consideration is to the vaccinator. Self inoculation, particularly into the finger pulp or tendon sheaths can result in loss of a finger (33). Immediate surgical attention should be given including incision and irrigation of the injected area (34).

Inactivation

The efficacy of the inactivation process should be verified by studying inactivation kinetics on a typical batch of harvest. Every batch must be demonstrated to be inactivated. The test should be carried out on samples taken from the bulk harvest after inactivation and immediately prior to final blending. To ensure no manufacturing accidents occur, samples of the product from final containers should also be tested, although this test is not very satisfactory because of the presence of the adjuvant.

Protection

As the protective capacity of inactivated ND vaccines can be established in laboratory trials there is usually no need for field trials. However, as these vaccines are often used for protection throughout the laying cycle, the duration of protection should be established. They may also be used in conjunction with live vaccine to overcome the inhibitory effects of maternal antibody in day-old chicks (35). The protective capacity in this situation should be verified before such use is recommended.

Each batch of final product should be subjected to a challenge test. A test using graded vaccine doses is recommended (36). Using this test, the dose of vaccine required to protect 50 % of birds (PD_{50}) can be calculated. It is recommended that the vaccine contains at least 50 PD_{50} per field dose. However, this value is influenced by the test design. Increasing the time of challenge from 17 to 21 days can improve the result (37), but because these vaccines are used for direct protection, an undue delay in development of protective response is unacceptable, and 21 days should be the limit in the potency test. The virulence of the challenge virus will also affect the results (22). A standard vaccine is of value in controlling assay variations. The standard should be calibrated against the WHO International Standard for Newcastle Disease Vaccine (Inactivated).

The single point assay recommended by the USDA (23) appears to be a less stringent test, and it certainly gives less information. The test involves a full dose of vaccine and challenge with an unspecified quantity of virus. The test criteria require only 90 % infection of the controls, which indicates a very ineffective challenge could be used, and 90 % protection of the vaccinates. In the PD_{50} assay, it is frequently observed that 90 % of vaccinates can be protected with as little as 1/50th of a vaccine dose.

It is unfortunate that a challenge test still has to be recommended for each batch of vaccine. However, work in progress at the Central Veterinary Laboratory to establish a correlation between protection and serological response to the graded vaccine doses in the PD_{50} assay may lead to the establishment of a serologically based potency test.

Antigen content

As an "in process" test, the antigen content of the harvest should be standardised prior to inactivation, by virus titration or HA tests.

Determination of HA content of the final product is an attractive alternative to an *in vivo* challenge tests as a measure of potency (38). However, extraction of antigen from emulsions cannot be done consistently from product to product (39), and variations

in the formulation of the final product will affect the protective capacity of the vaccine (40), so that antigen determination will not necessarily correlate with protection.

Extraneous viruses

If a non-SPF growth substrate is used, there is a potential for contaminants to be present in the product; such contamination may also arise from the manufacturing premises. Although it may be considered that SPF eggs cannot be used for manufacture of inactivated vaccines on economic grounds, the presence of contaminants should be avoided as far as possible. The effect of the inactivation procedure on potential contaminants should be examined. An outbreak of leukosis is thought to have resulted from residual live leukosis virus present in an inactivated ND vaccine (41). Strict controls should be taken during manufacture particularly to avoid contamination after the inactivation process. However, even though contaminants in the bulk harvest may be killed by the inactivation procedure, their presence is undesirable as they may cause seroconversion in the recipients. This may confuse diagnosis of disease or impinge on the import requirements of different countries. A recent case apparently occurred of seroconversion to Egg Drop Syndrome '76 in birds vaccinated with a contaminated ND vaccine (37).

Seroconversion to a range of common contaminants can be checked by standard serological tests such as ELISA (42).

Extraneous bacteria and fungi

Oil emulsion products are not the simplest products on which to conduct sterility tests. In some cases, the product may be filtered, but often the emulsion is not suitable for filtration. However, inoculation of the product into culture media can be successful, as can plating onto solid media. It is not usual to require tests for freedom from Mycoplasma to be done on inactivated vaccines.

Chemical and physical factors

A method to detect residual free formaldehyde in oil emulsion vaccines has been described (43).

Merthiolate is often used as a preservative in inactivated vaccines usually at a concentration of 0.013 % in the aqueous phase. Biological and chemical methods are available for assay, but results obtained with oil emulsion vaccines can be rather variable.

Other properties of the emulsion should be examined to ensure it is easy to use and sufficiently stable, such as rate of settling, viscosity and microscopic appearance before and after storage.

RECOMMENDED TEST METHODS

The tests suggested here are based on those recommended by the Ministry of Agriculture, Fisheries and Food (24), the British Pharmacopoeia (Veterinary) (36), the European Pharmacopoeia (44), the Code of Federal Regulations of the United States Department of Agriculture (23) and independent publications referred to previously. The status of these tests varies; the Government regulations are specifications for manufacturers whereas the Pharmacopoeias are for independent analysts assessing the final product.

Characterisation tests

Mean death time. Groups of 10 antibody-free eggs are inoculated with 10-fold dilutions of freshly harvested allantoic fluid prepared from the master seed virus. The eggs are candled at 8-hour intervals for 7 days. Using the level which contains the minimum lethal dose (i.e. the highest dilution at which all embryos are shown to be infected by 7 days) the mean death time is calculated.

Elution time. A standard HA titration using chicken red blood cells is kept at 4° and prevented from drying out. The titre is determined when the red cells have settled and twenty-four hours later.

Thermostability of haemagglutinin. Samples of clarified allantoic fluid are placed in sealed ampoules in a 56° waterbath. They are removed at intervals and chilled. The haemagglutination titre is determined using chicken red blood cells. The time for which the haemagglutinin is stable is recorded.

Clone morphology. The preparation is diluted so that discrete

plaques can be readily observed and compared. Cultures of chick embryo fibroblasts are inoculated and these are overlaid with agar medium containing, if required, DEAE dextran and magnesium ion additives. After incubation for 72 hours, a further overlay containing neutral red dye is added. Twenty-four hours later the plaques are observed and measured.

Safety tests

Safety test for live vaccine. A group of 10 SPF chicks is vaccinated intranasally with 10 field doses of vaccine. The chicks should be at the minimum age recommended for the product under test but not more than 3 weeks old. The chicks are observed for 3 weeks for mortality and clinical signs, especially severe respiratory signs. For vaccines claimed to be particularly mild, the effect on weight gain should also be assessed. In this case, a control group inoculated with diluent only is required, and the initial weights of the chicks must be equivalent in both groups.

Intracerebral pathogenicity index. The test is conducted on freshly harvested allantoic fluid containing approximately 10^9 EID_{50} per ml, diluted in a suitable liquid which does not contain antibiotics. Using a 26 gauge 4 mm needle, 0.05 ml of material is inoculated intracerebrally into each of 10 SPF chicks, 24 hours old. The chicks are inspected and scored at 24 hour intervals for 8 days. Healthy chicks score 0, sick chicks 1 and dead chicks 2. The total score is divided by the number of observations, 80, to give the ICPI.

Intravenous pathogenicity index. The test is conducted and the results calculated in a similar manner to the ICPI test, but using 6-week-old chickens inoculated intravenously, and a 10 day observation period, so the total number of observations is 100. Healthy birds score 0, sick birds 1, paralysed 2, dead 3.

Aerosol stress index. The aerosol is generated under standard conditions and applied to a group of 10 SPF chicks, 7 days of age. The chicks are inspected and scored at 24 hour intervals for 10 days. Birds showing no signs are given a score of 0, sneezing birds 1, birds with respiratory distress 2, sick birds 3 and dead birds 4. The total score is then divided by the number of observa-

tions, 100, to give the stress index.

Reversion to virulence. A group of 10 SPF chicks is vaccinated intranasally with the equivalent of a field dose of vaccine. The birds are placed in contact with an unvaccinated group of chicks for 5 days. The contact chicks are then removed and virus isolation is attempted from tracheal and or cloacal swabs. The group of contact controls is then placed in fresh housing in contact with a further group of uninoculated chicks. This procedure is carried out a total of at least 10 times. Virus recovered from the final passage is grown but not passaged in embryos. The characterisation tests are carried out on this material in parallel with the original vaccine material to see if there has been any enhancement of the characters associated with virulence, or evidence of any other genetic changes. The final group of chicks is retained for 21 days and examined for respiratory or other clinical signs.

Safety test for inactivated vaccine. The vaccine is administered to 10 SPF chicks 2 to 4 weeks of age, giving twice the field dose. The birds are observed for abnormal local and general reactions for a period of 14 days.

Inactivation test

Each of 10 SPF embryos, nine days of age are inoculated into the chorioallantoic sac with 0.2 ml of the treated vaccine harvest or final product. The embryos are incubated for 7 days, discarding any that die during the first 24 hours. Allantoic fluid is removed from embryos dying after 24 hours, and from any surviving the 7 days. Each fluid is tested for HA activity and separate pools are made of fluid from dead eggs and those that survived the 7 days. Each pool is inoculated into further groups of 10 embryos, which are treated and tested as above. The material fails the test if there is any evidence for the presence of live virus.

Potency tests

Potency test for live vaccines. A group of 100 SPF chicks, 5 days of age, is vaccinated intranasally with 1/10th of a field dose of vaccine. A further group is similarly vaccinated with a standard vaccine. A group of 10 unvaccinated chicks is retained. All

groups are separately housed. Three weeks later, the vaccinated chicks are divided into groups of 25 and challenged by the intramuscular inoculation of graded doses of the Weybridge Herts 33/56 strain. The challenge doses should be chosen to span the 50 % infection point, such at $10^{6.5}$, $10^{7.5}$, $10^{8.5}$ and $10^{9.5}$ ELD_{50} per dose. The birds are observed for 7 days, a record being kept of birds that die or are killed because of signs of ND. The quantity of challenge virus required to infect 50 % of the chicks (the CID_{50}) is calculated by Probit analysis.

Potency test for inactivated vaccines. Chicks free from antibodies to ND are vaccinated at about 3 to 4 weeks of age with 3 graded doses of vaccine using a micrometer syringe. The doses are chosen to span the 10 % to 90 % infected range, such as 1/25, 1/50 and 1/100 of a chicken dose. Graded doses of a standard vaccine are given to other groups of chicks. Ten unvaccinated control birds are retained. Three weeks later all birds are challenged by the intramuscular inoculation of 10^6 ELD_{50} of the Weybridge Herts 33/56 strain of challenge virus. The birds are observed for 10 days. Any showing clinical signs is killed and all birds which die or are killed are included as infected. The dose of vaccine required to protect 50 % of birds (PD_{50}) is calculated by Probit analysis. For the test to be valid, all the control birds should die within 6 days and the result for the standard vaccine should fall within its expected range.

Virus content

Each series of tests should include a test on a standard vaccine. A series of titrations carried out on the standard vaccine is used to determine the mean virus content and the 95 % tolerance limits, i.e. those values within which 95 % of titrations on the standard should occur. These values are used to validate the titrations on vaccine batches.

The test should be carried out on each vaccine batch using 3 samples of each separate filling and freeze-drying run. Each vial is reconstituted in 10 ml of peptone broth: this is regarded as the 10^{-1} dilution. Each preparation is then separately diluted up to 10^{-6} by making serial 10-fold dilutions transferring 0.5 ml

of material into 4.5 ml of peptone broth. Then 5-fold dilutions are made transferring 1 ml of material into 4 ml of peptone broth.

The range of dilutions spanning 0 to 100 % infection are assayed (usually $10^{-6.7}$ to $10^{-9.5}$). The dilutions are inoculated into groups of 7 SPF embryos, 9 days of age, injecting 0.1 ml, using a 1 ml syringe fitted with a 25 x 0.6 mm needle. The holes are sealed and the eggs are incubated at 37° for 7 days. The eggs are candled daily, eggs dying before 24 hours being discarded. After 7 days HA tests are done on all survivors after chilling. From the number of eggs at each dilution found to be HA positive, the EID_{50} per chick dose of vaccine is calculated using the method of Spearman and Karber. Each of the test samples should meet the required specification. The test is valid only if the result on the standard vaccine falls within the 95 % tolerance limits.

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 square centimetres.

Tests in embryos. Embryonated eggs, 9-11 days of age, are inoculated with 10 doses of neutralised vaccine, 10 onto the chorioallantoic 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 (45).

Tests in chick embryo fibroblasts. For the detection of reticuloendotheliosis virus, five cultures of chick embryo fibroblasts are each inoculated with 10 doses of neutralised vaccine. The cultures are passaged twice at 3-4 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 neutralised vaccine

are each inoculated into 10 cultures and these are subcultured at 3-4 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.

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 neutralised vaccine. After allowing adsorption for 1 hour, the cultures are incubated for a total of 20 days, subculturing at 4-5 day intervals. The cultures are examined for cytopathic effects and the cells and fluids tested for haemadsorption and haemagglutination respectively.

Test in chicks. A group of 20 chicks, 2 weeks of age, are each given 10 field doses of vaccine by eye drop, intramuscular, intratracheal 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. The test should include a serological test for freedom from avian encephalomyelitis virus, as it has been shown that intracerebral inoculation is not necessary to detect this agent (46).

Freedom from bacteria, fungi and mycoplasma

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 10 organisms of Bacillus subtilis, Clostridium sporogenes, Staphylococcus aureus and Candida albicans: these are incubated at 30°-32° and 20°-25° for not more than 7 days.

Representative containers of vaccine and diluent (1 % of the batch with a minimum of 3 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.

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.

The 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°-37° and 43° for 48 hours, subculturing at 24 and 48 hours onto desoxycholate citrate, brilliant green and bismuth sulphite agars; these are incubated at 37° for 48 hours. Any colonies are identified biochemically and serologically.

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°-37° for not more than 14 days in a humid atmosphere aerobically (air plus 5%-10% carbon dioxide) and anaerobically (nitrogen plus 5 %-10 % carbon dioxide).

The vaccine is tested in each medium incubated as above for not less than 28 days, subcultures being made at 3-4 day intervals or immediately there is a colour change. All cultures must be examined microscopically.

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