PATHOGENESIS AND CONTROL OF INFECTIOUS AVIAN ENCEPHALOMYELITIS IN THE CHICKEN

A THESIS PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF SYDNEY

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UNIVERSITY OF SYDNEY

1975

SUMMARY

An infectious avian encephalomyelitis virus (IAEV) was isolated and characterized by physico-chemical and serological tests, as well as by the disease and histological lesions it caused in chickens and chicken embryos. The behaviour of two viruses, the NSW-1 isolate and the van Roekel strain of IAEV in chickens, chicken embryos and tissue cultures were studied. Both viruses could be titrated in chicken embryos. The viruses propagated in chicken embryo neuroglial, chicken embryo kidney, chicken embryo fibroblasts and chicken embryo intestinal cell cultures, but failed to produce a cytopathogenic effect. Replication of IAEV was not detected in tracheal or intestinal organ cultures.

The pathogenesis of IAE was studied in normal and immunosuppressed chickens. Normal chickens became less susceptible to the development of clinical disease as they aged. Treatment of the chickens or chicken embryos with cyclophosphamide or testosterone respectively, disrupted the development of this age resistance. IAEV immunoglobulin inoculated at the time of challenge with the virus, or 48 hours after challenge prevented the development of the disease in both normal and immunosuppressed chickens. Maternal antibody to IAEV also prevented the development of clinical IAE in normal and immunosuppressed chickens. These results indicated that the bursal dependent lymphoid system was of considerable importance in the pathogenesis of, and immunity to, IAE.

The antibody producing capacity of one and seven day old chickens to IAEV was found to be inferior to that of older chickens. The production of specific serum neutralizing antibody was associated with the cessation of viraemia and the

waning of the virus titre in the brain. The interval before the detection of neutralizing antibody to the virus was shorter, and the intensity of antibody production was greater, the older the chickens were at the time of challenge with IAEV. This suggested that the development of clinical IAE was associated with an immunologically immature chicken.

The NSW-1 virus was found to be suitable for use as a vaccine. This virus induced an immune response when administered per os, by eye drop or intramuscularly. A virus dose - antibody response for each method of vaccination was determined.

TABLE OF CONTENTS

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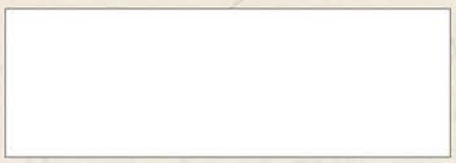
PREFACE

The work described in this thesis was carried out in the Department of Veterinary Medicine, University of Sydney between February 1971 and May 1974.

The experiments described were planned and carried out by the author with the assistance of the persons recorded in the acknowledgements. Information derived from the publications of other workers is acknowledged in the text.

No part of the work in this thesis has been submitted for any other degree.



Harvey Westbury.
July, 1975

ACKNOWLEDGEMENTS

This study was conducted with facilities generously provided by Professor J.R. Egerton and was financially supported by a grant from the Australian Chicken Meat Research Committee.

The author is indebted to Dr. B. Sinkovic for his enthusiastic support and guidance throughout these studies and for his helpful criticisms during the preparation of this thesis. The encouragement and assistance of the other members of the staff of the Department of Veterinary Medicine was also greatly appreciated. In particular, the help of Professor J.R. Egerton during the writing of this manuscript was of considerable assistance.

The writer is especially grateful to A.A. Tegel Pty.Ltd., Inghams Enterprises Pty.Ltd., Hazletts Dunrobin Poultry Stud, Arthur Webster Pty.Ltd. and to individual poultry farmers for the supply of IAEV susceptible fertile eggs.

The skilled technical assistance of Peter Liddy, Robin Small and Jackie Hughes is also gratefully acknowledged. Similarly the help of Harold Harris, and Roy Young was much appreciated.

Thanks are also due to Mrs.Val LaVelle for typing this thesis.

Finally I wish to thank Joan Westbury for displaying considerable patience, and providing a refreshing environment throughout this study.

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The antibody producing capacity of one and seven day old chickens to IAEV was found to be inferior to that of older chickens. The production of specific serum neutralizing antibody was associated with the cessation of viraemia and the

waning of the virus titre in the brain. The interval before the detection of neutralizing antibody to the virus was shorter, and the intensity of antibody production was greater, the older the chickens were at the time of challenge with IAEV. This suggested that the development of clinical IAE was associated with an immunologically immature chicken.

The NSW-1 virus was found to be suitable for use as a vaccine. This virus induced an immune response when administered per os, by eye drop or intramuscularly. A virus dose - antibody response for each method of vaccination was determined.

GENERAL INTRODUCTION

Infectious avian encephalomyelitis (IAE) or epidemic tremor is a highly contagious viral infection affecting chickens of all ages.

It was first described as a specific disease of the central nervous system (CNS) of young chickens (Jones, 1932), but is now known to be primarily an enteric infection. Invasion of the CNS by the virus is dependent upon its pathogenicity and the genetic susceptibility, age and immune status of the bird (Jungherr and Minard, 1942; Sumner et al., 1957b; Calnek et al., 1960; Calnek et al., 1961a; Zamberg, 1966).

In young chickens the disease is manifested by ataxia, paralysis and death, while in older chickens the infection may be sub-clinical or induce a temporary decline in egg production and/or hatchability (Taylor et al., 1955).

The histological lesions of the disease are those of a non-purulent, disseminated encephalomyelitis, characterized by local and diffuse gliosis, vascular lymphocytic infiltrations, neuronal satellitosis and central chromatolysis of neurones (Maas and Helmboldt,1962).

The virus can be transmitted to the hen's egg during the acute phase of infection of breeding flocks, resulting in congenitally infected chickens (Taylor et al., 1955). The virus can also spread to incontact chickens in the hatching incubator and on the farm (Calnek et al., 1960).

IAE was first recognized in Australia in 1939 (Hart, 1940), although its existence was first suspected in 1935

(Hungerford, 1969). Since then the disease has been diagnosed in all states of Australia (Seddon, 1966; Newton and Gray, 1969; Bray, 1972). IAE has been the cause of serious losses to the poultry industry throughout this period, although there have been no published estimates of these losses (Gilchrist, 1970; Lindsey, 1970; Bains, 1971). Despite these losses commercial IAE vaccines have not been available in Australia although such vaccines have been available in other countries (Schaaf, 1958; Calnek and Jehnich, 1959b; MacLeod, 1965; Bakos, 1966; Willemart, 1969). However, a number of reports have suggested that IAE vaccines have been only partially successful in inducing satisfactory immunity (Gentry, 1962; Schneider, 1967; Willemart and Dupond, 1969; Gentry, 1969; van der Heide, 1970).

Prevention of IAE in Australia has relied upon the chance infection of breeding flocks before the onset of egg production, or on the use of live, autogenous vaccines prepared from the tissues of chickens infected with infectious avian encephalomyelitis virus (IAEV). Whilst these vaccines have apparently been successful (Gilchrist, 1970; Lindsey, 1970) the continual reliance on this type of product is unacceptable to the poultry industry. This was the stimulus for research on the development of a suitable vaccine.

Related to this practical problem was the need to understand more completely the basis of immunity to the disease, so that a rational approach to immunization could be adopted. A major difficulty in research into IAE arises from the lack of a suitable *in vitro* assay technique for the virus. IAEV can only be assayed in embryonating eggs or in birds, provided these are obtained from a breeding flock that has no specific serum neutralizing antibodies to the virus (Sumner *et al.*, 1957a). These techniques are cumbersome, expensive

and less precise than virus assay techniques in cell culture. In addition, studies indicated that infection with IAEV was probably widespread in Australian poultry flocks (Jackson, 1971). Therefore the supply of chicken embryos and chickens susceptible to IAEV, and consequently suitable for research work, could be limited. A number of research papers have indicated that the virus could be grown satisfactorily in cell culture systems (Mancini and Yates, 1967; Abe, 1968; Matuka et al., 1968; Sato et al., 1971). As a result it was decided to investigate the growth characteristics of IAEV in various cell cultures and in other systems, in an attempt to develop assay techniques and to provide suitable systems for the multiplication of the virus for vaccine production and testing purposes.

Associated with this aim was a need to understand the pathogenesis of IAE. Little detailed information is available on such aspects as the growth of the virus in the organs of the chicken, the route(s) of invasion of the CNS by IAEV, the role of the immune system in the development of the disease or the effect of the age of the chicken on these factors. A study of these interactions was considered to be necessary to further understand the basis of immunity to IAE and consequently important to the development of vaccination programmes against the disease.

REVIEW OF THE LITERATURE

Infectious avian encephalomyelitis has been described as a natural disease of the chicken (Jones, 1932), pheasant (Mathey, 1955), quail (Hill and Raymond, 1962) and turkey (Hohlstein et al., 1970). The duck, pigeon and guinea fowl have been demonstrated to be susceptible to experimental infection with infectious avian encephalomyelitis virus (IAEV) (van Roekel et al., 1939; Olitsky and van Roekel, 1952; Greuel, 1965).

Characteristics of the virus

IAEV is a non enveloped picornavirus (Kraus and Ueberschar, 1966; van Steenis, 1968; Butterfield et al., 1969b; Melnick, 1971) whose physical and chemical properties have been mainly characterized by tests on the van Roekel (VR) isolate of the virus. This virus is highly adapted to growth in the chicken embryo and has lost some of the pathogenic characteristics of wild IAE viruses.

The infective nucleic acid of IAEV has been extracted using the cold phenol technique and found to be very sensitive to ribonuclease (van Steenis, 1968). Not all of the attempts to extract infective nucleic acid by this technique, or the hot phenol technique have been successful (Butterfield et al., 1969b; van der Heide, 1970). However, Butterfield et al. (1969b) found that treatment of the whole virus with ribonuclease at room temperature for 30 minutes resulted in a minor reduction in the infective titre of the virus.

The virus has been demonstrated to be resistant to the action of ether, chloroform, deoxycholate and to the proteolytic enzymes pepsin and trypsin (von Bulow, 1964; van Steenis,

1968; Butterfield et al., 1969b).

IAEV is stable for three hours at 37°C in buffered solutions between pH 2.3 and pH 10, but is less stable at pH 10.7 and is rapidly inactivated at pH 1.5 and pH 11.5 (van Steenis, 1968; Butterfield et al., 1969b).

Storage at 4°C for 180 days, at -20°C for 428 days, at -30°C for three years and at -60°C for six years, all preserve infectivity of the virus (Feibel et al., 1952; von Bulow, 1964; van Steenis, 1968).

Moderately high temperatures adversely affect the virus. Thus, Willemart (1965) found a 50 percent inactivation of the virus stored at 37°C in 48 hours, while van Steenis (1968) found a 90 percent reduction in infectivity of the virus kept at the same temperature for nine days. A 90 percent reduction in infectivity occurred in virus kept either at 56°C for 110 minutes or at 65°C for less than one minute (van Steenis, 1968). Virus kept at 56°C for 360 minutes was completely inactivated (Willemart, 1965).

The virus can be stabilized by divalent cations, particularly Mg^{++} at 65°C, and to a lesser extent at 56°C and 37°C (von Bulow, 1964; van Steenis, 1968; Butterfield et al., 1969b).

The decline in infective titre in a 1 in 4000 formalin in water solution at 37°C was 0.14 $\log_{10}$ units per hour, and at 40°C was 0.05 $\log_{10}$ units per day (Bakos, 1968; van Steenis, 1968). A one percent lysol solution at 37°C had only a slight effect on the infectivity of the virus (van Steenis, 1968). Ultraviolet light at an intensity of 100 μ watts per square cm. caused a 90 percent reduction in the infectivity of the virus in less than two minutes (van Steenis, 1968).

Lyophilisation of the virus has generally been associated with a reduction in the titre of the virus of approximately a

thousand fold (van Steenis, 1968; Brion et al., 1972). Von Bulow (1965) maintained the titre of freeze-dried virus using a ten percent glucose solution as a stabilizer. Similar results were obtained by Hitchner (1971).

IAEV passes through Seitz number 1 and 2 disc filters, Berkefeld N and V candles, and 50 mμ Millipore filters (Jones, 1934; Olitsky, 1939; Lindgren et al., 1957; Butterfield et al., 1969b). The virus is retained by 10 mμ Millipore filters (Butterfield et al.,1969b).

Olitsky and Bauer (1939) estimated the virus to be 20 to 30 mμ, while Butterfield et al. (1969b) reckoned it to be between 16.5 and 25 mμ.

In the only comprehensive electron microscopic study of the virus, Kraus and Ueberschar (1966) showed the virus to be icosahedral and devoid of an envelope. They estimated it to be 233Å ± 20Å in diameter. Ultracentrifugation at 120.000G resulted in a well defined pellet, while the specific gravity measured by density gradient centrifugation in a solution of caesium chloride was 1.33 (Butterfield et al., 1969b).

The virus has been propagated in embryonating chicken eggs, in chickens and in various cell culture systems.

For satisfactory growth, the eggs or chickens used must be obtained from a parent flock that does not have specific neutralizing antibodies (Sumner et al., 1957a). The failure, or irregular success of many of the early attempts to propagate the virus (Jones, 1934; Olitsky, 1939; Jungherr and Minard, 1942; Feibel et al., 1952) may be attributable to interference by these antibodies.

Embryonating eggs have usually been inoculated with IAEV by the yolk sac method (Wills and Moulthrop, 1956; Calnek and

Jehnich, 1959a; Moore and Flowers, 1959; Taylor and Schelling, 1960; Hoekstra, 1964; MacLeod, 1965). There have, however, been few critical studies of the most susceptible age of the embryo for inoculation with IAEV, although five to seven days has been most commonly used. Chicken embryos have also been challenged by the intra-ocular method (Sumner et al., 1957a) and by the chorio-allantoic membrane (CAM) method (Lawson and Gregg, 1969).

The criteria used for determining propagation of the virus in the chicken embryo have varied with the characteristics of the isolate of IAEV being used. Moore and Flowers (1959) developed a chicken embryo lethal isolate of the virus after 20 serial passages and they used the death of the embryo as their criterion of infectivity. Other isolates of the virus adapted to the chicken embryo, such as the VR isolate, induce changes in the embryo that are considered pathognomonic. These are paralysis or immobility of the embryo, skeletal muscular atrophy, dwarfing and mortality near the end of the incubation period (Wills and Moulthrop, 1956; Jungherr et al., 1956; Sumner et al., 1957a; Casorso and Jungherr, 1959). Such changes may be produced after seven to 20 passages of the virus in embryo (Sumner et al., 1957a; Willemart, 1965) although not all IAE viruses can be adapted to induce them (Butterfield et al., 1969b). Lesions induced in turkey embryos by the VR isolate are similar to those observed in chicken embryos (Deshmukh et al., 1971).

The criterion used for the detection of IAE viruses not adapted to the chicken embryo is the development of the disease in chickens hatched from embryonating eggs that were inoculated with the virus (Hoekstra, 1964; van Steenis, 1968).

The multiplication of the VR virus in chicken embryos has been studied by Hoekstra (1964), MacLeod (1965) and Burke et al. (1965). They found that this isolate attained its maximum concentration in the brain seven to nine days after yolk sac inoculation. MacLeod (1965) and Burke et al. (1965) also found the VR isolate in high concentrations in the viscera, the yolk sac, the chorio-allantoic membrane and in lower concentration in the fluid of the amnionic and allantoic sacs. Other IAEV isolates adapted to growth in the chicken embryo have been recovered from the liver and intestine (Hoekstra, 1964).

IAEV has also been propagated in chickens. Chickens have been usually challenged with the virus by intra-cerebral inoculation (Jones, 1932; van Roekel et al., 1938; Schaaf, 1958). Birds have also been infected by intra-peritoneal (Feibel et al., 1952), intra-muscular (Schaaf, 1958) and intra-venous (Sumner et al., 1957a; Groth and Greuel, 1961) inoculation. Chickens can also be infected by the oral route (Calnek et al., 1961a) and by aerosols (Calnek and Jehnick, 1959b).

The criteria used to confirm infection of birds with the virus have been the development of the clinical disease, of histological lesions or of specific serum neutralizing antibodies (van Roekel et al., 1941; Jungherr and Minard, 1942).

The virus has also been propagated in cell culture systems. IAEV has been grown in chicken embryo neuroglial (Mancini and Yates, 1967; Abe, 1968; Matuka et al., 1968; Kamada, 1971; Sato et al., 1971), chicken embryo fibroblasts (Mancini and Yates, 1968a; van der Heide, 1970) and chicken embryo kidney cells(Mancini and Yates, 1968b). However not all attempts at propagation of the virus in cell cultures have been successful (van Steenis, 1968; Butterfield et al., 1969b; Davis

and Lukert, 1971) and in some cases multiplication of other viruses has been mistaken for the growth of IAEV (Hwang et al., 1960).

The multiplication of IAEV in these cell cultures has not, however, been associated with the development of specific cytopathogenic effect (CPE) or inclusion bodies in the cells. Detection of the growth of IAEV in the cell cultures has been by the titration of the virus present in the cell culture maintenance medium or within the cells, in chicken embryos or chickens.

Electron microscopic examination of IAEV infected cell cultures have shown clusters of cytoplasmic vesicles indicative of virus multiplication (van der Heide, 1970) and viral antigen has been detected in the cytoplasm of IAEV infected cells using the fluorescent antibody technique (van der Heide, 1970; Kamada, 1971).

Attempts to develop alternative methods of assay for the virus have been unsuccessful. Feibel et al. (1952) and Halpin (1966b) were unable to develop either a direct or indirect haemagglutination test using a variety of erythrocytes. Attempts to develop a complement fixation test were also unsuccessful (Jungherr and Minard, 1942; Sato et al., 1969).

IAEV has been demonstrated to interfere with the growth of avian infectious bronchitis virus (IBV), Newcastle disease virus and influenza A virus in the allantoic sac of the chicken embryo (Yates et al., 1968). This interference was demonstrated by a lower titre of each of these viruses when they were grown in chicken embryos that had been previously infected with IAEV. This interference was most pronounced with IBV, though interference with the multiplication of even this virus was not

complete. Similar interference with the multiplication of IBV by IAEV in cell culture systems has been demonstrated by El-Zein et al. (1973).

Epidemiology

IAE is a widespread infection and has been recorded in all major poultry producing countries (van der Heide, 1970; Luginbuhl and Helmboldt, 1972).

Serological surveys of the incidence of IAE in a number of countries have shown the infection to be very common. These surveys have shown that almost all flocks become infected by the time they are six months old (MacLeod and Hemsley, 1965; Taylor and Schelling, 1960; Deshmukh et al., 1971).

An important factor contributing to the distribution of the infection is the transmission of the virus in the hatching egg (van Roekel et al., 1941; Jungherr and Minard, 1942; Schaaf and Lamoreux, 1955; Taylor et al., 1955).

The duration of transmission of the virus in the hatching eggs of an infected flock is usually seven to 21 days with only rare cases exceeding 28 days (Calnek et al., 1960; Hoekstra, 1964; Gylstorff and Kraus, 1964b; Zamberg, 1966; Willemart and Schricke, 1966).

The mechanism of infection of the egg is unknown. Because infected birds excrete the virus in their faeces during the transmission of the virus via eggs (Jungherr and Minard, 1942; Calnek et al., 1960), the possibility of external contamination has been considered (Calnek et al., 1961b). IAEV has also been recovered from the ovary of infected chickens (Jungherr and Minard, 1942) and demonstrated in ovarian tissue by the fluorescent antibody technique (Braune and Gentry, 1971b). Therefore, the possibility of transovarian transmission also exists.

Ingestion of the virus is also considered to be an important mode of infection (Calnek et al., 1960). Experimental challenge of chickens with IAEV by the respiratory route failed to reproduce the disease (Brion et al., 1972). Calnek and Jehnich (1959b) have, however, reproduced the disease by aerosol exposure.

The virus can be readily recovered from the faeces of infected chickens (Jungherr and Minard, 1942; Calnek et al., 1960) and a virus with many of the characteristics of IAEV has been recovered from the faeces of normal chickens (Burke et al., 1959b). Thus chickens hatched from eggs infected with IAEV, ie. congenitally infected chickens, may infect in-contact chickens both in the hatchery incubator or in the chicken brooding area (Calnek et al., 1960).

The persistent or latent carrier of IAEV has been referred to on a number of occasions in the literature about IAE (Guillon, 1961; Richey, 1962; Cheville, 1970). However, definitive work, demonstrating persistence of the virus in the bird for longer than 30 days, or persistent excretion of the virus from the bird, has not been published, although Jungherr and Minard (1942) claimed the virus persisted, for an unspecified time, in the gonadal tissue.

The virus is not transmitted by insect vectors (Taylor and Schelling, 1960) and a number of species of free flying birds have been demonstrated to be refractory to infection with the virus (van Steenis, 1971).

Pathogenesis

The method of invasion from the primary site of infection, probably the digestive tract (Calnek et al., 1960) to the

central nervous system has not been determined. Olitsky (1939) was unable to detect viraemia in experimentally infected chickens.

In a comprehensive study of the pathogenesis of the disease Braune and Gentry (1971b) used the fluorescent antibody technique (FAT) on selected tissues of chickens that had been challenged orally with a field strain of the virus. In chickens challenged at one day of age, specific fluorescence was first detected in the pancreas five days later. Positive fluorescent reactions were subsequently detected in the brain, proventriculus, ventriculus, intestinal tract, heart, lungs, kidney and liver on the sixth day and on subsequent days in the oesophagus, crop, skeletal muscles and ovary. These reactions persisted until the 13th day then decreased in prevalence and intensity until the 20th day, when only the pancreas, brain and ventriculus gave positive specific fluorescence. By the 30th day positive reactions could only be detected in the brain.

Chickens infected with the virus at six, 12, 18 and 24 weeks of age failed to develop specific fluorescence in any of the 22 different tissues examined, even though there was development of specific neutralizing antibody to the virus in the chickens. Because of this antibody Braune and Gentry (1971b) considered that the chickens had been successfully infected.

Similarly, van der Heide (1970) and Miyamae (1974) were able to demonstrate specific fluorescent reactions in the tissues of IAEV infected chickens. Van der Heide (1970) detected specific fluorescence in chickens that had been experimentally infected with IAEV at one day of age. He

obtained specific fluorescent reactions in the pancreas, proventriculus and heart on the fourth day after infection and these persisted through to the seventh day. Specific fluorescence was only detected in the brain on the sixth day after infection. Positive fluorescent reactions were not detected in chickens infected over one day of age.

Miyamae (1974) used the FAT to study the presence of IAEV in tissues of chickens of various ages obtained from seven different outbreaks of IAE. In one day old chickens, specific fluorescence was common in the liver, heart, spleen, skeletal muscle and in the muscle layers throughout almost all parts of the alimentary tract. It was less frequent in CNS tissue. Positive specific fluorescent reactions were most frequent in CNS tissue and the pancreas of ten to 30 day old chickens.

The above studies using the FAT agree substantially with the chronological development of histological lesions in IAE described by Springer and Schmittle (1968) and van der Heide (1970). They found lesions in the viscera of experimentally infected chickens before lesions could be detected in the CNS.

A number of factors influencing invasion of the CNS by IAEV have been defined. Variation in the virulence and tropism of isolates of IAEV has been demonstrated. Isolates have been termed viscerotropic if they infect young chickens without inducing severe CNS lesions, or neurotropic if they caused marked lesions in the CNS (von Bulow, 1964; Willemart, 1965; Luginbuhl and Helmboldt, 1972). The age of the chicken at the time of infection also has a significant effect. In chickens one to 35 days of age, the disease is characterized by neurological signs, while in older chickens it is usually sub-clinical (Jones, 1934; Olitsky, 1939; Jungherr and Minard, 1942; Calnek and Jehnich, 1959a). Cheville (1970) postulated that the

maturation of the immune system was a significant factor in this age susceptibility. He considered that CNS disease was a prominent manifestation only in immunologically immature chickens. Older chickens, with functional immune systems did not develop IAE due to the presence of more antibody producing cells.

Significant differences in susceptibility to the disease have been demonstrated between various breeds of chickens (Feibel et al., 1952). The Rhode Island Red, New Hampshire and White Rock breeds are considered to be highly susceptible; the White leghorn to be less susceptible. Brion et al. (1972) considered that husbandry factors had an effect on the development of the disease. These factors were not defined, but were postulated in an attempt to explain varying mortality in groups of chickens hatched on the same day from the same infected parent flock, but placed on different farms.

Cheville (1970) studied the influence of thymectomy and bursectomy on the development of IAE. He found the disease and microscopic lesions was most pronounced in the bursectomized chickens. In addition these chickens were susceptible to the disease regardless of their age. In another report, Cheville (1971) found that muscle necrosis and sarcolemmal proliferation at the site of inoculation of the VR isolate was marked in bursectomized chickens. Mononuclear inflammatory cell foci at these sites were rare. In contrast large foci of inflammatory cells with little muscle damage characterized the reactions in the normal and thymectomized birds.

The effect of the VR isolate on chicken embryos of varying ages was examined by van Steenis (1968). He concluded that the nervous system had to reach a certain stage of development before it became susceptible to the degenerative effect of the virus. However, this stage was not reached simultaneously by

different parts of the CNS. The later the part differentiated the later it began to show degeneration. Once the tissue reached a stage of susceptibility, it was very sensitive as shown by tissue dissolution.

Braune and Gentry (1971a) used the FAT to study the distribution of the VR isolate in the chicken embryo. Specific fluorescence were first found in the brain five days after inoculation of the virus. Subsequently reactions were detected in the heart, proventriculus, ventriculus, duodenum, pancreas, lung and kidney. Positive fluorescent reactions persisted up to 14 days after inoculation. Specific fluorescence were not observed in the tissue of chicken embryos that were inoculated with two field isolates of IAEV. On the third day after hatching however, fluorescence was observed in all the tissues examined, and the disease developed in some of the remaining chickens on the seventh to 12th day after hatching. They considered that these field isolates were present in either undetectable levels or in an altered antigenic form in the chicken embryo.

Clinical signs

The virus causes its most pronounced effect in chickens less than 35 days of age (Jones, 1932; Olitsky, 1939; van Roekel et al., 1939). In this age group the disease is characterized by ataxia, trembling, paralysis and death (Jones, 1932; Jungherr, 1939; van Roekel et al., 1939). Prior to the development of these signs, the disease may be manifested by non-specific signs such as a higher than normal mortality during transport from the hatchery, a high mortality during the first seven days of life and sometimes by a whitish diarrhoea (Verdruge, 1960; Willemart, 1965). The incubation period of the disease under

field conditions is usually ten to 20 days, although it may vary from one to 42 days (Jones, 1932; Jungherr, 1939; Willemart, 1965; Lindsey, 1970). Ataxia is the most common clinical sign. Forty to 75 percent of affected chickens exhibit this sign (Jungherr, 1939; Willemart, 1965). About 30 percent of these progress to paralysis (Willemart, 1965). The proportion of affected chickens with tremors rarely exceeds 20 percent (Jungherr, 1939; Willemart, 1969).

Not all infected chickens develop signs of IAE. From nine to 20 percent of naturally and experimentally infected chickens may have sub-clinical infections (Jungherr, 1939; Willemart, (1965).

The mortality associated with outbreaks of the disease may vary considerably. The pathogenicity of the virus, the susceptibility of the chickens involved, and whether congenitally infected chickens are hatched and/or brooded with IAEV susceptible or immune chickens have an important influence on the resultant mortality (Calnek et al., 1960).

In egg producing flocks, infection with IAEV may result in a significant reduction in egg production and/or hatchability (Taylor et al., 1955; Sumner et al., 1957b; Calnek et al., 1960; Richey, 1962; Ousterhout et al., 1963; Hemsley, 1964; Dorn and Kronthaler, 1964; Willemart, 1965; Halpin, 1967b;van der Heide, 1970). The period of decreased egg production may range from five to 56 days after infection with the decline ranging from nine to 40 percent. The hatchability of fertile eggs may also be affected. This decreased hatchability has been attributed to an increased embryonic mortality between the 14th and 21st day of incubation (Taylor et al., 1955; Guillon, 1961; Willemart, 1965).

However not all descriptions of IAEV infection in egg producing flocks have recorded these findings. Jungherr and Minard (1942), Zamberg (1966), Lindsey (1970) and van der Heide (1970) have reported outbreaks in which there was no decrease in egg production or hatchability. Van der Heide (1970) considered that these cases indicated that the virus was spreading slowly in a flock.

Natural infection of chickens between about 35 days of age and the time of sexual maturity usually results in sub-clinical infections (Calnek et al., 1960; Willemart, 1965; Brion et al., 1972).

However, it is possible to induce neurological signs of the disease in chickens of all ages by intra-cerebral challenge with IAEV (van Roekel et al., 1939; Kliger and Olitsky, 1940; Feibel et al., 1952; Schaaf, 1958).

An important sequel to an outbreak of the disease is the development of cataracts. Feibel et al. (1952) first described these eye lesions in the survivors of the disease. Similar observations have been made by Peckham (1957), Flowers et al. (1958), Bridges and Flowers (1958), Zander (1959), Barber and Blow (1963), Halpin (1967a)and Riggenbach (1969). In some instances the virus has been isolated from affected eyes (Zander, 1957; van Steenis, 1968).

In pheasants and quail the disease is similar to that observed in the chicken (Mathey, 1955; Hill and Raymond, 1962). In turkeys the disease is characterized by ataxia and trembling of the head and neck of affected poults, while a temporary decline in egg production and hatchability has been recorded in turkey breeding flocks (Hohlstein et al., 1970).

Pathology

There are no gross specific lesions of IAE. However young chickens may be dehydrated, exhibit atrophy of muscle groups and may eventually have ocular lesions (Bridges and Flowers, 1958; van Roekel, 1965; Mohanty and West, 1968b; Lindsey, 1970). Whitish pin point foci have been described in the musculature of the gizzard in some chickens (Jacquette, 1961) and Mohanty and West (1968b) have also recorded hydrocephalus.

A non-purulent, disseminated encephalomyelitis characterized by local and diffuse gliosis, vascular lymphocytic infiltration and central chromatolysis of neurones are the main histological lesions found in affected birds (Jones, 1934; Olitsky, 1939; Hart, 1940; Jungherr and Minard, 1942; Maas and Helmboldt, 1962). The significance of these in the many descriptions of the histopathology of IAE has varied. These variations have been dependent on the virulence and tropism of the virus causing the disease, the genetic susceptibility of the chickens and the stage of the infection at which the chickens were examined.

Neuronal degeneration has been considered to be the most striking lesion (Olitsky, 1939; Jungherr and Minard, 1942; Maas and Helmboldt, 1962; Mohanty and West, 1968b). It has been found throughout the CNS, but is most extensive in the pons and medulla regions of the brain, and in the ventral horn cells of the spinal cord, particularly in the region of the lumbo-sacral enlargement. The degenerating neurones are eventually surrounded by satellite oligodendroglia, and finally microglial cells phagocytose the neurone (Luginbuhl and Helmboldt, 1972).

Perivascular cuffing with mono-nuclear cells also occurs

throughout the CNS (Jones, 1934; Hart, 1940; Mohanty and West, 1968b). Luginbuhl and Helmboldt (1972) found it to be confined to Nucleus cerebrallaris when it occurred in the cerebellum.

Focal and diffuse gliosis is also observed through the brain and spinal cord. In the cerebellar molecular layer, gliosis is characteristically focal (Luginbuhl and Helmboldt, 1972). In the optic lobes, mesencephalon, N. cerebrallaris, medulla oblongata and less often in the corpus striatum gliosis is usually diffuse (Jones, 1934; Hart, 1940; Tyzzer and Sellard, 1941). Focal or diffuse gliosis in N. rotundus or N. ovoidalis of the brain is considered to be highly suggestive of IAE by Maas and Helmboldt (1962) and pathognomonic by Luginbuhl and Helmboldt (1972). A meningeal inflammatory cell reaction in the region of the blood vessels of the leptomeninges and choroid plexus has also been described (Brion et al., 1972). This reaction may disrupt the circulation of cerebro-spinal fluid and thus cause hydrocephalus (Ostendorf, 1956; Mohanty and West, 1968b).

Pathological changes in the dorsal root ganglia, particularly in chickens challenged intra-cerebrally with IAEV have been recorded (Olitsky, 1939; Bridges and Flowers, 1958; Butterfield et al., 1969a; Mohanty and West, 1973). These lesions consisted of neuronal degeneration, perivascular cuffing and interstitial foci of lymphocytes, large mononuclear cells and microglia. The histological changes were more intense however in the spinal cord than in the adjacent ganglia (Mohanty and West, 1973). Histological lesions of IAE have never been described in the peripheral nerves (Luginbuhl and Helmboldt, 1972).

The visceral lesions observed in IAE are mainly associated with the hyperplasia of lymphoid foci. These hyperplastic follicles are particularly noticeable in the musculature of the proventriculus and gizzard, in the pancreas and in the myocardium (Jones, 1934; Jungherr and Minard, 1942; Olitsky, 1948; Markson and Blaxland, 1958; Springer and Schmittle, 1968).

In chickens killed during the acute phase of the disease, there may be atrophy of the retinal pigment epithelium with some detachment of the sensory retina, even though there may be no degenerative changes in the sensory retina. Cataracts and anterior uveitis have only been observed in chickens with lens lesions. Glaucoma is common in eyes with long standing lesions in the anterior ocular segment (Peckham, 1957; Bridges and Flowers, 1958; van Steenis, 1968).

The lesions induced in chicken embryos infected with IAEV have been described by Wills and Moulthrop (1956), Jungherr et al. (1956); Casorso and Jungherr (1959) and van Steenis (1968). Chicken embryos affected by viruses adapted to the embryo become paralyzed and exhibit atrophy of the related muscles. The toes flex abnormally and arthrogryposis is also observed. The microscopic lesions are most conspicuous in the ventral horn cells of the spinal cord. Neuronal dissolution without a marked inflammatory response was described by Casorso and Jungherr (1959). Encephalomalacia and internal hydrocephalus also occurred.

The lesions in the turkey embryo affected by the VR isolate are similar to those in the chicken embryo, although the histological changes seem to be less severe (Deshmukh et al., 1971).

Diagnosis

Diagnosis of IAE can be obtained by histological examination of the tissues of affected birds, by virus isolation or by serological tests.

The clinical signs of the disease are not characteristic. However the sudden onset of ataxia, trembling and paralysis in chickens 11 to 21 days of age is suggestive of IAE. This is particularly so if further outbreaks of a similar disease occur in chickens hatched at the same time from the same parent flock, and if a fall in egg production and/or hatchability in the parent flock can be associated (Brion et al., 1972).

The histological lesions are not specific, and are inconsistent, so that several chickens must be examined to obtain the complete range of lesions. There is a good correlation between clinical signs and microscopic changes, although a detailed examination may be required (Jungherr and Minard, 1942; Luginbuhl and Helmboldt, 1972). Histological examination must include both the brain and spinal cord (Olitsky, 1948; Maas and Helmboldt, 1962; van der Heide, 1970). The hyperplasia of the lymphoid follicles, and the infiltration of visceral organs with mononuclear cells described in IAE also occurs in other diseases of poultry. In IAE these lesions are typically in the musculature of the proventriculus and gizzard, and in the pancreas (Maas and Helmboldt, 1962; Springer and Schmittle, 1968; van der Heide, 1970). Clinical signs and histological lesions are rare in growing and adult chickens (Calnek et al., 1961b; Brion et al., 1972). Histopathological examination of the tissues of chickens in these age groups is not recommended for diagnostic purposes (Maas and Helmboldt, 1962).

Isolation of the virus is rarely used in routine diagnosis (Brion et al., 1972). The main tissue used in virus isolation has been the brain, although the virus has been isolated from other organs (van Roekel et al., 1938; Jungherr and Minard, 1942; Calnek et al., 1960; van Steenis, 1968). Virus isolation has usually been attempted in chicken embryos, or by the intracerebral challenge of one day old chickens (Anon, 1971).

However little is known of the persistence and growth of IAEV in chickens. Jones (1934), van Roekel et al. (1939) and Jungherr and Minard (1942) have recorded difficulties in consistent isolation of the virus. Although they were unaware of the need to use chicken embryos or chickens that were free of neutralizing antibody to the virus, their difficulties could also have been due to a waning of virus titre in the tissues used. Schaaf (1958) recommended the use of brains of chickens that had shown the disease for only two days, while chickens showing only early clinical signs have been suggested by others (Anon, 1971).

Chickens that have been infected with IAEV develop specific serum neutralizing antibody (Olitsky, 1939; Feibel et al., 1952). The demonstration of these comprises the best diagnostic test for IAE in growing and adult birds. Standard virus-serum neutralization tests in chicken embryos have been most commonly used (Calnek and Jehnich, 1959a; Lawson and Gregg, 1969). Calnek and Jehnich (1959a) used the constant serum variable virus technique in the yolk sac to study the neutralization indices of undiluted sera of 1968 IAEV susceptible chickens. They found chickens with a neutralization index (NI) of less than $\log_{10}^{1.10}$ to be susceptible to IAE. Those with an NI of $\log_{10}^{1.10}$ or greater were completely resistant. By contrast, Lawson and

Gregg (1968) used the constant virus variable serum technique. They found the NI, expressed as the serum dilution that protected 50 percent of chicken embryos against the virus, to be 4 in susceptible chickens. The NI of the sera of chickens five weeks after infection with IAEV was 19 to 20.

The dilution of a serum before it is used in virus-serum neutralization tests can have marked effects on its neutralizing capacity (Horsfall, 1957). Von Bulow (1965) studied the effect of serum dilution on the neutralizing capacity of IAEV serum antibodies in the yolk sac of chicken embryos. He found that the relationship was not linear. A tenfold dilution of serum reduced its neutralizing capacity one hundred times, and a hundred fold serum dilution one thousand times.

The NI of the sera of infected birds remain at significant levels for several months (Schaaf, 1959; Calnek et al., 1961a). Sato and Choi (1972) examined the persistence of IAEV serum antibodies in experimentally infected chickens over a period of 14 to 34 months. They found that the NI attained in the first four weeks persisted through the test period. This suggested to them that the persistence of serum antibodies was dependent on the intensity of the initial antigenic stimulation.

Greuel (1963), Monreal (1963) and Willemart (1969) have recorded the NI of the sera of infected flocks to fall, so that the flocks became susceptible as judged by the neutralization indices of serum samples.

Because of the difficulties in obtaining large numbers of IAEV susceptible chicken embryos, in which neutralization tests must be undertaken, alternative serological tests have been developed. The most commonly used technique is the embryo susceptibility test (Sumner et al., 1957b; Taylor and

Schelling, 1960). This test may be undertaken by the variable or the constant virus technique. Von Bulow et al. (1963) compared the relationship between the NI obtained by the virus dilution embryo susceptibility test (EST) and the serum neutralization test. They found a poor relationship and ascribed this to the varying levels and antibody secreted into the yolk sac of the eggs of different hens. Consequently they recommended the constant virus technique of the EST.

When the constant virus technique of the EST is used the commonly accepted interpretations are (i) all the chickens affected by the virus - flock susceptible (ii) between 50 and 100 percent affected by the virus - a recent infection (iii) between 0 and 50 percent - flock immune (Anon, 1971).

The fluorescent antibody test has also been used in the diagnosis of IAE. Cheville and Monlux (1966) were the first to demonstrate specific fluorescence in the tissues of IAE affected chickens. Subsequently the FAT has been used by others to demonstrate the virus (Braune and Rothenbacher, 1967; Mohanty and West, 1968a, 1973; van der Heide, 1970; Braune and Gentry, 1971a; Ide, 1974; Miyamae, 1974). Van der Heide (1970) and Ide (1974) found the FAT to be a reliable diagnostic procedure provided the chickens being examined were exhibiting early clinical signs of the disease. By contrast, FAT reactions could not be detected in growing or mature birds infected with IAEV (van der Heide, 1970; Braune and Gentry, 1971b).

A fluorescent antibody blocking test for IAEV antibody has been developed by Davis and Lukert (1971). In comparison tests with the virus serum neutralization and embryo susceptibility tests, this blocking test was much less specific in detecting infection, as non-specific blocking occurred at

1:5 dilutions of the sera of six of 13 chickens known to be susceptible to IAE.

By contrast, Choi and Miura (1972) found a good correlation between an indirect fluorescent antibody test (IDFAT) and the virus-serum neutralization test. They claimed that the IDFAT was more sensitive.

An agar-gel preciptin (AGP) test has been used by Lukert and Davis (1971). The antigen was prepared from homogenized gastro-intestinal tract tissue of chicken embryos infected with the van Roekel isolate of IAEV. Precipitating antibodies were found in both hyperimmunized and normally vaccinated chickens, but not in control chickens. An AGP test has also been described by Ikeda (1968).

However the FAT and AGP test have not yet been widely applied, and have not been uniformly successful when used by other workers (Butterfield et al., 1969b; Brion et al., 1972).

Differential Diagnosis

The clinical signs of the disease are not specific and may be confused with other diseases. The most important of these are Newcastle disease (ND), nutritional encephalomalacia and Marek's disease (MD). It may also be confused with transient paralysis, equine encephalomyelitis virus infection of birds, and encephalitides caused by mycoplasmas, avian chlamydias and advenoviruses.

The microscopic lesions induced by Newcastle disease virus in the CNS are quite distinct with capillary haemorrhage, myelin degeneration, localized meningitis and peripheral chromatolysis of neurones (Jungherr and Minard, 1942; Luginbuhl and Helmboldt, 1972). Marked degenerative and inflammatory

lesions may also be found in the peripheral nervous system (Brion et al., 1972). These lesions are not characteristic of IAE.

Marek's disease most commonly affects chickens at least four weeks old and microscopic lesions in peripheral nerves and plexi, as well as diverse tumors, are commonly found (Helmboldt, 1972). Histologically, there may be marked perivascular infiltration with pleomorphic mononuclear cells in both the brain, spinal cord and pia mater (Maas and Helmboldt, 1962).

Encephalomalacia due to vitamin E deficiency may bear a striking clinical resemblance to IAE. However there are gross pathological changes in the cerebellum and haemorrhage in acute cases. In addition, there is demyelination, oedema and disruption of cellular elements, degeneration and necrosis of Purkinje cells, and pyknosis of cells in the granular layer. Hyaline thrombi may also be observed in capillaries in areas of necrosis (Pappenheimer et al., 1939; Jungherr, 1939; Guillon, 1961). In chronic cases of vitamin E deficiency there may be fibrosis of the cerebellum, especially in the molecular layer (Jungherr, 1939).

Transient paralysis is a disease of unknown aetiology characterized by the development of a paralysis that persists for only a few hours. There is spontaneous and complete recovery (Zander, 1957; Willemart et al., 1967; Walker and Grattan, 1968; Wight, 1968; Willemart and Dupond, 1969). In this disease there is considerable perivascular mononuclear cell infiltration in the CNS, both in the white and grey matter, as well as proliferation and vacuolation of endothelial cells of the capillaries. Atypical encephalomyelitis is a similar disease entity to transient paralysis, although the paralysis

appears more slowly. It is usually sporadic and most commonly occurs in six to 14 week old birds (Willemart and Dupond, 1969).

Mycoplasma and adenovirus infection resulting in encephalitis have been described, but are sporadic and may affect birds of any age (Raggi et al., 1959; Hwang et al., 1960; Maas and Helmboldt, 1962).

In turkeys IAE may be confused with Newcastle disease and enzootic encephalitis (Komarov and Kalmar, 1960).

Prevention

Effective control of IAE can be obtained by the immunization of all breeding and egg laying flocks (Calnek et al., 1961b).

Schaaf and Lamoreux (1955) reported the first successful attempts at the control of the disease. They vaccinated all breeding birds before the onset of egg production with a field isolate of IAEV by the wing web method. Such vaccinated chickens were less susceptible than controls when challenged intra-cerebrally (IC) with approximately 250 chicken lethal doses (CLD_{50}) of the isolate.

In further studies Schaaf (1958) compared the effectiveness of immunization by the intra-muscular, wing web and oral methods. He concluded that the intra-muscular (IM) route was superior, although severe post vaccination reactions were observed. The wing web method has been found superior by other workers (Zander, 1959; Lawson and Gregg, 1969). Calnek and Jehnich (1959b) obtained good results with either intra-muscular or wing web inoculation.

However, the oral administration of the vaccine has been most commonly used (Calnek et al., 1961a; Gylstorff and Kraus,

1964a; Schneider, 1967; van der Heide, 1970; Brion et al., 1972).

Calnek et al. (1961a) found that a satisfactory technique was the administration of vaccine virus per os to one percent of birds in a flock between the age of ten weeks and at least three weeks before the onset of egg production. Natural transmission of the virus from the vaccinated to the unvaccinated was rapid, with 65 to 93 percent of birds developing an immune response within four weeks. Transmission of the virus within flocks vaccinated at five to seven weeks of age was not complete. The slower response in these birds was related to the inhibitory effect of maternal antibody.

Of critical importance to the success of vaccination per os is the use of a virus that has retained its infectivity for the intestinal tract (Calnek et al., 1961a). Several well described isolates - 1143 (Calnek et al., 1961a), 2653 (von Bulow, 1964), HA (Hoekstra, 1964) and EA42 (Willemart, 1965) have been used in live virus vaccines. These isolates have a marked viscerotropism, are very contagious and have not been passaged a large number of times in the chicken embryo. These are considered to be the essential characteristics for an isolate to be suitable for use as an oral vaccine (Calnek,et al., 1961b).

Inactivated vaccines have also been used (Schaaf, 1959; Calnek and Taylor, 1960; Butterfield et al., 1961; MacLeod, 1965; Bakos, 1966; Zamberg, 1966; Willemart, 1969). These vaccines

have been used in situations where the spread of live virus is considered a hazard.

The methods used for evaluating the immunity induced by IAE vaccines have not been standardized. Four techniques have been used: the challenge of chickens with a known amount of virus sometime after vaccination (Schaaf, 1958; Butterfield et al., 1961); the estimation of the NI of serum (Calnek and Jehnich, 1959b; Lawson and Gregg, 1969); the embryo susceptibility test (MacLeod, 1965; Willemart, 1969) and the challenge of the progeny of vaccinated birds (Calnek et al., 1961a). Calnek et al. (1961a) considered that none of these could be strictly compared with the immunity necessary for protection against natural infection as they would all underrate the degree of immunity.

The dose of the virus used in the bird challenge procedures has varied from 100 CLD_{50} to 10,000 EID_{50} (Calnek and Jehnich, 1959b; MacLeod, 1965). The birds have been challenged either intra-cerebrally (Schaaf, 1958; Calnek and Jehnick, 1959b; MacLeod, 1965) or intra-muscularly (Butterfield et al., 1961) as early as 21 days (MacLeod, 1965), or as late as 270 days after vaccination (Butterfield et al., 1961). The criteria of infection after this challenge have been the development of the disease (Schaaf, 1958) or the development of histological lesions in the CNS (Calnek and Jehnich, 1959b; Butterfield et al., 1961).

The serum neutralization test has also been used to assess immunity (Calnek and Jehnich, 1959b; Lawson and Gregg, 1969). Calnek and Jehnich (1959b) found that all chickens having a NI of 1.1 or greater were protected against an IC challenge with 10.000 EID_{50} of IAEV. They considered that an NI of $\log_{10}^{1.1}$ or

greater was indicative of immunity. Similar criteria have been used by Butterfield et al. (1961), MacLeod (1965) and Willemart, (1969).

The embryo susceptibility test has also been frequently used as an indicator of immunity (Taylor and Schelling, 1960). Calnek et al. (1961a) found that the percentage of embryos resistant to challenge four weeks after vaccination ranged from 65 to 89 percent. MacLeod (1965) and Willemart (1969) found up to 90 percent immune three weeks after vaccination.

The challenge of the progeny of a vaccinated flock was also used by Calnek et al. (1961a). One day old chickens hatched from eggs collected four weeks after vaccination were all immune to an oral challenge with the virus.

Specific serum neutralizing antibodies have been shown to persist at immune levels for at least six months after vaccination (Butterfield et al., 1961; MacLeod, 1965; Zamberg, 1966). However, Gentry (1962) and Willemart (1969) have described cases of a fall in the NI to less than 1.1 in investigations of failures of IAE vaccination. Other failures have been studied by Schneider (1967), Gentry (1969) and Brion et al. (1972). Thus Schneider(1967) considered the technique of vaccination per os of a few birds to be inferior to the administration of the vaccine in the drinking water as natural transmission of the virus within the flock was slow. Gentry (1969) and Willemart (1969) found unsatisfactory use of the vaccine, either because of inactivation of the virus in the drinking water or as a result of poor storage and inferior quality control by the manufacturer, to be important factors in vaccination failures.

The persistence of maternal antibody is the important factor in the prevention of the disease in young chickens (Calnek et al., 1961a; Matsukura, 1970). Calnek et al. (1961a) detected IAEV maternal antibody four weeks after hatching, but not at six weeks. However it was not until ten weeks of age, that the serological responsiveness of chickens that received IAEV maternal antibody was comparable to that of chickens without this antibody. Similar poorer serological responses in the progeny of immune parents have been recorded by Matsukura (1970) and Brion et al. (1972). Matsukura (1970) found that IAEV maternal antibody decreased rapidly between seven and 14 days after hatching. Lawson and Gregg (1969) found IAEV parental antibody became low after the four weeks, but disappeared altogether only after the tenth week.

GENERAL MATERIALS AND METHODS

Eggs

The fertile eggs used in this study were classified as susceptible, if they were free of detectable levels of neutralizing antibody to IAEV and immune if they contained specific neutralizing antibody.

Flocks supplying susceptible eggs were tested every four weeks for their continuing susceptibility to IAE by either the virus-serum neutralization (SN) test or by the embryo susceptibility test.

The susceptible eggs were obtained at various times from commercial poultry farms in New South Wales, South Australia and New Zealand and from small flocks maintained by the author. The breeds used in these flocks were White leghorn x Australorp (WL x AO), White Rock x New Hampshire (WRxNH) or White leghorn (WL).

The immune eggs were obtained from a commercial poultry farm (WRxNH) that routinely vaccinated all breeding flocks with an autogenous IAE vaccine.

All the eggs were set in a commercial incubator maintained at approximately 37°C. The incubator trays were clearly labelled with the date of setting of the eggs, the date they were to be used and their immune status.

Chickens

The chickens used throughout the study were also classified as susceptible or immune.

The susceptible chickens were hatched from the eggs obtained from the flock supplying the susceptible fertile eggs that were being used routinely at the time of the experiment.

The immune chickens were obtained as one day old chickens from the poultry farm that supplied the immune eggs.

Accommodation and handling of chickens

(a) Chickens for experimental purposes were reared initially in electric battery brooders (see Figure 1) and subsequently in wire follow-on cages (see Figure 2). The chickens were provided with a commercial chicken feed and water ad libitum. Infected and uninfected chickens were kept separate at all times. When all chickens in a particular experiment had been removed, the room and all the facilities within it, were scrubbed with water and then steam cleaned. The cleaned room and equipment was then allowed to stand at least five days before chickens were again placed in the room. The effectiveness of this procedure in preventing virus contamination from experiment to experiment was demonstrated when susceptible chickens were placed in the room and they did not develop the disease or serum neutralizing antibodies to IAEV.

(b) Chickens for fertile egg production. Four flocks were established by the author on private properties in the vicinity of the University. Initially these consisted of 50 hens and 10 cockerels of the WL breed. The flocks were housed in sheds that had been wired to prevent access by wild birds. The chickens were placed on the farms at one day of age and maintained on the litter throughout their life and given feed and water ad libitum. The flocks were visited once a week by the author. The susceptibility of these flocks was assessed by the EST. These isolated flocks provided IAEV susceptible eggs for a two year period during this study. The flocks were designated as either WL/A, WL/B, WL/C or WL/D.

Figure 1

Electric battery brooder used in the rearing of chickens

Figure 2

Cages used to house chickens after removal from electric battery brooders

Viruses

The van Roekel isolate of IAEV was obtained from Mr. Ian Littlejohns of the New South Wales Department of Agriculture as a homogenate of chicken brain. The chicken embryo passage level of this isolate was not known.

The Newcastle disease virus (NDV) was obtained from Dr. R.J.H. Wells of the University of Melbourne.

The infectious laryngotracheitis virus (ILTV) used was the small plaque variant of a South Australian isolate (Tannock, 1965). The infectious bronchitis virus (IBV) was the T isolate (Cumming, 1963).

The NSW-1 isolate of IAEV was isolated by the author from an outbreak of the disease.

Egg inoculation methods

The methods used in the preparation of the chicken eggs for inoculation were essentially those described by Beveridge and Burnet (1946). For yolk sac inoculation the chicken embryos were incubated for seven days; for allantoic sac inoculation, nine days and for chorio-allantoic membrane challenge, 12 days. Before the shell was penetrated, the section to be cut was disinfected using an egg paint (see formula below) and a hole prepared with a punch or a circular drill. This hole was sealed with either paraffin wax (allantoic and yolk sac methods) or cellotape (CAM method) after inoculation.

All eggs were incubated at approximately 37°C in a commercial incubator (see Figure 3). Eggs were candled daily and all deaths were recorded on a daily basis.

Egg paint.	Mercuric chloride	1 gm
	Eosin	20 gm
	Alcohol 95 percent	1000 cc

Figure 3

Type of incubator used for the setting and hatching of eggs

Collection of tissues and fluids from chicken embryos

(a) Allantoic fluid: Before harvesting the fluid, the eggs were cooled at 4°C for three to five hours.

The fluid was harvested by opening the shell over the air cell and withdrawing the fluid with a sterile Pasteur pipette. The fluid was placed in sterile containers either as a pooled sample or individual egg aliquots and then centrifuged at 2000 rpm for ten to 15 minutes. The supernatant was then obtained and used as the virus stock.

(b) Chorio-allantoic membrane: The membrane was harvested by opening the egg directly beneath the "dropped" CAM and carefully removing the embryo without disturbing the CAM. Subsequently the membrane was withdrawn and the infected area cut away from the remainder. For passage, the CAM was macerated with scissors, homogenized in a tissue grinder and either used immediately or stored at -30°C.

(c) Embryonic tissues: The embryo was removed in a sterile manner from the egg and dissected with sterile, fine pointed scissors and forceps to obtain the prescribed tissues. For whole body homogenization of the embryo, the eyes, beak and legs were removed prior to use.

Criteria of infection with the van Roekel isolate of IAEV

Chicken embryos were considered to be affected by this virus if they exhibited (i) death 120 hours or more after inoculation of the virus and (ii) if the embryo was paralyzed.

Chickens were considered to be affected if they developed IAE or if the characteristic microscopic lesions were observed in the CNS.

Criteria of infection with the NSW-1 isolate of IAEV

Chicken embryos inoculated with the virus were allowed to hatch and the chickens then observed for ten days. The development of the disease within this time interval was the criterion of infection of the embryo. Chickens infected parenterally or orally with this isolate were considered to be infected if they developed IAE or if histological lesions of the disease were observed in the CNS during the specified time interval.

Disposal of infected material

All infected egg material was dropped into a five percent lysol solution or into plastic bags which were sealed and incinerated.

Carcases of infected chickens were also incinerated.

Preparation of virus stocks

Separate stocks of the van Roekel isolate were prepared from the brains of affected chickens and chicken embryos, and also from the viscera and skeletal musculature of affected chicken embryos.

The brains of affected chickens were removed when 50 percent of chickens showed clinical signs of IAE. The chickens were killed, decapitated and the brain extruded through the foramen magnum by squeezing the cranium between the fingers. A 40 percent brain homogenate (w/v) was prepared in phosphate buffered saline (PBS) containing 5,000 iu of benzyl penicillin sodium and 5 mgm streptomycin sulphate per ml. This homogenate was allowed to stand at 4°C for 30 to 45 minutes, and was then

resuspended and dispensed in five ml. aliquots and stored at -30°C. Before use, the homogenate was thawed, centrifuged at 2000 rpm for ten minutes and the supernatant used as the source of virus.

Similarly the brains of affected chicken embryos were removed and a ten percent homogenate (w/v) was prepared. Ten percent homogenates (w/v) of the viscera, and the musculature of affected chicken embryos were also prepared in the same way.

Titration of the van Roekel isolate

Tenfold serial dilution of virus were prepared in PBS. These dilutions were prepared by placing 0.5 ml. of the virus suspension into 4.5 ml. of PBS using a separate graduated pipette for transfer and mixing of the suspension. 0.1 ml. of each serial dilution was then inoculated into the yolk sac of five chicken embryos per dilution.

The inoculated embryos were incubated for 11 days Embryos exhibiting paralysis at the end of this time or those that died 120 hours after inoculation were considered as infected. The 50 percent end point of the embryo infective dose (EID_{50}) was calculated by the method of Spearman-Karber (Finney, 1952) and the titre expressed as EID_{50} per 1.0 ml. of inoculum.

Titration of non chicken embryo adapted virus

Tenfold serial dilutions of the virus were prepared and inoculated into the yolk sac as described above except that seven eggs were used per dilution.

The inoculated eggs of each dilution were then placed in

separate hatching trays (see Figure 4) so that dilution groups were not mixed. The chickens were hatched, wingbanded to identify dilution groups and the chickens were then all reared together. The chickens were observed for ten days for the development of IAE. The 50 percent end point of the chicken infective dose (CID_{50}) was determined by the Spearman-Karber method using the number of chickens in each group that developed the disease in the prescribed time interval.

Virus-serum neutralization test

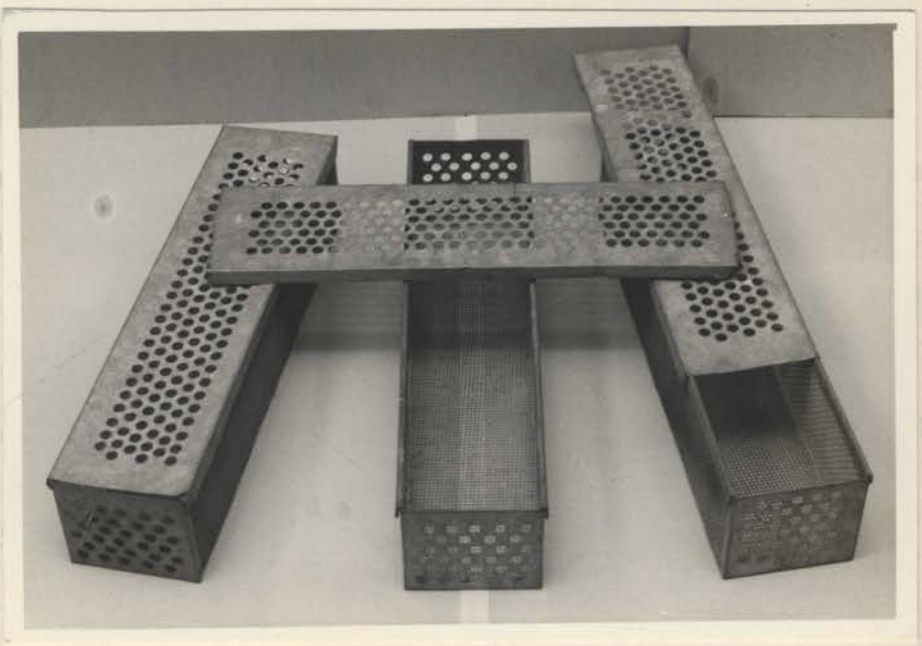
This test was performed in the yolk sac of embryonating chicken eggs using the constant serum variable virus technique.

Serial tenfold dilutions of the van Roekel isolate were prepared as previously described. Aliquots of 0.5 ml. of these dilutions were then placed in separate 5 ml. bottles with 0.5 ml. of chicken serum that had been inactivated at 56°C for 30 minutes. The virus-serum mixture was shaken and allowed to neutralize for 120 minutes at 4°C. A volume of 0.2 ml. of this mixture was then inoculated into the chicken embryo. The neutralization index was calculated as the difference in the end point of virus infectivity of the pre-challenge and post-challenge serum samples.

In tests to determine the NI of pre-inoculation sera, nutrient broth (*) was used. Where possible, undiluted serum was used in these tests. If insufficient serum was available then a two fold dilution in PBS was prepared.

* Nutrient Broth No. 2. Oxoid Ltd.

Figure 4



Hatching trays used to keep chickens from virus dilution groups separated

Embryo susceptibility test (EST)

The EST was performed using the constant virus procedure. Thirty five embryos from each test flock were inoculated with an estimated 100 EID_{50} of the VR isolate. The actual infectivity of the dose of virus used was titrated. The chicken embryos were incubated for 11 days, with deaths being recorded on a daily basis.

If all the test embryos showed signs of IAEV infection, the flock was considered susceptible, provided the test dose was within a range of 50 to 300 EID_{50}. The test was repeated if the virus dose was not within these limits. If only one or two inoculated embryos were normal, and the test dose was acceptable, the test was repeated as soon as eggs were available. During this time the flock was considered to be infected. If more than two embryos were normal in a test, the flock was considered to have been infected with IAEV.

The procedures for infection of chickens

(a) Intra-cerebral (IC) challenge. A fine needle was inserted through the cranium into the forebrain of the left side and 0.05 ml. of suspension inoculated. Most chickens were incapacitated for two to three hours after challenge and approximately five percent died immediately or within 36 hours. Chickens not behaving normally within 48 hours of inoculation were discarded from challenge groups.

(b) Intra-muscular (IM) challenge. Suspensions were inoculated either into the pectoral or the leg muscles.

(c) Oral (O) challenge. A plastic oesophageal tube 2 mm in diameter attached to a five ml. syringe was used to deposit the virus suspension into the crop.

(d) Wing web challenge. A double pronged vaccinating needle was punched through the fold of skin between the humerus and radius/ulna bones.

Bleeding of chickens

Young chickens were bled by decapitation. In chickens three weeks old and younger considerable difficulty was found in collecting blood samples from the brachial vein or by heart puncture.

Older chickens were bled from the jugular or brachial vein.

All collected blood was allowed to clot. The clot was cut and the sample refrigerated for two to 12 hours. The serum was decanted, centrifuged at 1,000 rpm for ten to 15 minutes and the supernatant harvested. The serum was treated at 56°C for 30 minutes and stored at -30°C.

Preparation of IAEV antisera

The van Roekel isolate was used in the preparation of IAEV specific antisera. It was prepared against a stock of this isolate that had been purified by the limit dilution technique and had been identified as an IAEV by neutralization by IAEV specific chicken antisera produced by Dr. G. van Steenis of the Rijks Instituut voor der Volksgezoadheid, The Netherlands (*).

Six week old IAE susceptible cockerels were used in the preparation of the IAEV antisera. The cockerels were bled from the brachial vein on three consecutive weeks prior to an intra-muscular inoculation with an estimated 1,000 EID_{50} of the virus. The birds were re-inoculated with an estimated 1,000 EID_{50} of virus at ten and 12 weeks of age respectively. At 14 weeks of age the birds were bled and

* Supplied by Dr. R.J.H. Wells

inoculated again intra-muscularly with an estimated 10,000 EID_{50} of virus and the NI of these serum samples was determined. If the NI were in excess of $\log_{10}^{3.0}$ the chickens were bled at 18 and 19 weeks of age, the serum collected, pooled and dispensed in one ml. amounts. These samples, together with the pre-inoculation sera were inactivated and stored at $-30^{o}C$.

The serum samples were tested for the presence of neutralizing antibody to ILTV and IBV, haemagglutination inhibition antibody to NDV and Mycoplasma gallisepticum (MG), agglutinating antibody to Salmonella pullorum and Mycoplasma synoviae (MS) and for precipitating antibody to Marek's disease virus (MDV). The techniques used in these tests were as described in Anon (1971). The MDV antigen was prepared by Mr. C.A.W. Jackson and the test performed according to the technique of Woernle (1966). The antigens used in the tests for S. pullorum, MG and MS were obtained commercially (*).

Histological examination of tissues

All tissue for histological examination was fixed in 12.5 percent formol saline for at least 48 hours. The fixed pieces were embedded in paraffin from which 5μ sections were cut and these were stained with haematoxylin and eosin.

Statistical analysis of the significance of results

Comparison of the mean score of two groups was made using a "t" test, corrected for small numbers in the test groups (Snedecor and Cochran, 1967).

* Burroughts Wellcome diagnostic reagents

More complex comparisons were made by an analysis of variance. In some instances it was necessary to make corrections for groups of unequal size, as non-specific deaths occurred in some treatment groups. Mr. C. Gray, Department of Mathematical Statistics, Commonwealth Scientific and Industrial Research Organization assisted with these analyses.

PART I

STUDIES ON INFECTIOUS AVIAN ENCEPHALOMYELITIS VIRUS

The culture systems that have been commonly used in the study of animal viruses include experimental animals, embryonated chicken eggs and tissue cultures. The choice of the system used depends on the virus being studied and the nature of the investigation.

IAEV has been cultivated in chickens (Jones, 1934; Olitsky, 1939; Schaaf, 1958), in chicken embryos (Wills and Moulthrop, 1956; MacLeod, 1965) and in various tissue cultures (Mancini and Yates, 1967, 1968a, 1968b). However, the multiplication of the virus in tissue cultures has not been associated with the development of specific cytopathogenic effect. Consequently the virus has only been assayed in birds or embryonating eggs, provided these contain no neutralizing antibody to IAEV (Sumner et al., 1957a). Chickens or chicken embryos without this antibody have not been readily available in Australia and as a result little research has been undertaken into the virus in this country. Therefore, adequately identified isolates, apart from the van Roekel virus, were not available at the beginning of this study.

The aim of the work described in this part was to isolate and characterize IAE viruses, and to study the growth characteristics of the virus in chicken, chicken embryos and in various cell cultures.

Chapter 1

THE PROPAGATION OF THE VAN ROEKEL VIRUS IN CHICKENS

Introduction

IAEV was originally isolated from the brains of IAE affected chickens by the intra-cerebral (IC) inoculation of tissue homogenates into susceptible chickens (Jones, 1934). Subsequently the virus has been isolated from the viscera and faeces of infected birds, and the disease reproduced by the challenge of chickens either *per os* or by other parenteral routes (Feibel *et al.*, 1952; Schaaf, 1958; Calnek *et al.*, 1961a).

The van Roekel isolate of IAEV has been extensively used in research and diagnostic work and is considered to be the standard reference isolate of the virus (Anon, 1971). Because of adaptation to the chicken embryo, the VR virus has lost the ability to induce the disease by contact exposure (Calnek *et al.*, 1961a), but will induce the disease in chickens challenged parenterally (van Roekel *et al.*, 1939; Feibel *et al.*, 1952; Calnek and Jehnich, 1959b).

The work in this section was undertaken to multiply the stock of the virus available and to study the effect of the VR virus on the chicken.

Materials and Methods

Sixty IAE susceptible chickens one day old were individually identified using metal wingbands. Ten chickens were challenged intra-muscularly, ten intra-cerebrally, ten intra-peritoneally (IP), ten sub-cutaneously (SC) and ten orally with the VR isolate. Ten chickens challenged IC with 0.05 ml.

of sterile PBS were the uninfected controls. The infectivity of the dose of virus used was subsequently titrated in chicken embryos.

The chickens were brooded together and examined daily for the onset of clinical signs of IAE. Chickens were removed as they became affected, killed and the brain and spinal cord removed for histological examination. All of the remaining chickens were killed and autopsied on the 28th day after challenge and the same tissues collected for histological study.

A further ten chickens, seven days of age, were inoculated with the VR virus by the IM method. These chickens were observed daily and when 50 percent showed IAE, all were killed, the brains removed and used to prepare a stock of the virus. The infective titre of this stock was titrated later in chicken embryos.

Results

All of the chickens in the group challenged IC developed the disease. The first clinical signs noted were incoordination and drooping of the wings. Most affected chickens eventually became paralyzed (see Figure 5). Only a proportion of the chickens in the group challenged IM, IP and SC developed IAE, although all exhibited microscopic lesions of the disease (see Table 1). Chickens in the group challenged orally failed to develop clinical disease or histological lesions. Uninfected control chickens maintained in-contact with infected chickens failed to develop the disease or microscopic changes of IAE. No differences were noted in the

Figure 5

Chickens clinically affected by IAE after an intracerebral challenge with the van Roekel virus;control chickens, standing; IAE affected, paralyzed

expression of the disease in the chickens challenged by the different procedures. The incubation period was 10 to 15 days.

Table 1

The development of IAE in chickens challenged with 845 EID_{50} of the VR virus

Method of challenge	Proportion affected		Number with IAE					
	Clinical signs	Histo. lesions	Incubation period (days) 10	11	12	13	14	15
Intra-cerebral	$10/_{10}$*	$10/_{10}$	1	4	3	1		1
Intra-muscular	$7/_{10}$	$10/_{10}$	1	2	4			
Intra-peritoneal	$8/_{10}$	$10/_{10}$		1	4	2	1	
Sub-cutaneous	$8/_{10}$	$10/_{10}$	1	1	1	3	2	
Oral	$0/_{10}$	$0/_{10}$						

* Number affected/$_{\text{number challenged}}$

Discussion

The parenteral challenge of IAE susceptible one day old chickens with the VR virus resulted in the development of clinical signs of the disease. There were no observable differences in the clinical disease in the chickens challenged by different methods, although there was a suggestion of a longer incubation period in the groups challenged IP and SC.

The histological lesions of IAE-neuronal degeneration, focal and local gliosis and perivascular cuffing were observed throughout the central nervous system, but were particularly noticeable in the cerebellum, medulla oblongata, and spinal cord. All chickens that developed clinical signs of IAE exhibited histological lesions.

The inability to cause the disease or induce microscopic lesions in chickens challenged orally with the VR isolate confirmed the findings of Calnek et al. (1961a). The failure to detect clinical IAE or histological changes, in the uninfected, incontact control chickens further confirmed the findings of Calnek et al (1961a) of the inability of this isolate to spread by contact exposure.

Summary

The VR virus induced IAE in chickens challenged parenterally with the virus, but not in chickens challenged orally. The incubation period of the disease was ten to 15 days There was a good correlation between the development of clinical signs of the disease and the detection of histological lesions.

Chapter 2

THE PROPAGATION OF THE VAN ROEKEL VIRUS IN CHICKEN EMBRYOS

Introduction

The VR isolate of IAEV has been successfully propagated in chicken embryos. The gross lesions induced in these embryos are paralysis, skeletal muscle atrophy, dwarfing and abnormal extension of the toes (Wills and Moulthrop, 1956; Jungherr et al., 1956). These lesions are considered to be pathognomonic and have been used as the end point of infectivity in titrations of the virus (Sumner et al., 1957a). The VR virus has been recovered from the brain, viscera, skeletal muscles and the embryonic membranes and fluids of affected embryos (MacLeod, 1965; Burke et al., 1965).

The aim of the work described in this chapter was to establish the characteristics of the growth of the virus under the prevailing laboratory conditions so that standardization of these fundamental tests could be achieved.

Materials and Methods

Fertile eggs. The susceptible eggs used were obtained from a commercial WLxAO flock.

Titration of the virus. The virus was titrated as described in the General Materials and Methods.

Accuracy of the determination of the end point of infectivity of the virus. A comparison was made of the virus content of embryos showing macroscopic signs of IAEV infection 11 days after inoculation, and those not showing signs of

infection. The embryos not showing the pathognomonic lesions were set aside and the brain removed. The brains of individual embryos were homogenized into a 10 percent suspension, and 0.20 ml of this inoculated into the yolk sac of ten chicken embryos. The brains of five uninoculated chicken embryos were used as controls.

These test and control embryos were examined 11 days after inoculation. Any embryo showing signs suggestive of IAEV infection were prepared for a further brain passage. Embryos obviously affected by IAEV on the first passage were considered infected and not passaged. The chicken embryo in the original titration, from which the brain suspensions were prepared, was scored as infected or uninfected on the basis of the detection of virus in either the first or second passage of that brain homogenate. This experiment was repeated three times.

The length of incubation of VR inoculated chicken embryos

Enough susceptible fertile eggs were placed in the incubator so that five separate virus titrations, of the one stock of virus, in the range 10^{-1} to 10^{-7} could be undertaken on the one day.

One complete titration of the virus was examined on the 9th, 10th, 11th, 12th and 13th day after inoculation, and the 50 percent end point of infectivity determined.

The age of the chicken embryo for propagation of virus

Enough susceptible fertile eggs were incubated so that, on a designated day, sufficient embryos aged three, five, six, seven, nine and ten days, were available to allow virus titrations in the range 10^{-1} to 10^{-7} to be undertaken in each age group. The embryos were incubated for eleven days.

The tests on the age of the chicken embryo and the length of incubation of the embryos, were not combined in a more complex experimental design because there were insufficient eggs to allow such an experiment on the one day.

Growth of the virus

Sixteen chicken embryos were inoculated with an estimated 100 EID_{50} of the VR isolate in the usual way. Ten chicken embryos inoculated with a suspension of chicken embryo brain containing no virus acted as controls. The actual infectivity of the dose of virus inoculated was subsequently determined by titration.

At intervals, eggs were removed from the incubator, opened and from each embryo, five ml. of allantoic fluid, a portion of CAM, and five ml. of yolk, were collected. The brain and viscera were also removed. Ten percent homogenates (w/v) of the brain, viscera and CAM were prepared and stored at $-30^{o}C$. These tissues were harvested after 24, 48, 72, 120 and 240 hours after inoculation.

Titrations of these suspensions were then prepared. The complete titration of only one tissue was performed on the one day, as insufficient eggs were available to permit the titration of all tissues on the one day.

Comparison of different diluents

Titrations of the infectivity of the one vial of the virus were prepared using the following diluents: phosphate buffered saline (PBS), PBS containing one percent normal chicken serum, nutrient broth, tryptose phosphate broth, distilled water, Hanks balanced salt solution and Medium No. 199.

Titrations were undertaken on vials of the virus containing

chicken embryo brain (CEB), chicken embryo viscera (CEV) and chicken embryo muscle (CEM) homogenates.

The neutralization of the van Roekel isolate by IAEV specific antiserum

The virus-serum neutralization tests were performed according to the technique described in the General Materials and Methods.

The antisera used were produced by Dr. G. van Steenis (GVS antiserum) and by the author (VR).

The effect of the dilution of serum on the neutralizing capacity of that serum.

Serial tenfold dilutions of the specific antiserum prepared by the author were made and were allowed to react in virus-serum neutralization tests as previously described. The NI of the sera were then determined. This test was repeated four times.

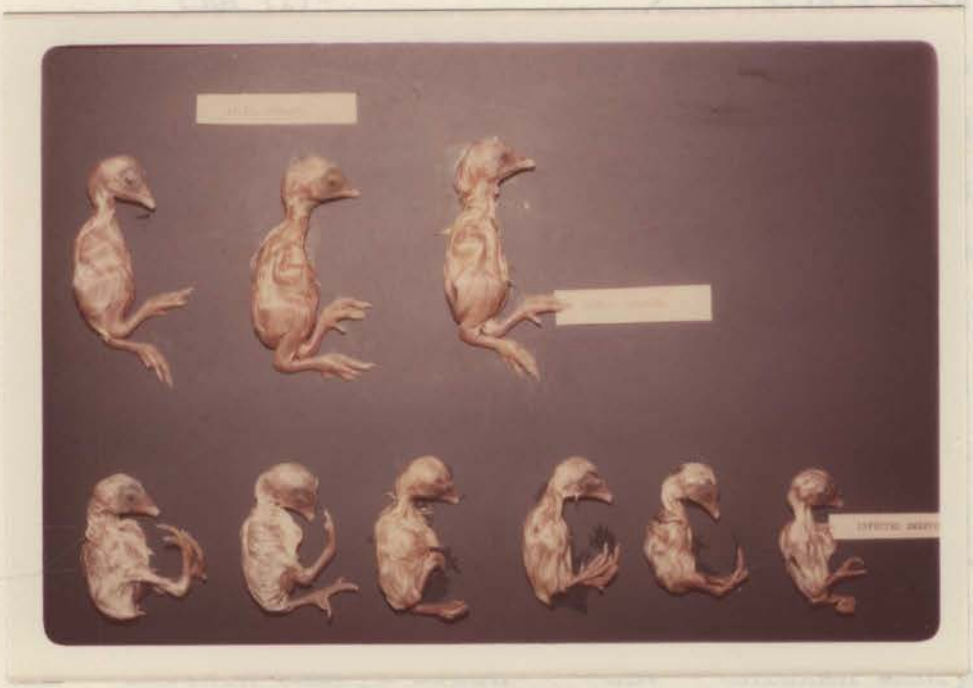
Undiluted antiserum was allowed to react with virus in separate tests for 60-120 and 180 minutes at 4°C.

Results

The titration of the VR virus

The characteristic lesions induced in chicken embryos affected by the VR virus are illustrated in Figure 6. The titre of virus in tissue homogenates prepared from infected chicken brains (CB), chicken embryo brains (CEB), chicken embryo viscera (CEV) and chicken embryo muscle (CEM) is recorded in Table 2 . Five successive titrations of the one vial of CEB over a one year period showed no significant decline in virus infectivity in vials stored at -30°C. The virus titre of CB, CEB and CEM were comparable, but that in CEV was lower

Figure 6



Chicken embryos affected by the van Roekel isolate of IAEV (18 days of incubation)

Table 2

The titre of the virus in tissues of chicken and chicken embryos infected with the VR virus

Tissue homogenate	Virus titre $\log_{10}$ EID_{50} per ml.±SD
CB	5.94 ± 0.24
CB	6.17 ± 0.24
CEB (1)*	5.78 ± 0.74
CEB (2)*	5.90 ± 0.44
CEB (3)*	6.35 ± 0.44
CEB (4)*	5.78 ± 0.36
CEB (5)*	5.92 ± 0.27
CEM	6.10 ± 0.44
CEM	5.97 ± 0.34
CEV	4.70 ± 0.41
CEV	5.15 ± 0.24

CB - chicken brain CEM - chicken embryo muscle

CEB - chicken embryo brain CEV - chicken embryo viscera

* Five successive titrations of the one vial over a 12 month period.

Accuracy of the determination of the end point of infectivity of the virus

The results of the comparison of the assessment of the virus titre based on macroscopic signs of infection and on virus isolation from the brain of the embryo are recorded in Table 3.

Table 3

A comparison of virus titre based on the development of lesion and virus isolation

	Virus concentration $\log_{10}$ EID_{50} per ml.	
	End point - gross lesion	End point - virus isolation
Expt. 1	5.96 ± 0.44	6.70 ± 0.42
Expt. 2	5.58 ± 0.36	7.16 ± 0.38
Expt. 3	5.90 ± 0.40	7.00 ± 0.52

Expt. - experiment

The virus could be readily recovered from chicken embryos not showing the characteristic lesions. These embryos were usually classified as sluggish or slow to respond to stimuli such as pinching of the toes with forceps.

Effect of incubation period on the titre of virus in inoculated chicken embryos

The virus titre was similar for all periods of incubation examined (Table 4).

Table 4

Titre of virus in chicken embryos incubated for different times

Length of incubation (days)	Virus titre $\log_{10}$ EID_{50} per ml. ±SD
9	5.54 ± 0.54
10	6.16 ± 0.48
11	6.10 ± 0.36
12	5.98 ± 0.44
13	4.60 ± 0.76

EID_{50} - Embryo infective dose

Effect of the age of the chicken embryo at inoculation on virus titre

No significant differences were found in the virus titres determined in embryos inoculated at five, six and seven days of age. The titres obtained in embryos inoculated at three, nine and ten days were lower ($P<0.01$) than those obtained on the other three days (Table 5).

Table 5

The effect of the age of the embryo on the end point of infectivity

Age of the embryo	Virus infectivity $\log_{10}$ EID_{50} per ml. ±SD
3	4.35 ± 0.28
5	5.32 ± 0.67
6	5.76 ± 0.74
7	5.94 ± 0.44
9	4.32 ± 0.56
10	3.96 ± 0.84

Effect of diluents on the titre of virus

No significant differences were detected between the titres obtained in titrations using the diluents tested (Table 6).

The mean titre of the CEV homogenate was lower ($P<0.01$) than that of the CEB or CEM homogenates, and this supported previous observations (see Table 2).

Table 6

The effect of different diluents on virus titre

Diluent	Virus concentration - $\log_{10}$ EID_{50} per ml.±SD		
	CEB	CEM	CEV
PBS	5.54 ± 0.34	5.46 ± 0.28	4.10 ± 0.24
PBS plus serum	5.78 ± 0.28	5.32 ± 0.44	4.88 ± 0.42
Nutrient broth	5.84 ± 0.44	5.57 ± 0.35	4.58 ± 0.27
Maintenance medium 199	6.10 ± 0.28	5.62 ± 0.38	4.06 ± 0.36
Hanks balanced salt soln.	5.68 ± 0.74	5.44 ± 0.41	4.90 ± 0.44
Tryptose phosphate broth	5.82 ± 0.28	5.63 ± 0.52	4.72 ± 0.20
Distilled water	5.72 ± 0.44	5.44 ± 0.44	4.86 ± 0.40

PBS - phosphate buffered saline

CEB - chicken embryo brain

CEM - chicken embryo muscle

CEV - chicken embryo viscera

The growth of the virus in the chicken embryo

The virus attained its highest concentration in the brain and viscera 240 hours after inoculation. The virus was also isolated from the CAM, allantoic fluid and yolk sac, but in lower concentrations (Table 7).

Table 7

The titre of virus in various embryonic tissues at intervals after infection with the virus

Time after inoculation (hours)	Virus titre - $\log_{10}$ EID_{50} per ml.±SD				
	Tissue				
	Brain	Viscera	Yolk sac	Allantoic fluid	CAM
24	0	0	0	0	0
48	1.70±0.44	2.14±0.62	0	0	1.70±0.25
72	2.96±0.36	3.92±0.41	1.36±0.34	0	2.15±0.40
120	5.58±0.42	4.36±0.28	NT	1.90±0.54	3.37±0.44
240	5.90±0.24	4.10±0.36	NT	2.54±0.36	*

CAM - chorio-allantoic membrane

NT - not tested

* - contaminated with bacteria

Virus challenge dose - 120 EID_{50}

The neutralization of the van Roekel isolate by IAEV specific chicken antisera

The neutralization indices contained in neutralization tests between the van Roekel virus and IAEV specific chicken antisera are detailed in Table 8. The NI of the GVS antiserum was $\log_{10}^{3.34}$ and that of the test antiserum $\log_{10}^{4.20}$ and $\log_{10}^{4.58}$. This antiserum did not react in tests with ILTV, IBV, NDV, MDV, M. gallisepticum and M. synoviae.

Table 8

The neutralization of the van Roekel virus by IAEV specific antisera

Virus titre				Neutralization index ($log_{10}x$)
	log_{10} EID_{50} per ml.±SD			
PBS	Antiserum	Pre-infection	Post-infection	
6.16±0.28	GVS	6.10±0.71	2.76±0.28	3.34
6.10±0.73	VR	5.90±0.48	1.70±0.40	4.20
6.30±0.68	VR	5.94±0.71	1.36±0.62	4.58

PBS - Phosphate buffered saline

The effect of dilution, and the time for neutralization on the neutralizing capacity of an antiserum

The neutralizing ability of an IAEV specific chicken antiserum at various dilutions, and at various times at 4°C is recorded in Table 9. The relationship between the neutralizing capacity of the serum and the dilution was not constant. The effect of dilution decreased as the serum became more dilute (see Graph 1).

No advantage could be demonstrated for a neutralization period greater than 120 minutes at 4°C. This was subsequently adopted as the standard time in all virus-serum neutralization tests.

GRAPH 1.

The effect of dilution of serum on the neutralizing capacity of IAEV chicken antiserum.

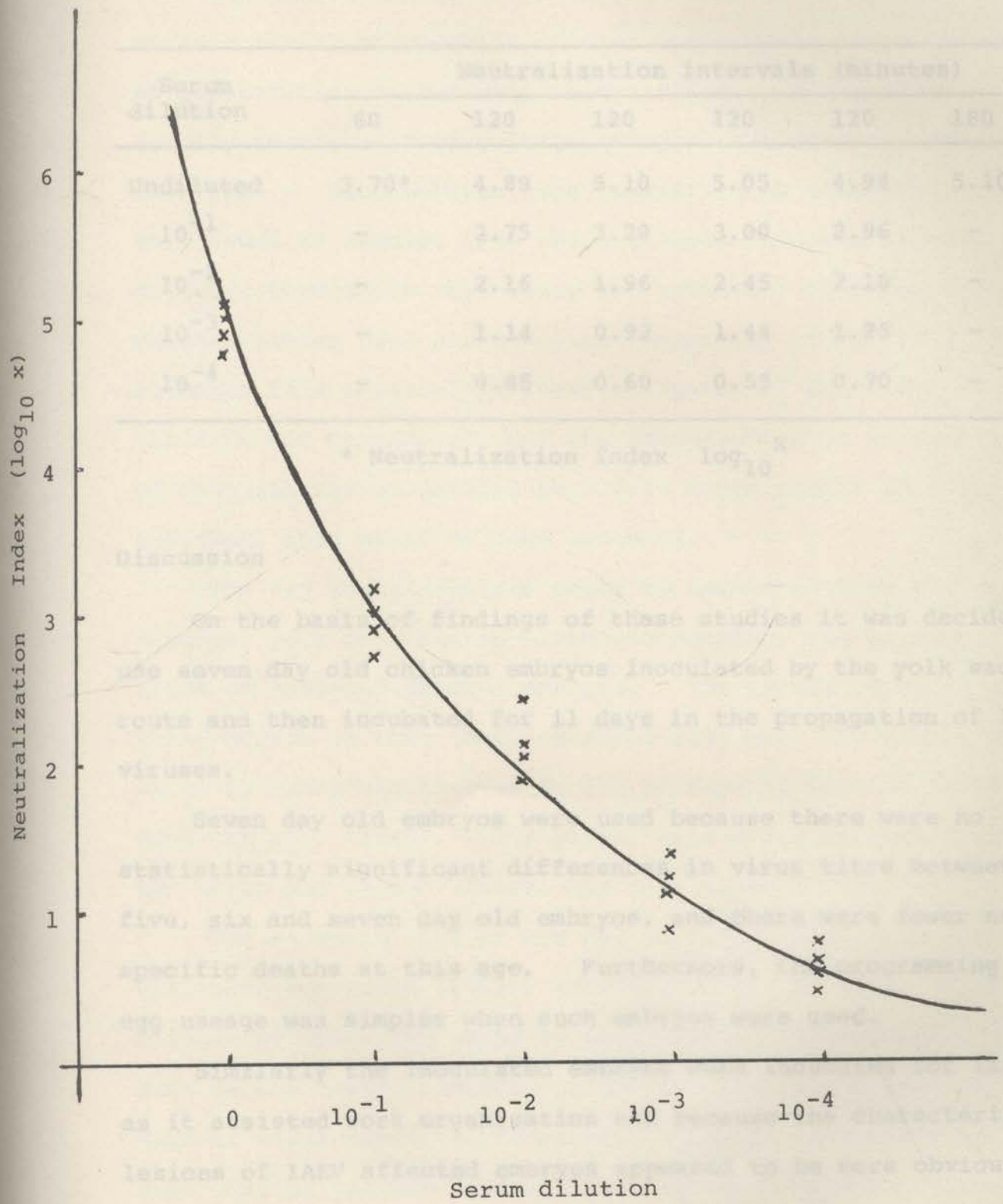


Table 9

The effect of dilution of IAEV antiserum, and the time of neutralization, on the neutralization index

Serum dilution	Neutralization intervals (minutes)					
	60	120	120	120	120	180
Undiluted	3.70*	4.80	5.10	5.05	4.94	5.10
10^{-1}	-	2.75	3.20	3.00	2.96	-
10^{-2}	-	2.16	1.96	2.45	2.10	-
10^{-3}	-	1.14	0.92	1.44	1.25	-
10^{-4}	-	0.85	0.60	0.55	0.70	-

* Neutralization index $\log_{10}{}^{x}$

Discussion

On the basis of findings of these studies it was decided to use seven day old chicken embryos inoculated by the yolk sac route and then incubated for 11 days in the propagation of IAE viruses.

Seven day old embryos were used because there were no statistically significant differences in virus titre between five, six and seven day old embryos, and there were fewer non-specific deaths at this age. Furthermore, the programming of egg useage was simpler when such embryos were used.

Similarly the inoculated embryos were incubated for 11 days as it assisted work organization and because the characteristic lesions of IAEV affected embryos appeared to be more obvious after this period. No significant effect on the virus by differer diluents was demonstrated. The beneficial effect of the addition of chicken serum to the diluent (Hoekstra, 1964) could not be

confirmed. As a result PBS was used as the standard diluent as it was readily available.

The use of paralysis of the chicken embryo as the end point of infectivity of virus in chicken embryos was found to result in conservative estimates of virus titre. Virus could be readily recovered from chicken embryos that were not completely paralyzed. Such embryos were classified as sluggish, or slow to respond to removal from the egg shell and pain stimuli. However, throughout this study the criterion of infection of the chicken embryo used was complete immobility of the live embryo. Although this resulted in lower estimates of the titre of the virus it was considered that the introduction of an assessment of sluggishness or partial paralysis could result in a subjective judgement that would be less accurate.

The van Roekel isolate could be recovered from the brain, viscera, yolk sac, chorio-allantoic membrane and allantoic fluid of infected chicken embryos. The titres of virus in these organs in this study compared well with the titres of virus in embryonic tissues in the studies of MacLeod (1965) and Burke et al., (1965). The virus reached its maximum titre in the brain and in the viscera ten days after infection.

The repeatability of the end points of titrations of the different embryonic tissues indicated that the titre of an aliquot of the virus could be predicted. Consequently, tests such as the embryo susceptibility test that required a certain dose of virus could be undertaken. Repeat titrations on the same vial of the van Roekel virus indicated that the virus could be stored at least one year at $-30^{\circ}C$ without a fall in titre.

The van Roekel isolate used in the virus serum neutralization test was neutralized by the IAEV specific antiserum provided by Dr. G. van Steenis. This provided additional evidence for the identity of the virus as an IAEV. Chicken antiserum prepared against this isolate by the hyper-immunization of chicken with a purified stock of the virus, neutralized the isolate. This antiserum contained no detectable antibodies to a range of common poultry pathogens. It was subsequently used in neutralization tests to identify other isolates of IAEV.

The dilution of the antiserum prior to its use in neutralization resulted in a significant fall in the neutralizing capacity of that serum. This effect was inconstant however, as it decreased the more dilute the serum became. This finding confirmed the results of von Bulow (1965). The extent of the decrease - dilution of whole serum to 1 in 10 resulted in a one hundred fold reduction in neutralizing ability - indicated that antiserum should not be diluted in determinations of a neutralization index. However, in studies utilizing baby chickens, it was frequently difficult to obtain an adequate serum sample and this necessitated the use of diluted serum. When this was necessary it was decided that serum dilution should be no more than two fold.

The virus-serum neutralization tests were incubated at $4^{o}C$ as a way to standardize the temperature of neutralization. At $4^{o}C$ no advantage could be demonstrated in the neutralizing ability of the serum after 120 minutes. This was adopted as the standard time interval for neutralization tests.

Summary

The van Roekel isolate induced lesions characterized by paralysis, atrophy of skeletal muscles, dwarfing and extension of the toes in affected chicken embryos. The virus could be titrated with good repeatability in chicken embryos using these characteristics as the end point in titrations. The virus could however be isolated from chicken embryos not exhibiting these lesions.

The virus was neutralized by IAEV specific chicken anti-serum and this indicated it was an IAEV. The dilution of the serum prior to a virus-serum neutralization test was demonstrated to have a significant effect on the neutralizing capacity of the serum. This effect was inconstant, decreasing as the serum became more diluted.

Chapter 3

THE ISOLATION AND CHARACTERIZATION OF A FIELD ISOLATE OF IAEV

Introduction

The van Roekel isolate of IAEV has lost the ability to induce disease in chickens by contact exposure (Calnek and Jehnich, 1959b; Calnek et al., 1960). It is therefore unsuitable to use in some studies of IAE and in the development of an oral vaccine. Consequently it became necessary to isolate an IAEV so that further studies into the disease and in the development of a live, oral vaccine could be commenced. An outbreak of IAE that occurred soon after the commencement of this study provided an opportunity not only to study the disease under field conditions, but also to isolate and characterize a wild IAE virus and to determine its distribution in the population.

Materials and Methods

Chickens and eggs. The susceptible chickens and embryonating eggs used in this section were obtained from a commercial WRxNH flock.

Origin of the field isolate. The field virus was isolated from the brain of a 12 day old chicken showing signs of IAE. The eggs from which this chicken hatched were derived from a 44 week old meat strain chicken flock of approximately 6000 birds. Two strains of birds, a DW and a TM strain were maintained on this farm. The DW strain provided a broiler chicken sire line, and the TM strain a broiler chicken dam line. The percentage hen day production of the two groups was recorded daily on the farm. The chickens were maintained under deep

litter conditions and were given feed and water ad libitum.

The eggs collected from the DW and TM strain were kept separated, both on the farm and in the hatchery. Consequently, accurate figures on the egg production and hatchability of the eggs of the two strains were available. During the period of the outbreak DW chickens hatched from this flock were distributed to four farms. Daily total mortality counts were made of these chickens. These could be used to calculate a percentage mortality of the birds received at each of the four farms. This was done when the DW chickens were nine weeks of age. The DW chickens hatched from the flock were reared on new wood shaving litter under high pressure gas brooding equipment and given feed and water ad libitum. The conditions on the four farms were apparently similar. The outbreak was confirmed to be IAE by histological examination of the brain and spinal cord of affected chickens.

Virus isolation

Twenty susceptible one day old chickens were challenged intra-cerebrally with 0.05 ml. of a ten percent homogenate of brain tissue from the affected chicken.

The IC challenged chickens were observed and when 50 percent showed clinical signs of IAE, the chickens were killed, the brains removed and a ten percent brain tissue homogenate was prepared. This preparation was used as the initial stock of the virus, hereafter termed the NSW-1 isolate.

Serial passage of the NSW-1 isolate in chicken embryos

0.2 ml. of a twofold dilution of the chicken brain stock was inoculated into 15 seven day old susceptible embryos by the yolk sac route. The inoculated embryos were incubated for 11 days

at 37°C. The eggs were then opened and the embryos examined for signs of IAE. If no signs were present the brain of the chicken embryos were removed and a ten percent suspension (w/v) was prepared. A twofold dilution of this suspension was then inoculated into the yolk sac of a further 15, seven day old embryos.

This procedure was repeated until all of the inoculated embryos that survived 11 days of incubation showed lesions of IAE. At this passage level the isolate was considered to be adapted to chicken embryos.

Identification of the NSW-1 virus

In tests used to identify and characterize the field isolate, the virus was used as a brain suspension of the second passage of the virus in embryonating eggs.

Titration of the NSW-1 virus

Titrations were undertaken by the technique described in the General Materials and Methods for isolates of the virus not adapted to the chicken embryo.

Virus-serum neutralization tests

These were performed according to the technique described in the General Materials and Methods, with the following modifications. Seven embryos were inoculated with each virus dilution, and the end point of infectivity was determined as in titrations of the infectivity of isolates not adapted to grow in the chicken embryo. IAEV specific antisera supplied by Dr. G. van Steenis (GVS), and one produced by the author (VR) were used. Both of these were paired serum samples.

Tests for essential lipids in the virus

Both the van Roekel and the field isolate of IAEV were used in these tests. The van Roekel isolate preparation was a chicken embryo brain suspension.

(a) Ether sensitivity - This test was performed according to the technique of Andrews and Horstman (1949).

(b) Chloroform sensitivity - This test was undertaken according to the procedure of Feldman and Wang (1961).

pH stability test

This test was performed using the technique of Dimmock and Tyrrell (1964). The virus was placed in the buffered solution at 37°C for 60 minutes. At the end of this time, the pH was adjusted to 7.2 by the addition of equal volumes of 1M phosphate buffer (pH 7.2) for the solutions of pH 3.4 or less, and 1M phosphate buffer (pH 6.9) for high pH values. The infectivity of the isolate was then titrated. Tests were conducted on both the van Roekel and the NSW-1 viruses.

Thermostability test

The test was undertaken at 56°C for varying lengths of time. The virus preparation was diluted tenfold. Six, 1 ml. aliquots were placed in a refrigerator at 4°C, and six aliquots of one ml. were placed in a water bath at 56°C. One vial was removed for each of these at 30, 60, 120, 180, 240 and 300 minutes after the commencement of the test and the infectivity of the virus titrated. This format was based on the technique of Wallis and Melnick (1962). Both the van Roekel and the field isolate of IAEV were used.

Haemagglutination test

The haemagglutination of an 0.5 percent suspension of thrice washed chicken, turkey, duck, guinea pig, rabbit, sheep and rat red blood cells by IAEV was tested in micro-haemagglutination trays using the technique of Halpin (1966b). The haemagglutination of the chicken and turkey red blood cell types was tested using a NDV isolate.

Formation of pocks on the CAM

The virus suspension was centrifuged at 2000 rpm for ten minutes and then 0.2 ml. was inoculated onto the CAM of ten, 12 day old embryonating eggs. After five days incubation, the CAM's were harvested and examined for lesions. If none were found, the CAM's were homogenized to a 25 percent suspension (w/v), and the supernatant inoculated onto a further ten CAM's. If these were negative, one further passage was undertaken. Virus suspensions not producing lesions on the CAM after three serial passages were considered free of pock forming agents.

Infectivity for chickens of the NSW-1 virus

Sixty, one day old susceptible chickens were challenged with an estimated 1,000 CID_{50} of the field isolate. The actual dose of virus was subsequently titrated in chicken embryos. Ten uninfected chickens housed separately were kept as controls. Ten chickens were challenged by each of the following routes - IC, IM, ID, SC and O. The ten control chickens were challenged IC with a brain suspension of a chicken embryo that was not infected with the virus.

Prevalence of infection with IAEV

The embryo susceptibility test was used to determine the prevalence of IAE infected flocks in Australia. Only flocks

isolated from other poultry farms, and which had not used autogenous IAE vaccines, or controlled exposure system to limit the disease, were tested. Thirty five flocks in New South Wales, Queensland, Victoria, South Australia and New Zealand were examined. The EST was performed according to the technique described in the General Materials and Methods.

Results

Clinical description of the outbreak of IAE

The mean egg production over a seven day period, expressed as the percentage of hens laying eggs, for both the DW and TM strains is recorded in Table 10. The percentage egg production over a ten week period is tabulated.

Table 10

Egg production in a flock infected with IAEV

Week number	Shed 1		Shed 2	
	TM strain	DW strain	TM strain	DW strain
41	56.5*	51.4	62.7	56.7
42	57.9	55.8	64.6	60.0
43	54.3	52.4	62.5	47.1
44	42.7	25.2	46.5	36.2
45	36.9	18.7	43.7	24.1
46	38.5	26.3	40.8	26.3
47	38.9	34.3	41.9	30.9
48	38.7	32.1	40.4	33.6
49	50.7	49.3	57.2	53.3
50	51.4	48.3	57.2	57.5
51	50.3	48.0	56.0	50.0
52	48.4	46.8	52.0	49.8

* Mean number of birds laying per week - expressed as a percentage

The decline in egg production was 35 to 40 percent in the DW strain, and 20 to 25 percent in the TM strain. The production of both strains in sheds 1 and 2 was markedly affected for five weeks, and never recovered to pre-infection levels.

The hatchability of the eggs supplied from both sheds to the farms hatchery during this time is recorded in Table 11. The week number detailed in the table refers to the week of egg collection rather than to the week of hatching. The hatchability can therefore be correlated with the percentage egg production of that week.

Table 11

The hatchability of eggs produced during the outbreak of IAE

Week number	% Hatchability	
	TM strain	DW strain
41	82.8	80.0
42	87.0	80.5
43	85.0	78.0
44	84.0	61.0
45	87.5	67.0
46	85.5	74.0
47	86.3	74.5
48	85.0	76.5
49	82.0	69.8
50	82.4	69.0
51	81.0	69.0
52	83.5	68.8

The figures presented in Table 11 are the average of three hatches per week for each strain of chicken.

The hatchability of the TM strain was unaffected by the infection. Progeny of this strain nevertheless developed the disease. The hatchability of the DW strain declined by 18 percent, and never regained pre-infection levels of hatching.

Affected chickens hatched from this infected breeding flock were first detected during week 43 and subsequently in weeks 44, 45 and 46. No clinical cases of the disease were reported in chickens hatching from week 47 and onwards.

The percentage mortality to nine weeks of age, in the DW chickens hatching from weeks 41 to 48 is recorded in Table 12.

Table 12

The percentage mortality in the progeny of IAEV infected DW strain chickens

Week number	Hatch No.	% mortality	Week number	Hatch No.	% mortality
41	1	3.0	45	1	31.8
	2	4.2		2	42.0
	3	2.9		3	23.6
42	1	4.5	46	1	16.1
	2	4.1		2	11.3
	3	3.9		3	14.8
43	1	5.1	47	1	6.2
	2	3.6		2	5.8
	3	10.2		3	3.9
44	1	22.4	48	1	5.0
	2	18.6		2	3.5
	3	25.5		3	3.6

During the outbreak on the breeding farm, one day old DW strain chickens were distributed from it to four rearing farms. The

DW strain chickens supplied to the rearing farms from the infected breeding farm comprised 90 percent or more of the intake of DW strain chickens onto these farms for that particular period. This provided an unusually fortunate opportunity to assess the affects of IAE, and the viability of chickens, as commonly more than one breeding farm contributes chickens to a rearing farm. The rise in mortality was pronounced from the third hatch of week 43. The mortality continued at a high rate in all subsequent hatches up to week 46. The mortality then returned to a rate similar to that of the period before IAE was diagnosed. The highest mortality was produced in the chickens hatched during week 45. IAE affected chickens were detected from the third hatch of week 43 through to the third hatch of week 46.

The average percentage mortality, on a weekly basis, of one of the farms detailed in Table 12 during weeks 44, 45 and 46 is recorded in Table 13.

Table 13

The percentage week mortality in DW strain chickens infected with IAEV

Week number	Age of chickens infected with IAEV (weeks)								
	1	2	3	4	5	6	7	8	9
44	11*	10	52	10	1	2	4	5	3
45	7	3	58	6	5	3	3	5	5
46	4	14	12	44	9	9	4	1	2

* % mortality

Most of the mortality in weeks 44 and 45 occurred during the third week of life of the progeny. In week 46,

however, most of the mortality occurred in the fourth week.

Prevalence of infection with IAEV in Australian flocks

Thirty five separate flocks were surveyed. Five of the 35 flocks examined were found to be susceptible to IAEV at the first test of the flock. A re-test of these flocks 12 weeks after the initial test revealed that three had subsequently been infected. The managers of these flocks had not been aware of this infection.

The origin, age of the flock at the initial test, the virus dose used and the immune status of these flocks is recorded in Table 14. Five turkey flocks were also surveyed. These were found to have been infected with IAEV.

Serial passage of the field isolate in chicken embryos

The lesions characteristic of IAE in the chicken embryo were first detected in one of 12 embryos on the fourth passage. Subsequently 10 of 13 showed changes of IAEV infection on the fifth passage. All the surviving embryos were effected on the sixth passage. The embryos not exhibiting the characteristic sign of paralysis on the fifth passage showed slowness in their movement and this suggested that they were also affected by the virus. Nevertheless the virus was considered to have become adapted to grow in the chicken embryo on the sixth serial passage.

Virus-serum neutralization tests with the field isolate

The results of the neutralization tests using IAEV specific antisera and the field isolate of the virus are recorded in Table 15. The field isolate was neutralized by both the GVS and the test antisera.

Table 14

The prevalence of IAEV in breeding flocks

Origin	Age (wks)	Virus dose (EID_{50})	Result	Origin	Age (wks)	Virus dose (EID_{50})	Result
NSW	26	180	Immune	NSW	50	120	Susceptible
NSW	27	200	Immune	NSW	50	140	Immune
NSW	28	80	Immune	NSW	50	220	Immune
NSW	28	150	Immune	NSW	50	220	Immune
NSW	30	200	Susceptible	NSW	52	180	Immune
NSW	30	110	Immune	NSW	55	260	Immune
NSW	32	260	Immune	NSW	55	100	Immune
NSW	32	260	Susceptible	SA	32	140	Susceptible
NSW	34	260	Immune	SA	34	220	Immune
NSW	41	300	Immune	SA	36	200	Immune
NSW	44	180	Susceptible	QLD	28	120	Immune
NSW	44	100	Immune	QLD	28	120	Immune
NSW	44	196	Immune	QLD	32	240	Immune
VIC	28	100	Immune	QLD	42	240	Immune
VIC	28	160	Immune	NZ	26	240	Immune
VIC	30	180	Immune	NZ	28	300	Immune
VIC	33	240	Immune	NSW	36(T)	150	Immune
VIC	34	240	Immune	NSW	38(T)	210	Immune
VIC	41	180	Immune	NSW	40(T)	280	Immune
				NSW	35(T)	190	Immune
				NSW	38(T)	100	Immune

NSW - New South Wales, SA - South Australia, QLD - Queensland, VIC - Victoria, NZ - New Zealand

T - turkey test

Table 15

The neutralization of the NSW-1 isolate by IAEV specific antisera

		Virus titre $\log_{10}$ EID_{50} per ml. ± SD		Neutralization index
PBS	Antiserum	Pre-infection	Post-infection	$\log_{10}$ x
4.50±0.54	GVS	4.48±0.84	1.26±0.26	3.22
4.46±0.84	VR	4.38±0.76	0	4.38
4.26±0.68	VR	4.10±0.68	0	4.10

Tests for essential lipid in the virus

The infectivity of the VR and the field isolate was unaffected by treatment with either ether or chloroform. The titres of virus obtained before and after treatment with these lipid solvents are recorded in Table 16.

Table 16

The effect of ether and chloroform on the VR and NSW-1 isolate of IAEV

Virus isolate	Virus titre - $\log_{10}$ CID_{50} per ml.±SD (NSW-1) / $\log_{10}$ EID_{50} per ml.±SD (VR)		
	Control	Ether	Chloroform
VR	5.55 ± 0.65	6.05 ± 0.59	
NSW-1	4.81 ± 0.71	4.56 ± 0.86	
VR	6.05 ± 0.28		5.90 ± 0.48
NSW-1	4.58 ± 0.74		4.86 ± 0.73

pH stability of the virus

The VR isolate was stable in the range pH 2.50 to pH 10.0. It was rapidly inactivated at pH 1.50 and at pH 10.50 and greater.(Table 17).

The NSW-1 isolate was stable in the range pH 3.50 to pH 10.0. It was less stable at pH 2.50, and was inactivated at pH 1.50 and pH 10.50 or greater.(Table 17).

Table 17

The effect of pH on VR and NSW-1 virus isolates.

pH value	Virus titre	
	VR - $\log_{10}$ EID_{50} per ml.±SD	Test - $\log_{10}$ CID_{50} per ml.±SD
1.50	0.75 ± 0.34	1.16 ± 0.28
2.50	6.38 ± 0.65	2.91 ± 0.44
3.50	6.45 ± 0.52	4.78 ± 0.71
5.00	6.30 ± 0.62	4.85 ± 0.76
6.00	6.36 ± 0.22	4.50 ± 0.54
7.00	6.50 ± 0.20	4.76 ± 0.56
7.50	6.05 ± 0.22	3.96 ± 0.48
8.50	6.43 ± 0.55	4.41 ± 0.59
10.00	6.20 ± 0.55	4.60 ± 0.40
10.50	4.55 ± 0.55	3.20 ± 0.62
11.50	0.90 ± 0.48	0.00

Thermostability of the virus isolates

The results of the effect of heat (56°C) for the specified time intervals on the infectivity of the the VR and the field virus are recorded in Table 18.

The VR isolate was 86 percent inactivated after 120 minutes at 56°C, and 90 percent after 180 minutes. The test

virus was 82 percent inactivated after 120 minutes at 56°C.

Table 18

Thermostability of the virus isolates

Duration of temp. exposure (minutes)	Virus					
	VR			NSW-1		
	Virus titre - $\log_{10}$ EID_{50} per ml. ±SD			Virus titre - $\log_{10}$ CID_{50} per ml. ±SD		
	4°C	56°C	Difference	4°C	56°C	Difference
0	6.15±0.71	6.30±0.62	-	4.12±0.59	4.58±0.44	-
30	6.21±0.58	5.11±0.59	1.10	NT	NT	-
60	6.18±0.65	4.80±0.38	1.38	4.90±0.74	3.55±0.52	1.35
120	6.25±0.34	0.90±0.48	5.35	4.11±0.59	0.75±0.34	3.36
180	5.91±0.44	0.55±0.52	5.36	NT	NT	-
240	6.10±0.62	0	6.10	NT	NT	-
300	5.46±0.48	0	5.46	NT	NT	-

NT - Not tested

Haemagglutination by the VR and field isolate

Neither of the isolates tested haemagglutinated the erythrocytes derived from chickens, turkeys, ducks, guinea pigs, rabbits, sheep or rats, either at room temperature or at 4°C. Chicken and turkey red blood cells were haemagglutinated by NDV.

Growth on the chorio-allantoic membrane

Neither the VR or the field isolate induced pocks or thickening of the CAM after three serial passages.

Challenge of chickens with the field isolate

The field isolate induced the disease in chickens challenged by the IC, IM, IP, SC and oral routes. Not all

challenged chickens however developed the disease. All the chickens that had clinical signs of IAE showed the characteristic histological lesions. Two chickens in the orally challenged group and one in the group challenged subcutaneously developed sub-clinical infections with the virus. The number of chickens that developed the disease, the number with histological lesions and the incubation period in individual birds in chickens challenged with the field isolate by different routes are recorded in Table 19.

Table 19

Development of IAE in chickens challenged with the field virus

Method of challenge	Proportion affected by IAE		Incubation period (days)							
	Clinical signs	Histo lesions	11	12	13	14	15	16	17	18
Intra-cerebral	$^{10}/_{10}$*	$^{10}/_{10}$		2	1	2	4	1		
Intra-muscular	$^{9}/_{10}$	$^{9}/_{10}$	1	1	2	3	0	1	1	
Intra-peritoneal	$^{9}/_{10}$	$^{9}/_{10}$				2	4		3	
Sub-cutaneous	$^{8}/_{10}$	$^{9}/_{10}$		2	1	0	2	2	0	1
Oral	$^{8}/_{10}$	$^{10}/_{10}$		2	2	1	3			

Challenge dose 477 CID_{50} *Number affected/number challenged

Discussion

Effects on egg production and hatchability recorded in this outbreak of IAE in a breeding flock are similar to those observed in other countries (Taylor et al., 1955; Hemsley, 1964; Halpin, 1967b). The depression in egg production observed during this outbreak was, however, more severe than in many other descriptions (Hemsley, 1964; Willemart, 1965, van der Heide, 1970). The marked fall in egg production in this flock may have been related to the higher susceptibility of

heavy breeds (TM and DW) to the disease (Feibel et al., 1952). It may have also been associated with the age of the flock. Any change in husbandry, nutrition, climate or the onset of a disease during the 30 to 50 week age range is likely to induce a decline in egg production in both these strains of chickens (Best, 1972). The egg production of both strains was affected for approximately five weeks, ie. up to week 49. However, chickens affected by IAE were not detected after week 46. The effect of the virus on egg production, therefore, persisted longer than the effect on hatchability or on the production of diseased chickens. Not all descriptions of egg producing flocks affected with IAEV have included a decline in egg production (Zamberg, 1966; Willemart and Schricke, 1966; Lindsey, 1970).

The hatchability of eggs produced by the TM and DW strains was affected differently. The hatchability of the DW strain declined 13 percent, and never regained pre-infection levels of hatching after the outbreak. A decrease in hatchability in IAEV infected breeding flock has also been described by Taylor et al (1955), Hemsley (1964) and Calnek et al. (1960). The hatchability of the TM strain was not affected however. This difference could not be accounted for by malfunctioning of hatchery equipment used to hatch the DW strain eggs as eggs from other strains in the same incubator hatched normally. In addition egg transmission of the virus in the TM strain was confirmed by the development of the disease in TM strain progeny hatched in separate incubators.

The disease in the young chickens was closely studied only in DW strain. The highest mortality was detected in chickens hatched in week 45, the second week of transmission

of the virus. Affected chickens were detected in weeks 43, 44, 45 and 46. This suggested transmission of the virus through the hatching egg for approximately three weeks. The duration of the transmission of the virus through the egg has been seven to 21 days, with only rare cases exceeding 28 days (Calnek et al., 1960; Hoekstra, 1964; Gylstorff and Kraus, 1964b; Zamberg, 1966; Willemart and Schricke, 1966). The mortality in affected chickens hatched from the DW strain occurred mainly between 14 and 21 days of age, except in week 46 when most occurred between 21 and 28 days of age. A similar delay in the age group most affected by the disease has been observed by Lindsey (1970) in association with chronic IAEV infections.

A survey of the prevalence of IAE showed it to be widespread in Australia. Of 35 flocks tested, only five were free of infection, and three of these subsequently became infected. The widespread occurrence was demonstrated by the detection of the infection in all States of Australia tested and by the fact that only flocks with some geographical isolation, and that attempted no vaccination programme against the disease, were tested. The flocks tested must also have been infected before the age of the onset of egg production, as none of the managers of the immune flocks experienced outbreaks of clinical disease in young chickens. The detection of infection in the turkey flock tested provided further information on the contagiousness of the virus, as the turkey flocks tested had been in isolation from other poultry for many years. The disease has not been described in turkeys in Australia.

The virus isolated from the outbreak was identified as an

IAEV isolate. This identification was based on neutralization tests using IAEV specific antisera and on various bio-physical tests. The virus isolate reproduced the disease in chickens challenged either orally, or by the IC, IM, IP and SC routes. The reproduction of the disease by challenge with the oral method contrasted with the inability of the van Roekel virus to induce the disease by this method. The results obtained in these tests corresponded to the findings of van Steenis (1968), Butterfield et al. (1969b) and van der Heide (1970). The virus was subsequently termed the NSW-1 isolate of IAEV.

Further characterization of the isolate could have been obtained by electron microscopic studies. A number of attempts to study the morphology of the van Roekel isolate, using the highest titred virus preparation available, were unsuccessful. This lack of success, both with concentrated and unconcentrated virus preparations, could have resulted from the relatively low titre of the virus in preparations. In addition, the impurity of the virus suspensions presented difficulties in seeing the virus. The titres obtained with the NSW-1 isolate were never as high as those obtained with the van Roekel isolate and therefore the inability to see virus particles in these preparations was not unexpected. There is a need to undertake further studies in the morphology of the virus to assist the classification of IAEV.

Summary

A virus was isolated from chickens with clinical signs and histological lesions characteristic of IAE. The parent flock of these birds had experienced a decline in egg production and hatchability and apparently excreted the virus for

three weeks. The virus was identified as an IAEV isolate by neutralization tests with IAEV specific antisera and by various bio-physical tests. The virus isolate, termed NSW-1 was adapted to produce IAE specific lesions in chicken embryos after six serial passages and reproduced the disease in susceptible chickens challenged either by the IC, IM, IP, SC or oral routes.

Infection with IAEV was found to be widespread in Australian poultry farms.

Chapter 4

THE PROPAGATION OF IAEV IN TISSUE CULTURES

Introduction

Dilute suspensions of virus particles can induce discrete lesions in sheets of sensitive tissue culture cells. This system provides a means for quantitation of virus preparations and is one of the keystones of modern virology. An effective "plaque test" was first developed for western equine encephalitis virus by Dulbecco (1952) and was promptly adapted to use with poliovirus (Dulbecco and Vogt, 1954). Tissue culture systems have subsequently been widely used for the propagation and quantitation of many animal viruses.

IAEV has been propagated in a number of cell culture systems of avian origin (Mancini and Yates, 1967; Abe, 1968; Mancini and Yates, 1968a, 1968b; Matuka et al., 1968; van der Heide, 1970; Sato et al., 1971; Fukuso, 1972). The multiplication of the virus in these cell systems has not however, been associated with the development of specific lesions. An in vitro assay technique for IAEV would have many theoretical and practical advantages, particularly if the virus could be assayed in tissue cultures derived from birds that were not naturally susceptible to the virus or from those with IAEV neutralizing antibodies.

The aim of this investigation was to study the growth characteristics of the van Roekel and NSW-1 isolates of IAEV in various cell culture systems and to determine whether suitable assay procedures could be developed.

Materials and Methods

Viruses

The van Roekel and NSW-1 isolates of IAEV were used. The van Roekel isolate was a 10 percent (w/v) suspension of chicken embryo brain and had a titre of $10^{5.5}EID_{50}$ per ml. The NSW-1 isolate was used as a 50 percent suspension of the brain from chicken embryos infected with the second passage of the virus in embryonating eggs. The titre was $10^{4.6}$ CID_{50} per ml.

On certain specified occasions van Roekel virus was used as a ten percent suspension of the whole chicken embryo or of the viscera from affected embryos. These had titres of $10^{5.30}$ and $10^{4.90}$ EID_{50}/ml. respectively.

Eggs

The susceptible eggs both in the preparation of tissue cultures, and in the titration of IAEV were obtained from a WL flock.

Cell culture techniques

(a) Chicken embryo neuroglial (CEN) cells. Thirteen or 14 day old chicken embryos were used. The embryos were removed from the eggs, decapitated and the heads placed in a sterile Petri dish. The brain of the embryo was then removed, and placed in sterile PBS at 37°C in a 20 ml. screw topped bottle. Twelve to 15 brains were usually used.

The brains were washed three times in sterile PBS and then macerated with scissors. The macerated tissue was subsequently trypsinized in 0.25 percent trypsin for 20 minutes at room temperature using 10 ml. of trypsin per brain. The trypsin-

cell suspension was shaken vigorously for 15 seconds and then filtered through 12 layers of sterile gauze.

The filtered suspension was poured into two large centrifuge tubes and centrifuged at 800 rpm for ten minutes. The supernatant was harvested aseptically and the cells evenly suspended in 30 ml. of Hanks Balanced Salt Solution (HBSS) containing 20 percent sterile foetal calf serum.

Ten ml. of this suspension was pipetted into a sterile 10 ml. graduated centrifuge tube and centrifuged at 1500 rpm for three minutes. The packed cell volume (PCV) was then determined. HBSS containing 20 percent foetal calf serum was added to the cell suspension to give a 1 in 100 suspension of cells. Tissue culture dishes (60 mm in diameter) were seeded with five ml. of this suspension per dish. These containers were incubated at 37°C in a humidified five percent CO_2 in air atmosphere for four days. After this time the tissue culture medium was changed to HBSS containing 10 percent foetal calf serum. Cell cultures were usually confluent on the eighth or ninth day after seeding and the cells were usually challenged at this time.

The growth medium for CEN cells consisted of HBSS, 20 percent foetal calf serum, 0.5 percent lactalbumin hydrolysate, yeast extract, 100 iu sodium penicillin G per ml., 100 mgm streptomycin sulphate per ml. and 25 gmg amphotericin B per ml. The maintenance medium consisted of a similar solution but with ten percent foetal calf serum and without lactalbumin hydrolysate (see Appendix 1.1).

This technique was based on that of Mancini and Yates (1967).

(b) Chicken embryo fibroblast (CEF) cells. Ten day old chicken embryos were used. The eyes, beak and limbs were removed from the embryo and the remaining portion of the embryo finely minced using curved, sharp pointed scissors. The minced tissues were washed at least three times in sterile PBS at 37°C. The tissue was trypsinized in 0.125 percent trypsin, using approximately 20 ml. of trypsin per embryo, for 30 minutes in a water bath at 37°C. During tyrpsinization it was stirred with a magnetic stirrer. Chilled calf serum (4°C) was added at the end of this time at a rate of five percent. The dispersed cells were filtered through 12 layers of sterile gauze, and centrifuged at 500 rpm for 10 minutes. The supernatant was aspirated and the cells resuspended in 50 ml. of CEF growth medium. The cells were counted in a haemocytometer and the CEF seeded at a rate of 1×10^6 cells per ml. for stationary vessels and 4×10^6 cells per ml. for rolling vessels. Cell sheets were usually confluent within 24 hours. The cell culture medium was then changed to a maintenance medium.

The CEF growth medium consisted of Medium 199, 5 percent calf serum, sodium bicarbonate, 100 iu sodium penicillin G and 100 mgm streptomycin sulphate per ml, and 25 mgm amphotericin per ml. In the maintenance medium the calf serum was reduced to one percent (see Appendix 1.2).

(c) Turkey embryo fibroblast (TEF) cells. These cell cultures were prepared according to the technique for CEF. Twelve day old turkey embryos were used, and the cells seeded at 1×10^6 cells per ml. of medium.

(d) Duck embryo fibroblasts (DEF) cells. DEF tissue cultures were prepared by the technique described for CEF. Twelve day old duck embryos were used and the cells seeded at 2×10^5 cells per ml. of medium.

(e) Chicken embryo intestinal (CEI) cells. Eighteen day old embryos were used in the preparation of these tissue cultures. The intestinal tract was removed aseptically, and the portion between the duodenum and ileo-caecal valve used. It was finely minced using fine pointed sharp scissors. The minced cells were washed at least three times in sterile PBS at 37°C. The tissue suspension was then trypsinized using 0.25 percent trypsin solution. The dispensed cells were then used in the way described for the preparation of CEF cultures.

(f) Chicken embryo kidney (CEK) cells. The kidneys of eighteen day old chicken embryos were removed and placed in a 20 ml. screw topped sterile bottle containing sterile PBS at 4°C. The kidney tissue was washed three times in PBS and transferred to a sterile Petri dish. Any connective tissue was then carefully removed. The tissue was minced and trypsinized at 37°C for 30 minutes using a 0.25 percent trypsin solution. The trypsin-cell suspension was shaken vigorously for three minutes, sterile chilled calf serum added at a rate of five percent and the suspension then filtered through 12 layers of sterile gauze. The cells were dispensed into a sterile centrifuge tube and centrifuged at 500 rpm for 10 minutes. The supernatant was collected and the cells resuspended in 30 ml. of CEK growth medium.

Ten ml. of this suspension was pipetted into a ten ml. graduated centrifuge tube and centrifuged at 1500 rpm for three minutes. The packed cell volume (PCV) was estimated. The cell stock was diluted to a final dilution of 1 in 200 in CEK growth medium and dispensed in 60 mm cell culture dishes at 5 ml. per dish. The cultures were incubated in a humidified atmosphere of 5 percent carbon dioxide in air at 37°C. Confluent monolayers, usually established after two days incubation, were used in these studies. The growth medium and maintenance medium consisted of HBSS, tryptose phosphate broth, five percent foetal calf serum and antibiotics (see Appendix 1.3).

(g) Chicken kidney (CK) cells. Chickens aged two to five weeks of age were used. The birds were killed by the injection of air into the brachial vein. The kidneys were removed aseptically and minced. The finely minced tissue was washed three times in sterile PBS at 37°C and trypsinized at 37°C for 30 minutes using a 0.125 percent trypsin solution. The dispersed cells were washed twice in CK growth medium and then filtered through 12 layers of sterile gauze. The cells were centrifuged at 500 rpm for ten minutes, the supernatant removed and the cells resuspended in a known quantity of CK growth medium. The cells were counted in a haemocytometer and cell culture dishes seeded at a rate of 8×10^5 cells per ml. The cells were incubated in a humidified atmosphere of 5 percent carbon dioxide in air at 37°C.

The growth and maintenance medium consisted of Medium 199, foetal calf serum, tryptose phosphate broth, sodium bicarbonate and antibiotics (see Appendix 1.4).

(h) HeLa and VERO cell cultures. The HeLa cells were obtained from the Commonwealth Serum Laboratories, Parkville. The VERO cells were provided by Dr. G. Gard, Veterinary Research Station, Glenfield.

On receipt, the cells were trypsinized with a trypsin-versene solution for three to five minutes at room temperature. The cells were then centrifuged, resuspended in a known volume of growth medium, counted and seeded at a rate of 10^6 cells per ml. The growth medium for the HeLa cells consisted of Medium 199, 10 percent foetal calf serum, sodium bicarbonate and antibiotics. The maintenance medium contained two percent calf serum. For VERO cells the growth medium contained five percent foetal calf serum, the maintenance medium two percent foetal calf serum.

Tracheal organ cultures

The tracheas were removed from 14 day old WL chickens that were completely susceptible to IAEV. The tracheas were placed in HBSS, the adventitial coat carefully removed and the trachea cut into transverse explants 1.0 to 1.5 mm thick. These explants were washed two to four times in culture medium that consisted of HBSS, ten percent calf serum, 0.5 percent lactalbumin hydrolysate and antibiotics (see Appendix 1.5). Two ml. of culture medium was placed in 60 mm unwettable sterile plastic culture vessels, with four explants per dish. The tracheal organ cultures were incubated at 37°C in a humidified CO_2 incubator. The culture medium was changed every second day. The epithelial activity was observed using an inverted microscope. Criteria of infection with the virus

was based on ciliary activity, mucus production and epithelial cell desquamation.

The tracheal organ cultures were infected with 100 EID_{50} of the van Roekel virus or approximately 100 CID_{50} of the NSW-1 isolate. The cultures were washed three times after infection. The maintenance medium was added after these washings.

Intestinal organ culture

The intestinal tract from the duodenum to the ileo-caecal valve was removed from 20 to 21 day old unhatched live chicken embryos. This portion of the intestinal tract was placed in HBSS at 37°C, washed twice with HBSS and then cut into transverse portions approximately 1.0 mm wide. Five to 10 explants were placed in 60 mm unwettable sterile plastic dishes together with 2 ml. of maintenance medium. The cultures were treated and maintained in the same way as tracheal organ cultures.

The intestinal organ cultures were infected with approximately 100 CID_{50} of the NSW-1 isolate. The criteria of infection of the explants with the virus was - the recovery of the virus in quantities greater than used to infect the organ culture and differences in mucus production and activity of the villi between the infected and control organ cultures.

Growth curve studies of IAEV in cell cultures

The medium was removed from the cell culture and the cells infected with either an estimated 1,000 EID_{50} of the van Roekel, or 1,000 CID_{50} of the NSW-1 isolate of IAEV. The actual dose of virus used was titrated subsequently in chicken embryos. The virus was allowed to

adsorb for 60 minutes at 37°C. The excess fluid was removed and the cells washed three times with 1.0 ml. of the appropriate cell culture maintenance medium before 5.0 ml. of maintenance medium was added. The virus in the fluid recovered from the cells after the adsorption period was titrated in an attempt to determine the dose of virus actually adsorbed.

The supernatant fluid from two infected and two uninfected cell culture dishes were harvested at 12, 24, 48, 72, 96, 120 and 144 hours after infection and stored at -30°C until used. The dose of virus in these samples was titrated in chicken embryos. A complete growth curve study of one isolate in a cell culture system was titrated in chicken embryos on one day. These tests were performed on virus obtained from the 5th passage of the virus in the various cell cultures.

In another growth curve study, comparisons were made between the multiplication of the virus in CEK cell cultures prepared from known IAEV immune and susceptible embryos. The technique used was the same as described previously although supernatant fluid was not harvested 144 hours after infection.

Serial passage of the virus in cell cultures

The cell cultures were infected with either the van Roekel or NSW-1 isolates in the same way as described above. The infectivity of the virus dose used to infect the cell cultures was titrated in chicken embryos.

The supernatant fluid was harvested from two infected and two control cell cultures 96 hours after infection with van Roekel or 120 hours after infection with NSW-1 isolate. These were used either immediately to infect further cell cultures or were stored at -30°C. To infect further cells, one

tenfold dilution of the aliquot was prepared and used to infect the next cell culture passage. Portion of this aliquot was used to challenge ten chicken embryos seven days old by the yolk sac or given intra-muscularly to five, one day old IAEV susceptible chickens. The infected and control cell cultures were examined daily with an inverted microscope for the development of a specific cytopathogenic effect. In addition, two infected and one control cell culture were covered with an agar overlay (see Appendix 1.6) 96 hours after infection with the virus.

At the 4th, 10th and 15th passage of the van Roekel isolate and the 9th passage of the NSW-1 isolate in CEF and CEK cells, tissue cultures were grown on coverslips in Leighton tubes. These cells were fixed and stained with haematoxylin and eosin 120 hours after infection and were examined for the development of inclusion bodies in the cells.

Effect of various physical and biochemical treatments on IAEV in cell cultures

These tests were performed using CEK cell cultures and either van Roekel or NSW-1 isolates of the virus.

(a) Temperature of incubation. CEK cell cultures infected with either of the virus isolates used were incubated at 35°C, 39°C and 41°C.

(b) Diethylaminoethyl-dextran (DEAE-D). This compound was incorporated into the diluent used in the preparation of virus for infection of the cell culture at a rate of 100 µgm per ml. The virus was allowed to stand in this solution for 30 minutes at 4°C before use.

DEAE-D was also incorporated in the cell culture maintenance medium at 200 µgm per ml.

(c) Treatment with certain amino acids. L cystine and L cysteine were incorporated in the maintenance medium in separate tests. A 10 mM stock of L cystine was prepared by dissolving the amino acid in 1N HCl and then neutralizing with 0.1N NaOH. This stock was diluted tenfold when incorporated into the maintenance medium. A 10 mM stock of L cysteine was prepared and used in the same way.

(d) Addition of Mg^{++} to cell culture medium. A cell culture medium containing 25 mM of $MgCl_2$ was prepared.

In all these tests, cell cultures were examined at 24 hourly intervals up to 168 hours after infection for the development of CPE. At the end of this period, the supernatant fluid was harvested and either used immediately or after storage at $-30^{o}C$ to infect ten susceptible one day old chickens. These chickens were inoculated IM with 0.2 ml. of untiluted cell culture medium. The inoculated chickens were observed for the development of IAE. Tissues from five chickens in each group were examined histologically for microscopic lesions of the disease.

Specific interference between IAEV and NDV

This test was performed according to the technique of Carver et al. (1967).

The cells used were CEK cultures infected with an estimated 1,000 EID_{50} of van Roekel or 1,000 CID_{50} of NSW-1 isolate of the virus.

The infected cultures were incubated at 37°C for 72 and 120 hours. The medium was removed and the cells infected with an estimated $10^{5.0}$ $TCID_{50}$ of NDV. This virus was allowed to adsorb for 60 minutes at 37°C, when the cells were washed three times with sterile PBS at 37°C and then exposed to 1.0 ml. of a specific NDV chicken antiserum for 30 minutes at 37°C. The cells were again washed three times with PBS and 5 ml. of maintenance medium was added and the cells incubated at 37°C for 36 hours.

The cells were tested for the development of haemadsorption using the procedure of Cunningham (1966).

Tests for cell-associated virus

These tests were undertaken in both CEF and CEK cell cultures. The cells were infected with an estimated 1,000 EID_{50} of van Roekel virus. The actual dose used was titrated after use.

Two cell cultures were harvested at intervals of 48, 96 and 120 hours after infection. At these times the supernatant was harvested, and pooled and stored at -30°C until it was titrated. The cell sheet was then washed three times with sterile PBS at 37°C and 3 ml. of maintenance medium added to the cultures. The cells were scrapped from the surface of the dish. The suspensions of cells from the two cultures were pooled and frozen at -30°C. Both the respective supernatant fluid and cell suspension preparations were frozen and thawed three times. After this they were centrifuged at 5,000 rpm for ten minutes and titrations of the infectivity of the virus present prepared in chicken embryos. One uninfected control cell culture was treated in the same way on each occasion.

Results

Growth of the virus in cell culture

The concentration of virus detected in the fluid harvested at various time intervals after infection of the different cell culture systems tested is recorded in Table 20.

Both van Roekel and NSW-1 isolates grew in CEN, CEF, CEK and CEI cell cultures. This was demonstrated by a greater than one hundred fold increase in the titre of the virus between the time of infection and the last estimation of the virus in the cell culture fluid. No specific cytopathogenic effect was observed in any of the cell cultures in which the virus multiplied

The virus was not detected in either control cell cultures or in infected DEF, TEF, DEF, CK, VERO or HeLa cell culture systems. The virus was not detected isolated from the cell culture fluid at any time after the initial infection of these cell systems.

Adsorption of the virus

There were differences between the titre of virus obtained from the tissue cultures in which the virus multiplied and in which it did not. Between 80 to 100 percent of the virus was unadsorbed into DEF, TEF, CK and HeLa tissue cultures. The virus, however, seemed to become associated with VERO cells as an estimated 63 percent remained after adsorption. However multiplication of the virus was not detected in this cell line. More virus adsorbed to cell culture systems in which it was demonstrated to have propagated, than to systems in which it did not grow (see Table 21).

Table 20

Multiplication of IAEV in various cell culture systems

Virus isolate	Cell system	Virus dose	Virus titre - van Roekel; $\log_{10}$ EID_{50} per ml. ± SD NSW-1; $\log_{10}$ CID_{50} per ml. ± SD						
			Time after infection (hours)						
			12	24	48	72	96	120	144
van Roekel	CEN	630*	-	1.90±0.48	3.25±0.34	4.45±0.71	5.25±0.65	5.71±0.59	5.96±0.48
van Roekel	CEF	630	-	1.70±0.39	2.16±0.28	2.58±0.44	4.24±0.52	4.50±0.62	4.91±0.71
van Roekel	CEK	630	-	0.30±0.39	2.43±0.56	4.41±0.44	5.[illegible]1±0.59	5.50±0.48	5.55±0.52
van Roekel	CEI	1995	-	0.75±0.34	2.75±0.52	3.63±0.48	3.80±0.56	4.91±0.43	5.80±0.56
NSW-1	CEN	3467+	-	-	1.73±0.48	2.35±0.59	2.75±0.76	3.65±0.86	3.95±0.90
NSW-1	CEF	3467	-	-	1.43±0.56	2.70±0.74	3.21±0.59	4.87±0.65	5.31±0.76
NSW-1	CEK	3467	-	-	1.10±0.48	2.41±0.44	2.90±0.78	4.50±0.68	4.26±0.68
NSW-1	CEI	1690	-	0.70±0.39	2.41±0.44	2.75±0.52	3.51±0.65	4.56±0.83	4.65±0.98

CEN - chick embryo neuroglial

CEK - chick embryo kidney

CEF - chick embryo fibroblast

CEI - chick embryo intestine

* EID_{50} - embryo infective dose

\+ CID_{50} - chick infective dose

Table 21

Adsorption of IAEV in various cell cultures

1. VR virus

Cell type	Virus challenge dose	Virus recovered	% unadsorbed
CEN	630*	224*	36
CEF	630	398	63
CEK	630	316	50
CEI	1995	1122	56
DEF	1995	1778	89
TEF	1995	1698	85
CK	1260	1023	81
VERO	12590	7943	63
HeLa	1698	1622	96
2. NSW-1 virus			
CEN	3467†	1738†	50
CEF	3467	1622	47
CEK	3467	1995	58
CEI	1698	813	48
DEF	2240	2138	95
TEF	2240	1995	89

* EID_{50} † CID_{50}

Multiplication of IAEV in cell cultures derived from susceptible and immune chicken embryos

The virus content of the tissue culture medium at specified times after challenge of cell cultures produced from susceptible and immune embryos is recorded in Table 22.

Table 22

Multiplication of the van Roekel isolate in immune and susceptible CEK cell cultures

Origin of cells	Challenge dose (EID_{50})	Virus titre ± SD*				
		24 hours	48 hours	72 hours	96 hours	120 hours
Immune	707†	1.90±0.28	3.25±0.34	4.30±0.34	5.31±0.28	5.50±0.20
Immune	550	1.30±0.44	3.80±0.56	4.24±0.52	5.71±0.40	5.55±0.28
Susceptible	707†	1.16±0.48	3.95±0.65	4.56±0.44	5.50±0.20	5.80±0.34
Susceptible	550	1.00±0.56	3.63±0.43	4.65±0.48	5.21±0.68	5.31±0.43

* $\log_{10}$ EID_{50} per ml.

CEK - chick embryo kidney

† duplicates

The virus multiplied equally well in both cell cultures. The titre of virus produced in the "susceptible" cell cultures was not significantly different to that produced in the "immune" cell cultures. Specific CPE was not observed in either type of culture.

Serial passage of the virus in cell cultures

Van Roekel isolate was passed 15 times in CEN, CEF, CEK and CEI cell cultures. No specific CPE was observed up to 168 hours after infection in any of the cultures. The virus was detected in the suspension passaged on each occasion by the development of lesions in chicken embryos.

NSW-1 isolate was passed 11 times in CEN, CEF, CEK and CEI cells without the development of specific CPE. The virus was demonstrated in the passaged material by the development of IAE in chickens challenged intra-muscularly with the passage suspension.

The viruses were not passaged more than five times in cell cultures in which multiplication was not demonstrated.

Effect of physical and biochemical treatment on the virus in cell cultures

The addition of DEAE-D, L cysteine, L cystine or $MgCl_2$ either separately or combined did not induce the formation of CPE in cell cultures infected with IAEV. Similarly incubation at $35^{o}C$, $39^{o}C$ had no apparent effect on the development of CPE.

Specific interference between IAEV and NDV in tissue culture

Haemadsorption was not observed in CEK cultures infected with either van Roekel or NSW-1 isolates using suspensions of either chicken, turkey, duck, guinea pig, sheep or rabbit red blood cells.

Uniform haemadsorption of 0.5 percent chicken erythrocytes was observed in CEK cultures infected with $10^{5.2}$ tissue culture infective doses ($TCID_{50}$) of NDV, 36 hours after infection. Forty-eight hours after infection of CEK with this dose of virus, specific CPE was detected. Haemadsorption 36 hours after infection with 10^5 $TCID_{50}$ of NDV was not observed in CEK cells treated with NDV chicken antiserum prior to the test for haemadsorption.

Specific interference between IAEV and NDV was not detected in these studies. Uniform haemadsorption was observed in cultures that were superinfected with NDV, 72 or 120 hours after infection with van Roekel or NSW-1 isolates.

Cell associated virus

The titre of the virus in the supernatant and in cell suspensions of CEK or CEK cultures that had been infected with van Roekel virus are recorded in Table 23.

Cell associated virus was detected in both CEF and CEK cultures. However,it was not present in high concentrations. The virus was also detected in the supernatant fluid. This suggested that release of the virus from infected cells was occurring.

Table 23

Cell associated virus in cell cultures infected with van Roekel virus

Cell type	Virus Source	Virus titre ± SD*		
		48 hours	96 hours	120 hours
CEF	Supernatant	2.58±0.54	4.16±0.65	5.46±0.52
	Cell suspension	1.83±0.28	0.95±0.52	0.75±0.34
CEK	Supernatant	2.30±0.28	4.71±0.56	5.15±0.59
	Cell suspension	1.33±0.56	0.70±0.28	1.00±0.39

* $\log_{10}$ EID_{50} per ml. CEK-chick embryo kidney

CEF - chick embryo fibroblast

Organ culture studies

(a) Chicken tracheal organ cultures. No changes were detected in either the action of the cilia, mucous production or in the *nature of the epithelium in organ cultures challenged with* either van Roekel or NSW-1 isolates. The virus was not detected in the culture maintenance medium 96 hours after challenge. The virus was also not detected in organ cultures homogenized in tissue grinders 96 hours after challenge with the virus.

(b) Intestinal organ cultures. The intestinal organ cultures rapidly degenerated, the villi became coarse and covered in mucus 24 hours after preparation. The cells also failed to take up a neutral red stain 24 hours after preparation. No differences were detected between cultures that had been challenged with IAEV and those that had not. The virus was not recovered from the organ culture maintenance medium or the organ culture itself 72 hours after virus challenge.

Discussion

Van Roekel and NSW-1 isolates multiplied in CEN, CEF, CEK and CEI cultures. This propagation was demonstrated by a 1,000 fold increase in titre of the virus in the cell culture medium over the 144 hour period after infection. Propagation of the virus was not detected in TEF, DEF, CK, HeLa or VERO cell cultures.

Comparison between these results and those of others is difficult. The validity of the claim of the growth of IAEV in cell cultures by some other workers is difficult to substantiate because of inadequate description of the techniques used. In some studies the dose of the virus used to challenge the cells was not titrated, and cells were not apparently washed after adsorption of the virus. A determination of the challenge dose of virus, requires not only a titration of the initial dose of the virus but also quantitation of the virus that remains unadsorbed. In this study, 36 to 96 percent of virus in different tests was not adsorbed after 60 minutes at 37°C. Van Steenis (1968) found that the virus could survive for up to nine days at this temperature. This emphasises the need to remove unadsorbed virus from cell cultures used in growth studies of IAEV.

Also the titres of virus obtained in this study are difficult to compare with those obtained by other workers. In most studies of IAEV in tissue cultures, the virus content of the cell culture medium has been determined at specified intervals from the same cell culture dish (Mancini and Yates, 1967; Abe, 1968; Mancini and Yates, 1968a, 1968b). This technique would not contribute to as high titres of virus as the procedure used in this study of harvesting cell culture

medium from different cell cultures at regular intervals.

Most studies of the growth of IAEV in tissue cultures have used isolates of the virus that have been adapted to grow in chicken embryos (Mancini and Yates, 1967; Abe, 1968; Mancini and Yates, 1968a, 1968b; Matuka et al., 1968). Fukusho (1972) and Sato et al. (1971) have demonstrated that IAEV not adapted to grow in the chicken embryo also multiply in cell cultures. Similarly, the NSW-1 isolate used in this study failed to induce lesions of IAE in chicken embryos, but did grow in some cell cultures.

The propagation of the virus in these cell cultures was, however, not associated with the development of specific cytopathogenic effect. Examination of haematoxylin and eosin stained CEK cultures grown on cover slips and infected with fourth, tenth and 15th passage of VR virus, and the ninth passage of NSW-1, showed no changes in the cells. Serial passage of the virus in the various cell line in which the virus multiplied also failed to induce CPE. This finding was similar to the results of other workers (Mancini and Yates, 1967; Abe, 1968; Mancini and Yates, 1968a, 1968b; van der Heide, 1970; Kamada, 1971; Sato et al., 1971). Apatenko (1972) has however described a CPE in cell cultures infected with IAEV and Halpin(1966a) reported multiplication and CPE in monkey kidney cells infected with an IAE like virus.

A number of techniques have been used to enhance the growth of or to improve plaque formation of enteroviruses in cell cultures. The addition of $MgCl_2$, L cysteine, cystine and pancreatin to the cell culture medium have been used to

enhance the growth, stability and plaque formation of polioviruses (Dubes, 1956; Pohjanpelto, 1958; Wallis et al., 1966). Halstead et al. (1970) found that poliovirus was stabilized by a lowered oxygen potential and pH in the medium. Similarly the polycation, diethylaminoethyl-dextran has been used to enhance the plating effeciency of polio and encephalomyocarditis virus in cell cultures (Pogano and Vateri, 1965; Vateri and Pogano, 1965). The temperature of incubation and the use of $MgCl_2$, L cysteine, cystine and DEAE-D as a additives to the cell culture maintenance medium failed to induce CPE in cells challenged with IAEV. Similarly infection of cells at different stages in the confluence of the cell culture monolayer also failed to induce CPE formation. Cell cultures derived from the chicken embryo intestinal tract were used because the intestinal tract has been demonstrated to be a rich source of virus (Burke et al., 1965; Lukert and Davis, 1971). The virus multiplied in these CEI cells, but did not cause CPE.

The inability to induce CPE in the cell cultures could also have been related to retention of the virus in the infected cells. Although cell associated virus was detected in cells infected with VR virus, the relative proportion of released and associated virus indicated that retention of the virus in the cell was not the cause of lack of cytopathogenicity of IAEV.

Because of the relative ease with which CEK cells could be prepared in comparison to CEN, and as there were no significant differences in titres of virus obtained using the

different cell cultures, CEK cells were used preferentially in some studies. No significant differences were detected in the titre of virus produced in CEK cell cultures derived from immune or susceptible chicken embryo. The growth of IAEV in cells derived from immune chicken embryos has not been described previously. This may be of considerable practical significance, as it removes the necessity to use IAEV susceptible chickens or chicken embryos in the multiplication of the virus. The supply of such embryos is often a major problem in undertaking research projects in IAE. The ability to grow and assay the virus in cell cultures derived from immune chickens or chicken embryos would aid laboratory work with this virus.

Non-cytopathogenic viruses have been assayed in cell cultures by a system of specific interference between the growth of one virus and another. This test was first used to assay Rubella virus (Marcus and Carver, 1965). Subsequently other viruses have been assayed in this manner (Beard, 1967; Marcus and Carver, 1967; Carver et al., 1967). Specific interferences between IAEV and NDV was not detected in this study, even though IAEV was known to be multiplying in the culture up to the time of super-infection with NDV. Interference between IAEV and NDV in chicken embryos has been demonstrated, although this was not absolute (Yates et al., 1968). The failure to detect interference between these isolates could be related to a small number of cells in the cell sheet that become infected with IAEV. Yates et al. (1968) also demonstrated interference between IAEV and IBV in chicken embryos. The possibility of interference between IAEV

and IBV in cell culture as an assay technique for IAEV was not investigated because of the lack of an IBV isolate which would produce CPE in cell culture.

IAEV was not recovered from organ cultures of chicken trachea or chicken embryo intestine. Pathological changes that could be attributed to the virus were also not observed. The rapid degeneration of the intestinal organ cultures inhibited a thorough investigation of this system for assay of the virus. The intestine is a difficult organ to maintain *in vitro* (Browning and Trier, 1969; Rubenstein and Tyrrell, 1970; Stenhouse, 1970), probably because of its naturally high cell turnover. However,several different viruses have been grown in intestinal organ cultures including polio, Sendai and encephalomyocarditis (Rubinstein and Tyrrell, 1970); ECHO (Stenhouse, 1970); Rubella (Mitus *et al.*, 1970) and transmissible gastro-enteritis virus of swine (Rubinstein *et al.*, 1970). In general the yield of virus has been poor. However,the system does have potential for the isolation of previously unisolated viruses (Dolin *et al.*, 1970) and for cultivating viruses that have never been grown *in vitro* (Rubinstein *et al.*, 1971). Therefore further investigation of intestinal organ cultures for cultivation of avian enteric viruses, and IAEV in particular, should be justified.

Summary

Infectious avian encephalomyelitis virus was propagated in CEN, CEF, CEK and CEI cell cultures. Van Roekel virus reached titres of approximately $\log_{10}$ 5.50 EID_{50} per ml. after 144 hours cultivation, while NSW-1 isolate reached $\log_{10}$ 4.50 CID_{50} per ml.

Specific cytopathogenic effect was not observed in cells in which the virus propagated. CPE could not be induced by serial passage of the virus in the cultures or by alterations to the bio-physical conditions of the cell cultures. The virus could not be assayed using specific interference techniques with NDV, nor could growth or pathological changes be detected in organ cultures of chicken trachea or chicken embryo intestine.

No significant differences in the titre of virus produced in CEK cultures prepared from IAEV immune or susceptible chicken embryos.

CONCLUSIONS

As a result of the findings recorded in these studies it was decided to standardize the chicken embryo propagation of the virus on the seven day old chicken embryo inoculated by the yolk sac route and incubated for eleven days at 37°C.

The criterion used to determine the end point in titrations of the VR virus in chicken embryos was complete paralysis, even though the virus could be isolated from chicken embryos that were classified as slow or sluggish. This was adopted in an attempt to reduce subjectivity in assessment and therefore to reduce the variation already inherent in quantal end point determinations.

For titrations of the NSW-1 virus, inoculated embryos were hatched and the chickens observed for the development of the disease within ten days of hatching. This system resulted in consistent estimations of the end point of infectivity of the same stock of the virus. In all the titrations undertaken using this technique, the disease was not observed in chickens less than ten days of age that had not been inoculated with the virus prior to hatching. This confirmed the observation of Hoekstra (1964) that only congenitally infected chickens developed the disease within ten days of hatching. The constant use of this procedure in the assay of the NSW-1 virus emphasised the need for an alternative, preferably an in vitro assay technique for IAEV.

NSW-1 isolate was characterized as an IAEV. The disease outbreak from which it was recovered resembled descriptions of IAE, the virus was neutralized by IAE specific antisera and it possessed biophysical characteristics of the

virus.

Both van Roekel and NSW-1 isolates could be propagated in the chicken embryo. The virus could be readily isolated from the brain, viscera and body of infected chicken embryos. This indicated that these parts of the embryo should not be neglected when stocks of the virus are being prepared, particularly if IAEV susceptible chicken embryos were scarce.

Repeat titrations of the same vial of the van Roekel virus indicated that a vial could be used up to four times without a significant decline in the infectivity of the virus. This allowed economies to be made in the use of titrated vials of the virus.

Van Roekel virus was pathogenic for chickens, provided that they were challenged parenterally with the virus. Microscopic lesions of IAE were observed in all chickens that developed the disease. Sub-clinical infections with IAEV were also detected, particularly in chickens challenged by the IM, IP or SC routes. NSW-1 virus infected chickens, challenged either parenterally or per os, developed clinical IAE. This indicated that this isolate was suitable for studies in the pathogenesis of IAE and for the development as a vaccine.

IAEV was demonstrated to multiply in various tissue cultures obtained from chicken embryos. However, CPE failed to develop in these cultures. The inability to assay IAE in vitro is a continuing frustration to research work in IAE. The available assay techniques depend on a constant supply of susceptible chickens or chicken embryos. They are tedious and less accurate than plaque or pock counting techniques. The demonstration of the propagation of IAEV in a range of cell culture systems by various workers suggests that an in vitro

assay technique could be developed either by finding a susceptible line of cells, or a cytopathogenic IAEV isolate or by providing a suitable environment for the development of CPE in currently used cell cultures.

PART 2

THE PATHOGENESIS OF INFECTIOUS AVIAN ENCEPHALOMYELITIS

The study of the pathogenesis of viral infections has come to assume increasing importance in recent years, since it offers the prospect of a more rational basis for understanding the character of diseases. In numerous viral infections the significance of the clinical, pathological and epidemiological features is likely to remain obscure until more is known about the manner in which the virus enters the body, spreads and gives rise to illness.

In general, viral infections have been arbitarily divided into local infections, in which the virus produces its effects predominantly at the portal of entry into the body, and generalized infections, in which the virus, after primary multiplication near or at the site of entry, becomes dispersed through the body with the establishment of multifocal secondary or tertiary sites of multiplication in other organs or tissues. The readiness with which a virus can generalize is an important underlying influence determining the character and severity of the disease it produces. Many factors may influence the generalization of a virus and these include the pathogenicity of the virus, the genetic susceptibility, immunocompetence and specific immune status of the host.

Since its recognition as a disease of chickens in 1932, IAE has been studied most extensively in relation to the efficacy of vaccines and vaccination procedures for the control of the disease. Following the development of these vaccines and

vaccination programmes control of the disease was obtained, although some failures of vaccination have been recorded. The prospects for improving control of the disease are in part dependent on the development of improved vaccines, but also depend upon a better understanding of the pathogenesis of the infection.

The aim of this investigation was to clarify and extend knowledge on the pathogenetic patterns of this infectious disease.

Chapter 1

EFFECT OF AGE OF THE CHICKEN AND THE ROUTE OF CHALLENGE ON THE DEVELOPMENT OF IAE

Introduction

Although infection with IAEV may occur in chickens of all ages, the loss in productivity associated with the development of the disease are most pronounced in young birds (Jones, 1932; Calnek et al., 1961b). These losses occur from paralysis and death of chickens. IAE was first described as a specific disease of the CNS of young chickens (Jones, 1932). It is now thought to be primarily a gastro-intestinal tract infection with only secondary invasion of the brain and spinal cord by the virus (Calnek et al., 1960).

Several methods of challenge of chickens with the virus have been used to reproduce the disease (Jones, 1932; Olitsky, 1939; Jungherr and Minard, 1942; Feibel et al., 1952; Schaaf, 1958). These studies demonstrated that inoculation of the virus IC consistently induced the disease in chickens of all ages. By contrast the disease could not be consistently reproduced in chickens challenged by other parenteral routes or per os.

However, the effect of the method of challenge with the virus and the age of the chicken on the development of the disease have not been studied in a systematic manner.

The aim of this chapter was to study these aspects of the pathogenesis of IAE and concurrently to examine the development of specific serum neutralizing antibody in the chicken.

Materials and Methods

Chickens. The chickens used were obtained from WL/A flock and hatched at the University of Sydney.

Eggs. The fertile eggs were obtained from WL/A flock

Virus. The NSW-1 isolate was used as a 10 percent (w/v) brain suspension of the 2nd passage in chicken embryos. This stock had a titre of $10^{4.6}$ CID_{50} per ml.

Van Roekel isolate was used in the virus serum neutralization tests, and for intra-cerebral challenge of chickens.

Chicken challenge procedures. Chickens were challenged either by the IC, IM, IP or oral routes. Non-specific deaths in chickens challenged IC were replaced by other IC inoculated chickens so as to maintain group sizes.

Experimental design. Groups of chickens were challenged at the specified ages by various routes as illustrated in Table 24. Control chickens were challenged with an equivalent quantity of a brain homogenate from uninoculated IAEV susceptible chicken embryos.

Table 24

Age and number of chickens, challenged with NSW-1 isolate by different routes

Age of challenge (days)	Route of inoculation							
	Intra-cerebral		Intra-muscular		Intra-peritoneal		Oral	
	Virus	Control	Virus	Control	Virus	Control	Virus	Control
1	10	5	10	5	10	5	10	5
7	10	5	10	5	10	5	10	5
14	10	5	10	5	10	5	10	5
21	10	5	10	5	10	5	10	5
28	10	5	10	5	10	5	10	5

The chickens were reared in an isolated room from one day of age. The chickens were removed from this unit as required and following infection they were maintained in a room remote from the rearing unit. The control chickens were challenged and maintained in isolation.

Pre-inoculation serum samples were obtained from chickens in the 21 to 28 day groups only. Previous experience indicated the difficulty of obtaining pre-inoculation sera in adequate quantities from chickens in the one, seven and 14 day groups. The pre-inoculation sera used in the serum neutralization tests for these age groups was obtained from chickens of the same age that were not inoculated.

The inoculated chickens were examined daily for the onset of the disease. The chickens were observed at rest and also when disturbed by the observer. Chickens developing clinical signs of the disease were recorded and marked. These were checked daily for progression or remission of the disease.

Chickens were autopsied as they became comatose or at 28 days after challenge, whichever was sooner. The chickens were bled prior to autopsy, and the serum was collected, inactivated at 56^{o} for 30 minutes and then stored at -30^{o}C. The brain, proventriculus, ventriculus and pancreas were removed for histological examination.

The brain was examined for microscopic lesions along a mid-line saggital section. If lesions were not apparent in this section, five further sections 200 μ apart were cut. If lesions were not detected in these six sections, the brain was considered to have no visible lesions of IAEV infection.

Chickens not developing IAE in the group challenged per os were not autopsied on the 28th day. A serum sample was

obtained by bleeding from the brachial vein and the chicken was then challenged IC with a calculated dose of 10,000 EID_{50} of van Roekel virus. The actual dose of the challenge virus was subsequently titrated. These chickens were observed for a further 28 days.

Virus serum neutralization tests. The test was performed using the technique described in the General Materials and Methods.

Results

Challenge of chickens with the NSW-1 virus

Chickens became less susceptible to the development of IAE the older they were at the time of challenge. This was demonstrated by an inability to induce clinical signs of IAE in chickens 28 days of age challenged by either the IM, IP or oral methods. None of the chickens in uninfected control groups developed clinical or histological signs of IAE

(a) One day old group. IAE developed in all the chickens challenged with the virus, with the exception of one chicken in the IM and IP groups. All chickens exhibited microscopic lesions, except the two that failed to develop clinical disease.

The disease that developed in this age group was classified as paralytic. All of the diseased chickens eventually developed complete paralysis within the 28 day observation period.

The incubation period of the disease in the various groups were - IC route, 12 to 16 days; IM route, 11 to 16 days; IP route, 14 to 19 days and oral route, 12 to 18 days.

The results are summarized in Table 25.

Table 25

Response of one day old chickens to challenge with NSW-1 isolate by various routes

Method of challenge	Virus dose (CID_{50})	Incubation period in days									Number of birds affected	
		Number of chickens									Clinical (10 birds per group)	Histo-
		11	12	13	14	15	16	17	18	19		
IC	450		4	1	3	1	1				10	10
IM	4500	1	1	2	1	1	3				9	9
IP	4500				2	1	1	2	1	2	9	9
Oral	4500		3	2	2	1			2		10	10

IC - intra-cerebral, IM - intra-muscular, IP - intra-peritoneal
CID - chick infective dose

Seven day old group. The disease developed in all groups challenged with the virus. However not all of the chickens in the group developed the disease. Furthermore the disease that developed varied from paralytic to mild. Some chickens in all of the groups developed paralysis - IC, 5; IM, 3; IP, 3; Oral 4. The remainder with clinical signs exhibited ataxia or tremor.

All of the chickens that developed clinical signs of the disease exhibited histological lesions. Chickens not showing the disease also developed microscopic lesions of IAE. These were classified as sub-clinically infected birds. The incubation period of the disease in the various groups was - IC, 11 to 19 days; IM, 15 to 24 days; IP, 20 to 26 days and oral 12 to 21 days.

The results of this group are shown in Table 25.

Table 26

Response of seven day old chickens to challenge with the NSW-1 isolate by various routes

Route of challenge	Virus dose (CID_{50})	Incubation period in days — Number of chickens																Number of chickens affected	
		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Clinically (10 chickens per group)	Histologically (10 chickens per group)
IC	600	2	1		1		2	1	1	2								10	10
IM	6,000					1			1			1	2	1	1			7	10
IP	6,000										1		3		1		1	6	10
Oral	6,000		1		1			2	1	1	2	1						9	9

IC - intra-cerebral, IM - intra-muscular, IP - intra-peritoneal

Group challenged at 14 days of age. IAE developed in all groups challenged with the virus, although the number of chickens affected in the IM, IP and orally challenged groups was smaller than that in the day one and seven groups. Affected chickens in these groups only exhibited ataxia. By contrast three of the affected chickens in the IC group developed paralysis.

Histological lesions were observed in all affected birds. Sub-clinically infected birds were also detected. The incubation periods of the disease in the groups were - IC, 13 to 20 days; IM, 20 to 23 days; IP, 24 to 27 days and oral, 18 to 21 days.

The results of this group are tabulated in Table 27.

21 day old group. The disease developed only in the IC, IM and IP challenged groups. The clinical signs of the disease were generally mild, although two chickens in the IC group developed paralysis.

All affected chickens developed histological lesions. Microscopic changes of IAE were also observed in chickens not clinically affected.

The incubation periods in the various groups were - IC, 14 to 21 days; IM, 24 days; IP, 26 days.

The results of the challenge of 21 day old chickens are summarized in Table 28.

28 day group. Clinical signs of the diseases developed only in the chickens challenged IC. All affected chickens became paralyzed and showed histological lesions of IAE. However, microscopic lesions were observed in chickens challenged by the

Table 27

Response of 14 day old chickens to challenge with the NSW-1 isolate by various routes

Route of challenge	Virus dose (CID_{50})	Incubation period in days														Number of chickens affected	
		Number of chickens														Clinically	Histologically
		11	14	15	16	17	18	19	20	21	22	23	24	25	26	27	(10 chickens per group)
IC	550	1		2	1	3		1	2								10	10
IM	5,000								1			3					4	9
IP	5,000											1				1	2	10
Oral	5,000						1			1							2	2

IC - intra-cerebral, IM - intra-muscular, IP - intra-peritoneal

Table 28

Response of 21 day old chickens to challenge with the NSW-1 isolate by various routes

Route of challenge	Virus dose (CID_{50})	Incubation period in days — Number of chickens													Number of chickens affected (10 chickens per group)	
		14	15	16	17	18	19	20	21	22	23	24	25	26	Clinically	Histologically
IC	780	2		1		1	1		1						6	10
IM	7,800											1			1	8
IP	7,800													1	1	7
Oral	7,800														0	-

IC - intra-cerebral, IM - intra-muscular, IP - intra-peritoneal

IC, IM and IP routes that did not develop the disease. The incubation period of the disease in affected chickens varied between 15 and 25 days. The results of this age group are summarized in Table 29.

Serological tests on chickens challenged with the virus by various routes and at different ages

The mean neutralization index (NI) and the standard deviation of the mean, of the sera obtained four weeks after challenge of chickens with NSW-1 isolate by different routes and at specified ages are recorded in Table 30.

The mean NI of the pre-inoculation sera obtained from the 21 and 28 day groups was 0.27±0.28. The mean NI of the uninfected control chickens was 0.27±0.20.

Differences were recorded in the mean NI of the sera obtained from the different age groups. An analysis of variance of these results indicated that some of these differences were statistically significant. The mean NI of the 21 and 28 day old groups was greater than that of the one, seven and 14 day groups ($P<0.01$). The mean NI of the 14 day group was greater than the mean NI of the one day group ($P<0.05$) (Table 31).

The mean NI of the groups challenged by either IC, IM or oral was greater than the mean NI of the group challenged IP ($P<0.01$).

Intra-cerebral challenge of chickens surviving oral infection with NSW-1 virus.

The results of the challenge of chickens that had previously been infected per os with the NSW-1 isolate are recorded in Table 32.

Table 29

Response of 28 day old chickens to challenge with the NSW-1 isolate by various routes

Route of challenge	Virus dose (CID_{50})	Incubation period in days											Number of chickens affected	
		Number of chickens											Clinically	Histologically
		15	16	17	18	19	20	21	22	23	24	25	(10 chickens per group)	
IC	1,000	1			1			1				1	4	10
IM	10,000												0	6
IP	10,000												0	5
Oral	10,000												0	-

IC - intra-cerebral, IM - intra-muscular, IP - intra-peritoneal

Table 30

Mean neutralization indices of the sera of chickens 28 days after challenge with NSW-1 virus

Age at challenge	Mean neutralization index				Age group mean NI
	Route of virus challenge*				
	IC	IM	IP	Oral	
Day 1	2.59±0.45	2.54±0.44	2.21±0.45	2.53±0.54	2.48±0.46
Day 7	2.73±0.22	2.55±0.57	2.38±0.26	2.70±0.55	2.59±0.43
Day 14	2.88±0.30	2.79±0.28	2.43±0.90	2.60±0.45	2.69±0.50
Day 21	3.18±0.34	3.11±0.38	2.94±0.28	3.19±0.34	3.10±0.34
Day 28	3.31±0.26	2.94±0.17	2.86±0.33	3.53±0.31	3.16±0.38
Route mean NI	2.93±0.42	2.78±0.43	2.58±0.51	2.91±0.58	

* IC - intra-cerebral, IM - intra-muscular, IP - intra-peritoneal

Table 31

Statistical significance of differences between NI of sera of chickens challenged at different ages

Mean NI of 28 day group	>	mean NI of 14 day group	P<0.01
" " " " " "	>	mean NI of 7 day group	P<0.01
" " " " " "	>	mean NI of 1 day group	P<0.01
Mean NI of 21 day group	>	mean NI of 14 day group	P<0.01
" " " " " "	>	mean NI of 7 day group	P<0.01
" " " " " "	>	mean NI of 1 day group	P<0.01
Mean NI of 14 day group	>	mean NI of 1 day group	P<0.05

Chickens that had a NI of their sera of 1.80 or greater prior to challenge resisted an intra-cerebral dose of between 9,500 to 17,600 EID_{50} of van Roekel virus. Chickens with NI of 1.00 or less succumbed to a similar intra-cerebral challenge. Not all such chickens developed the disease.

All of the chickens that had been infected with the NSW-1 isolate 28 days before the intra-cerebral dose of van Roekel virus resisted the challenge.

Table 32

Intra-cerebral (IC) challenge with VR virus of chickens that had previously been infected with NSW-1 isolate per os

IC challenge dose (EID_{50})	Age at challenge with VR virus	Number challenged	Number affected	Mean NI of sera (*)	Range of NI
9,500	35(a)	1	0	2.70	-
9,500	35(b)	5	5	0.44±0.39	0.00-1.05
9,800	42(a)	8	0	2.90±0.47	1.80-3.34
9,800	42(b)	5	4	0.16±0.21	0.00-0.55
17,600	49(a)	10	0	3.19±0.34	2.41-3.80
17,600	49(b)	5	4	0.36±0.39	0.00-1.06
13,000	56(a)	10	0	3.53±0.31	2.47-3.80
13,000	56(b)	5	3	0.34±0.21	0.14-0.62

(a) - group previously infected with the NSW-1 virus

(b) - group not infected with NSW -1 virus

(*) - NI of sera prior to IC challenge

Discussion

The results obtained in this study confirmed the findings of Jones (1932), van Roekel et al. (1938), Olitsky (1939) and Jungherr and Minard (1942) of the consistency of IC route of inoculation with the virus for inducing the disease regardless of the age of the chicken. Feibel et al. (1952) and Schaaf (1958) have demonstrated chickens 103 and 444 days of age to be susceptible to IC challenge with the virus.

By contrast chickens challenged by IM, IP and oral procedures became less susceptible to IAE as they became older. This was demonstrated by the smaller proportion of chickens in each group that developed clinical disease, by a decreasing severity of the disease, and finally by an inability to reproduce the disease in certain age groups challenged by various methods. In addition there was an increase in the incubation period of the disease in older chickens challenged either parenterally or per os.

This increase in resistance to the disease was, however, not related to an insusceptibility of the cells in the CNS to the virus as chickens of all ages challenged IC developed the disease. Nor was it due to a failure of invasion of the CNS by the virus as microscopic lesions of IAE were evident in all age groups of chickens that were challenged IC, IM or IP. However the damage produced in the CNS by the virus in chickens not exhibiting clinical signs must have been insufficient to cause overt disease. This may have been associated with curtailment of virus multiplication in the CNS.

It has been known for a long time that viruses that regularly killed following IC inoculation, may produce only sub-clinical infections following challenge by other routes of

inoculation (Lennette and Koprowski, 1944). More recently it has become clear that in many such instances, the virus invaded the CNS but failed to kill the host even though the virus reached titres much greater than the minimum lethal intracerebral dose (El Dadah and Nathanson, 1967; Doherty, 1969; Thind and Price, 1969; Weiner et al., 1970). After challenge by routes other than IC, invasion of the CNS by the virus is apparently delayed and it has been suggested that this lag allows the hosts defences to prevent a potentially lethal infection (Nathanson and Cole, 1971).

The effect of the age of the chicken and the route of challenge with the virus is also reflected in the serological response. The mean NI of the sera of the 21 and 28 day groups was significantly greater ($P<0.01$) than that of the other age groups. These differences were not associated with a shorter time interval between virus challenge and post mortem in the younger, more severely affected age groups. Even in the one day group challenged IC eight of the 10 affected chickens were post mortemed on the 28th day after infection. The differences may be related to a poorer humoral response in the younger chickens. This inferiority could influence the outcome of an IAEV infection as a poorer antibody response could allow the virus to invade, multiply rapidly and overwhelm the CNS. This concept of a race between the multiplication of the virus and the immune response may be important in the pathogenesis of IAE.

The mean NI of the pre-inoculation sera of 80 IAEV susceptible chickens was 0.27 ± 0.28. The susceptibility of these chickens was established by IC, IM, IP or oral challenge with NSW-1 isolate. The success of this challenge was assessed by the development of clinical signs or histological lesions of

IAE or by the production of IAEV serum neutralizing antibodies in the chickens. The mean NI of the sera of a further 97 chickens was 0.27±0.20. The susceptibility of these chickens was, however, only established by the successful challenge of chickens reared in co-habitation.

Calnek and Jehnich (1959a) determined the mean NI of the sera of 168 IAEV susceptible chickens to be 0.14±0.13. This suggested to them the absence of non specific neutralizing substances to the virus in the serum of chickens. Although the mean NI of the sera of susceptible chickens obtained in this study was higher than this, the result substantiated the claim of Calnek and Jehnich (1959a) of insignificant levels of non-specific neutralizing substances in normal chicken serum.

Chickens with an NI of 1.80 or greater resisted an intracerebral challenge with from 9,500 to 17,600 EID_{50} of van Roekel virus. Chickens with an NI of 1.00 or lower succumbed to a similar challenge. Calnek and Jehnich (1959a) found that chickens with a NI of 1.10 or greater always resisted an IC challenge with up to 10,000 EID_{50} of van Roekel virus. This indicated a good correlation between the serum neutralization test and susceptibility to live virus challenge of chickens. A NI of 1.10 has subsequently been widely used in the assessment of immunity to IAE (Butterfield et al., 1961; MacLeod, 1965; Willemart, 1969). The results obtained in this study confirmed the association of functional host immunity and the presence of circulating neutralizing antibody to the virus. In this study however, the lowest NI of serum of resistant chickens available for challenge was 1.80. Therefore the significance of an NI of 1.10 demonstrated by Calnek and Jehnich (1959a) was not confirmed.

Summary

The age of the chicken and the route of challenge with the virus had a marked effect on the development of IAE and on the production of specific virus neutralizing antibody. Chickens one to 56 days of age were consistently susceptible to the development of clinical signs of the disease after an IC challenge, whereas chickens challenged by other routes developed a decreasing susceptibility to IAE as they aged. This decreased susceptibility was demonstrated in terms of the number of chickens that became diseased, the severity of the clinical signs of the disease and by the length of the incubation period.

Chickens challenged at one, seven and 14 days of age showed poorer serological responses to the virus than those infected at 21 and 28 days of age. The mean NI of the sera of 80 IAEV susceptible chickens was 0.27±0.28. These chickens succumbed to a challenge with the NSW-1 isolate of the virus.

Chapter 2

THE EFFECT OF IMMUNOSUPPRESSION ON THE DEVELOPMENT OF IAE

Introduction

The lymphoid system of the chicken has been functionally and morphologically separated into two components, namely the bursa dependent system which is responsible for the development of humoral immune responses and the thymus dependent system which is responsible for cellular immunity (Glick et al., 1956; Warner et al., 1962; Cooper et al., 1966a).

The selective removal of one or other of these systems has been used to study the significance of cellular or humoral immune responses in the development of immunity to a number of diseases (Chang et al., 1959; Kemmes and Pethes, 1963; Peterson et al., 1964; Pierce and Long, 1965; Peterson et al., 1966; Foster and Moll, 1968; Cooper et al., 1966b; Payne and Rennie, 1970; Rose and Long, 1970; Cheville and Richards, 1971; Portnoy et al., 1973).

Results obtained in this study (Part 2, Chapter 1) demonstrated that chickens challenged by the oral route with NSW-1 virus were less susceptible to IAE when infected after 14 days of age. This age related resistance to the development of the disease could provide a suitable system for investigation of the significance of components of the immune system in the development of IAE.

Cheville (1970), working with van Roekel virus inoculated IM, has demonstrated that neonatal bursectomy adversely affects the development of this resistance.

The work described in this chapter was designed to extend and clarify knowledge of the effect of immunosuppression on

the development of IAE in chickens challenged per os with IAEV.

Materials and Methods

Chickens. The susceptible chickens used were hatched at the University of Sydney from eggs obtained from WL/A flock.

Eggs. The fertile eggs were obtained from WL/B flock.

Immunosuppressive treatments

(a) Hormonal bursectomy. This was performed in ova at nine days of incubation by the injection of 5.0 mgm (0.10 ml. of a 50 mgm/ml. solution in corn oil) of testosterone proprionate (T) into the allantoic sac of 200 embryonating eggs. Control fertile eggs were inoculated in the same way with 0.1 ml. of sterile corn oil. The inoculated fertile eggs were hatched and the chickens wing banded for identification purposes.

(b) Cyclophosphamide treatment. Cyclophosphamide* (CY) was injected IP as a freshly prepared aqueous solution containing 4 mgm/0.1 ml. 0.1 ml. of this solution was inoculated daily for four consecutive days beginning on the day of hatching. Control chickens were injected IP with 0.1 ml. of sterile distilled water. All chickens were identified by wing bands.

Virus. The chickens were challenged per os with an estimated 100 CID_{50} of the NSW-1 virus. The virus was used as a chicken embryo brain suspension of the second passage of the virus. The actual dose of virus used was titrated after challenge. Control chickens were challenged with a 50 percent (w/v) brain suspension of IAEV susceptible chicken embryos.

* Endoxan-Astra, Mead Johnson, Caringbah, N.S.W.

Van Roekel virus was used in the virus-serum neutralization tests.

Experimental design.

This is presented in Table 33.

Table 33
Number of chickens used, their age and the immunosuppressive treatment administered

Immunosuppressant treatment	Number of chickens used (days of age at time of challenge)					
	1	7	14	21	28	35
Nil	16	16	16	16	16	16
Cyclophosphamide	16	16	16	16	16	16
Testosterone	16	16	16	16	16	16
Corn oil	-	10	-	10	-	10
Distilled water	-	10	-	10	-	10

Half the chickens in each group were infected with the virus, the remainder being the uninfected control group.

Chickens were transferred from the isolated rearing room to a separate room for virus challenge. Control chickens were kept in the rearing room throughout the experiment. Chickens 21, 28 and 35 days of age were bled prior to challenge with the virus to obtain a serum sample.

The chickens were challenged per os and then observed for 28 days for the development of clinical disease. Chickens were weighed, bled, killed and autopsied as they became comatose, or 28 days after challenge, whichever was sooner. An equal number of uninfected control birds were autopsied on each occasion birds were examined. The brain, spleen, bursa of

Fabricius and thymus were removed, excess fat and connective tissue teased from their surface and the organ weighed. Portion of each organ was then placed in 12.5 percent formal saline.

In an additional study, five CY, five T and five normal chickens were killed at one, seven, 14, 21, 28 and 35 days of age. The blood from each chicken was heparinized and used in a Simonsen assay (see below).

Histological examination. The spleen was cut into two equal portions and the cut surface used in tissue examination. The number of germinal centres present in four sections spaced 200 μ apart in one portion were counted. The mean of these counts was used as the number of germinal centres in a section of spleen. All tissues were examined for evidence of IAE or for effects induced by immunosuppressive therapy.

Serological examination. Individual serum samples were used to determine the IAEV neutralizing antibody titre of all challenged chickens. Pre-inoculation samples were only obtained from the 21, 28 and 35 day old groups. A serum pool obtained from IAEV susceptible chickens of the same age as those challenged was used as the pre-inoculation serum sample for the younger groups. These serum pools were obtained from other normal, CY or T treated chickens as the serum pair dictated.

Simonsen Assay. The test was performed according to the

technique of Szenberg and Warner (1962b). 0.1 ml. of a dilution of whole heparinized blood was inoculated onto the CAM of 12 day old embryonating eggs. Ten to 18 eggs were used per blood sample. The eggs used in this test were from a WR x NH flock. Eggs inoculated with the blood were incubated for four days at 37°C. The CAM's were then harvested and the number of pocks on each membrane counted. The mean of the count of the ten to18 eggs used for a blood sample was then calculated.

Simonsen assay were undertaken on blood obtained from normal chickens and from CY or T treated birds. These were of the same genotype as those challenged with the virus. The chickens were bled at one, seven, 14, 21, 28 and 35 days of age.

Effect of passive immunoglobulin inoculated IP on the development of IAE. The experimental design is summarized in Table 34.

Table 34

Design of experiment to determine the role of immunoglobulin to IAEV in protecting normal and immunosuppressed chickens against IAEV

Age of chickens (days)	Time after challenge Ig inoculated (hours)	Number of chickens			
		Normal		CY treated	
		Normal Ig	Anti-VR Ig	Normal Ig	Anti-VR Ig
1	0	6	6	6	6
1	48	6	6	6	6
7	0	6	6	6	6
7	48	6	6	6	6
14	0	6	6	6	6
14	48	6	6	6	6

Ig - immunoglobulin

Chickens were challenged by the oral route with an estimated 100 CID_{50} of the NSW-1 virus, the actual dose was titrated after the challenge. Afterwards they were inoculated IP with 1.0 ml. of immunoglobulin which was prepared by precipitation of anti-IAEV and normal chicken serum with Na_2SO_4 as described by Orlans et al. (1961). The IAEV antisera was obtained from chickens that had been hyper-immunized with van Roekel virus, and it had an NI of $\log_{10}^{4.20}$. The normal chicken serum was obtained from susceptible chickens and had an NI of $\log_{10}^{0.32}$.

Chickens were examined daily for the onset of the clinical signs of the disease. They were bled, killed and autopsied as they became comatose, or 28 days after challenge, whichever was sooner. The brain, proventriculus, ventriculus, bursa of Fabricius, spleen and thymus were removed for later histological examination. Serological tests were undertaken for the detection of IAEV neutralizing antibody.

Persistence of serum neutralizing capacity in chickens inoculated IP with immunoglobulin. Thirty IAE susceptible chickens one day of age were treated with CY as previously described. Fifteen were inoculated IP with 1.0 ml. of the IAEV immunoglobulin described above. Fifteen were inoculated IP with 1.0 ml. of the IAEV negative immunoglobulin. Five chickens in each group were bled at seven, 14 and 21 days of age. The neutralizing capacity of the serum from the chickens was determined individually using virus-serum neutralization tests. The mean NI of each age group was then calculated

Results

Development of the disease

The normal infected chickens developed an age resistance to the development of clinical IAE. This confirmed previous findings (Part 2, Chapter 1).

In contrast, immunosuppressed chickens remained highly susceptible to the development of the disease, regardless of their age at the time of infection with the virus. (Table 35).

Evidence of infection was not detected in any of the uninfected control chickens.

(a) One day group. All chickens in the groups challenged with the virus developed the paralytic form of the disease. The shortest incubation periods were - normal group, 12 days; CY group, 11 days and the T group, 10 days. Histological lesions of IAE were detected in all the chickens that developed the clinical disease.

(b) Seven day group. The clinical disease developed in all chickens, however, the normal chickens exhibited only ataxia, whereas immunosuppressed birds developed paralysis. The incubation periods were - normal, 12 days; CY group, 10 days; T group, 10 days. Microscopic changes of the disease were obvious in all infected birds.

(c) Fourteen day group. Only two of the eight chickens in the normal infected group developed clinical IAE. The incubation period was 19 days. However seven chickens showed microscopic lesions of the disease. By contrast, all of the chickens in the CY group developed the paralytic disease. Only two of six chickens in the T group developed the paralytic lesions, although three others showed inco-ordination. All of the chickens in the

Table 35

Development of IAE in normal and immunosuppressed chickens challenged at different ages with the NSW-1 virus

Chick treatment	Age of chickens at time of challenge with virus (days)																	
	1			7			14			21			28			35		
	No. affected		Days PI first seen	No. affected		Days PI first seen	No. affected		Days PI first seen	No. affected		Days PI first seen	No. affected		Days PI first seen	No. affected		Days PI first seen
	Clinic	Histo		Clinic	Histo		Clinic	Histo		Clinic	Histo		Clinic	Histo		Clinic	Histo	
Nil	8	8	12	8	8	12	2	7	19	0	6	0	0	2	0	0	2	0
Cyclo-phosphamide	8	8	11	8	8	10	7	7	15	6	6	18	5	5	22	3	4	18
Testosterone	7	7	10	7	7	10	5	6	14	4	4	19	4	4	19	4	4	21
Corn oil	-	-	-	5	5	11	-	-	-	0	3	0	-	-	-	-	2	0
Distilled water	-	-	-	5	5	13	-	-	-	0	5	0	-	-	-	0	3	0

Clinic - clinically affected

Histo - affected histologically

PI - post infection

Virus challenge dose (CID_{50})

days	
1	- 180
7	- 260
14	- 300
21	- 160
28	- 360
35	- 280

CY and T treated groups showed histological changes of IAE.

(d) Twenty-one day group. Clinical IAE was not observed in the normal infected chickens. However, six of eight of the chickens showed histological changes of the disease. However, in the CY and T treated groups the paralytic disease was present in all affected birds. All chickens that showed IAE, had histological lesions of IAEV infection.

(e) Twenty-eight day group. Clinical signs of the disease were not observed in the normal infected chickens, although two exhibited histological lesions of IAE. Five of six of the chickens in the CY group developed the paralytic form of the disease. Four of six in the T group developed this form of IAE also. All of the chickens that showed clinical IAE had microscopic changes of the disease.

(f) Thirty-five day group. Two of the eight chickens in the normal infected group showed histological lesions of IAE; none showed clinical signs. Three of five in the CY group, and four of the four in the T group showed the paralytic disease. All chickens that developed clinical IAE showed microscopic lesions. Sub-clinically affected chickens were detected in the normal infected chickens and in the CY treated groups.

Effect of the disease on weights of certain organs

(a) Chicken body weight. There were no significant differences in the body weight of the chickens in the normal, CY and T treated, uninfected control groups.

The body weight of the normal chickens infected at one day of age was significantly lower ($P<0.001$) than of birds of the same age not infected with the virus. There were no significant

differences between normal chickens in the infected and uninfected groups for all the other aged chickens tested.

By contrast, the body weight of the chickens in the CY and T groups that were infected with the virus, were significantly lower ($P<0.001$), than the uninfected groups in all the different age categories.

The body weight of the normal infected group was significantly different ($P<0.001$) to that of the CY and T treated, infected groups for all age groups. These results are summarized in Table 36.

(b) Chicken brain weight. The differences in weights of the brains recorded between the infected and uninfected and the normal and immunosuppressed chickens were not statistically significant (Table 37).

(c) Spleen weight. The mean weight of the spleen from the chickens in the CY and T treated infected groups was significantly lower ($P<0.001$) than that of the normal infected group for all the different age groups. No significant differences were found in the weight of the spleens from the uninfected chickens in the 7, 14 and 21 day age groups. The spleens of the CY and T treated uninfected chickens in the one, 28 and 35 day groups was significantly lower ($P<0.01$) than that of the uninfected normal chickens.

The weight of the spleen in the different categories studied are recorded in Table 38.

(d) Germinal centre numbers in the spleen. The number of germinal centres in spleens of chickens treated with either CY or T was significantly lower ($P<0.001$) than that of untreated chickens. There was a regeneration of the germinal centres in the spleen of the CY and T treated chickens in the older

Table 36

Body weight of normal and immunosuppressed chickens infected with NSW-1 virus at different ages

	Chick treatment	Age of chickens at time of challenge with virus (days)					
		1	7	14	21	28	35
Infected	Nil	148.6±14.4*	245.9±38.6	357.1±36.4	458.0±44.3	524.6±83.0	776.9±59.6
	Cyclophosphamide	100.0±27.2	180.4±34.2	219.0±68.1	270.5±62.3	325.7±61.8	516.4±120.8
	Testosterone	99.2±19.3	183.6±22.2	183.5±24.1	258.5±31.8	287.3±28.3	641.8±37.1
	Corn oil	-	243.7±71.8	-	520.3±81.8	-	812.5±67.2
	Distilled water	-	262.4±56.2	-	476.2±75.4	-	850.3±32.4
Uninfected	Nil	219.7±12.2	273.7±6.1	345.0±12.3	463.9±62.8	578.4±52.8	801.9±44.3
	Cyclophosphamide	210.6±17.8	285.8±31.1	311.4±27.2	408.8±17.5	574.8±65.9	757.4±32.8
	Testosterone	170.6±25.4	239.6±14.2	332.7±42.1	419.8±19.9	583.2±15.1	742.6±28.4
	Corn oil	-	284.6±18.4	-	506.4±55.4	-	774.8±55.6
	Distilled water	-	276.7±34.6	-	571.6±48.6	-	789.4±61.7

* Weight in grams, measured as chickens became comatose, or 28 days after challenge.

\- Not examined

Table 37

Brain weight of normal and immunosuppressed chickens infected with NSW-1 isolate at different ages

	Chicken Treatment	Age of chickens at the time of challenge (days)					
		1	7	14	21	28	35
Infected	Nil	1821.2±260.8*	2061.8±148.0	2043.0±181.2	2173.2±179.3	2434.3±103.7	2573.7±116.6
	Cyclophosphamide	1795.6±181.9	1796.5±53.3	1897.3±196.0	2142.7±149.8	2310.9±210.8	2458.7±290.0
	Testosterone	1853.2±211.4	1907.0±79.3	1963.4±70.3	2230±35.8	2287.6±126.1	2560.6±150.1
	Corn oil	-	1976.7±97.6	-	2167.2±51.9	-	2571.0±189.7
	Distilled water	-	1850.4±80.4	-	2185.4±62.4	-	2497.4±160.2
Uninfected	Nil	1868.3±350.3	2033.6±51.9	1998.0±148.5	2213.3±108.0	2363.5±129.0	2451.3±175.8
	Cyclophosphamide	1843.3±85.9	2040.5±58.6	1965.2±88.7	2266.3±173.6	2346.8±122.7	2510.3±178.6
	Testosterone	1885.4±104.6	2062.0±20.0	1901.5±54.3	2251.4±206.1	2426.0±86.3	2466.5±204.0
	Corn oil	-	1941.5±58.4	-	2314.7±106.0	-	2511.7±176.3
	Distilled water	-	2014.7±60.5	-	2300.1±124.4	-	2606.1±187.4

* Weight in milligrams, measured as chickens became comatose, or 28 days after challenge.

\- Not examined

Table 38

Spleen weight in normal and immunosuppressed chickens infected with NSW-1 isolate at different ages

	Chicken treatment	Age of chicken at time of challenge (days) 1	7	14	21	28	35
Infected	Nil	320.8±118.7 *	459.8±118.7	728.5±185.2	833.0±73.2	1011.5±30.3	1436.2±337.9
	Cyclophosphamide	78.0±50.1	223.7±71.8	271.7±131.5	358.3±17.6	245.2±82.1	748.0±399.4
	Testosterone	70.0±34.5	197.5±30.7	207.6±16.3	373.3±103.9	512.0±46.8	650.8±116.3
	Corn oil	-	397.4±111.7	-	589.8±131.4	-	1187.3±263.4
	Distilled water	-	367.5±89.4	-	487.4±108.6	-	1512.5±184.4
Uninfected	Nil	519.3±53.0	437.9±19.5	630.8±94.3	636.5±111.7	765.7±171.1	947.0±146.4
	Cyclophosphamide	180.8±52.4	427.7±113.7	534.3±144.7	681.5±44.7	658.7±147.1	608.0±137.4
	Testosterone	138.6±19.9	341.8±47.4	565.2±47.2	614.7±47.8	596.3±124.4	680.6±113.4
	Corn oil	-	341.7±21.4	-	589.4±71.4	-	1087.4±289.4
	Distilled water	-	307.7±36.0	-	498.7±98.7	-	1011.4±171.4

* Weight in milligrams, measured as chickens became comatose, or 28 days after challenge.

\- Not examined

Table 39

The number of germinal centres in a section of spleen from normal and immunosuppressed chickens infected with NSW-1 virus at different ages

	Chicken treatment	Age of the chicken at the time of challenge with virus (days)					
		1	7	14	21	28	35
Infected	Normal	6.6±4.8	27.9±18.8	27.5±14.7	27.3±9.2	28.3±10.3	22.3±7.1
	Cyclophosphamide	0	0	0	2.2±2.1	2.0±2.1	5.6±3.5
	Testosterone	0	0	0.3±0.8	0	0.7±1.0	2.3±2.6
	Corn oil	-	31.2±15.3	-	29.8±6.4	-	29.2±9.4
	Distilled water	-	36.4±12.6	-	24.6±7.8	-	31.4±10.2
Uninfected	Normal	6.3±3.4	14.1±9.1	16.1±8.0	17.1±9.3	19.4±8.1	19.7±9.8
	Cyclophosphamide	0	0	0	3.1±3.7	3.4±2.7	8.4±3.6
	Testosterone	0	0	1.6±0.9	0.5±1.1	2.4±2.3	2.8±2.9
	Corn oil	-	16.4±6.8	-	17.2±8.2	-	23.6±10.1
	Distilled water	-	19.3±7.2	-	11.4±9.6	-	18.7±11.4

Counted as chickens became comatose, or 28 days after challenge

- Not examined

Table 40

Mean weight of the bursa of Fabricius from normal and immunosuppressed chickens infected with NSW-1 isolate at different ages

	Chicken treatment	Age of the chicken at the time of challenge with the virus (days)					
		1	7	14	21	28	35
Infected	Normal	1276.3±333.6	1456.7±565.5*	1911.0±566.5	1848.3±370.2	2073.7±313.8	3343.5±213.4
	Cyclophosphamide	69.0±29.0	218.7±58.7	93.0±36.5	163.7±81.7	128.7±24.7	260.3±90.9
	Testosterone	61.3±13.8	86.2±12.9	88.5±18.6	133.0±24.3	115.0±15.5	263.5±55.9
	Corn oil	-	1410.5±201.3	-	1876.2±139.4	-	3037.2±911.2
	Distilled water	-	1213.7±155.8	-	1742.0±156.2	-	3146.8±316.3
Uninfected	Normal	903.8±95.2	935.0±199.1	2471.8±287.6	2054.2±98.1	2426.3±560.4	2787.2±803.6
	Cyclophosphamide	126.2±15.5	230.9±24.3	79.0±21.5	181.5±20.5	494.7±171.9	262.3±34.0
	Testosterone	138.6±27.9	195.6±22.1	108.7±9.2	154.0±25.9	258.5±25.6	384.5±30.3
	Corn oil	-	889.4±80.6	-	1970.4±141.4	-	2941.5±870.4
	Distilled water	-	901.5±66.7	-	1670.4±88.6	-	2765.2±991.4

* Weight in milligrams, measured as chickens became comatose, or 28 days after challenge

\- Not examined

Table 41

Mean weight of the thymus from normal and immunosuppressed chickens infected with NSW-1 isolate at different ages

	Chicken treatment	Age of the chickens at the time of challenge with the virus (days)					
		1	7	14	21	28	35
Infected	Normal	1928.8±295.7*	1165±514.5	1881.7±466.5	2314.0±415.5	4079.8±1918.6	6019.2±1445.5
	Cyclophosphamide	1116.0±110.4	694.2±282.7	821.6±411.9	1230.8±537.2	2762.4±464.9	3202.0±1265.4
	Testosterone	873.0±257.4	716.3±109.6	1738.2±267.4	1423.0±189.6	2185.4±507.2	3335.4±1416.0
	Corn oil	-	1410.5±316.7	-	2411.3±230.4	-	5110.5±976.8
	Distilled water	-	1241.6±426.7	-	1976.5±306.2	-	4875.3±702.7
Uninfected	Normal	1803.5±111.3	1381.5±122.9	2053.2±167.6	2215.3±178.9	4306.7±1050.7	6360.7±1407.8
	Cyclophosphamide	1776.2±264.3	1516.4±115.5	2312.0±208.3	2070.3±297.2	3048.3±521.4	4198.4±1114.6
	Testosterone	1690.3±115.6	1478.1±98.6	1640.7±251.7	2158.4±303.4	2870.7±248.6	4656.0±1078.4
	Corn oil	-	1142.6±284.4	-	-	-	N.W.
	Distilled water	-	1214.7±189.4	-	-	-	N.W.

* Weight in milligrams, measured as chickens became comatose, or 28 days after challenge

\- Not examined

age groups.

The number of germinal centres in the spleen of the normal chickens increased throughout the observation period. The number of germinal centres in the spleen of the normal infected chickens was significantly greater ($P<0.001$) than that in the normal uninfected chickens in all age groups except the one day group.

The germinal centre counts are summarized in Table 39.

(e) Bursa of Fabricius weight. Treatment with either CY or T resulted in a significant weight loss ($P<0.001$) of this organ compared to untreated controls.

The differences between the weight of the infected and uninfected normal chickens bursa of Fabricius were not significant.

These results are tabulated in Table 40.

(f) Thymus weight. The differences recorded between the mean weights of thymus of infected and uninfected, normal and immunosuppressed groups were not statistically significant.

These results are summarized in Table 41.

Histological lesions in organs of chickens infected with the NSW-1 virus

(a) Brain lesions. No lesions were observed in groups of birds not exposed to IAEV irrespective of the immunosuppressant treatment used.

(i) Normal chickens. Histological lesions were detected in all groups of chickens that were infected with the virus. However, not all of the chickens dosed with the virus developed microscopic lesions of IAE. The older the normal chickens were at the time of challenge, the less likely was the development of

histological lesions (see Table 35). Histological changes indicative of IAE were also seen in chickens that failed to develop the clinical disease.

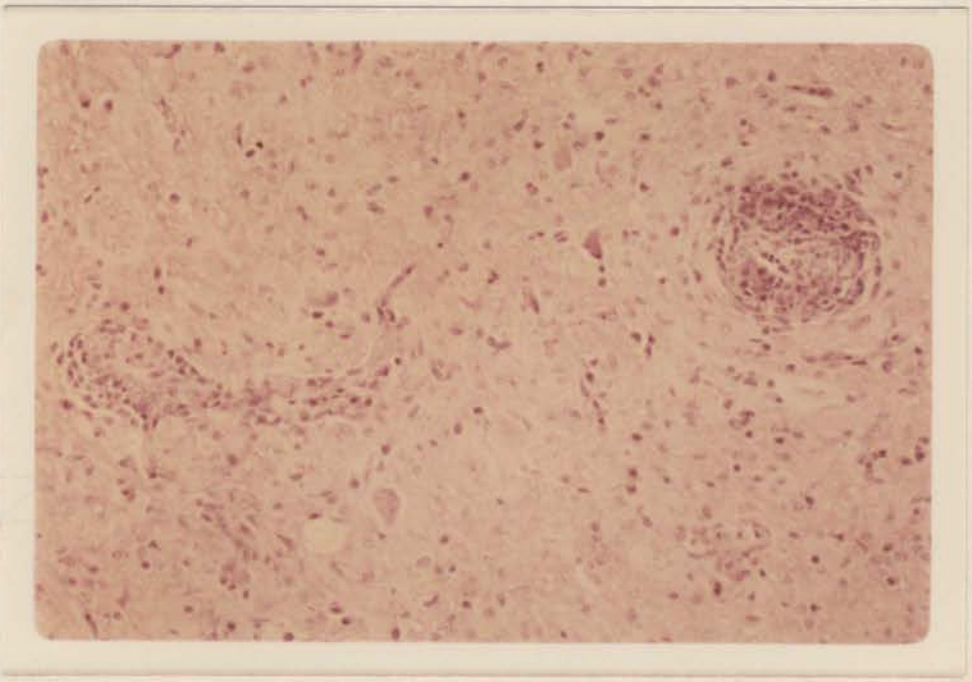
The lesions observed in the brain of infected chickens consisted of neuronal degeneration, focal and local gliosis, neuronophagia and perivascular cuffing (see Figure 7). The extent and severity of the microscopic lesions that developed were apparently not related to the clinical severity of the disease, as marked histological lesions were apparent even in sub-clinically affected birds.

(ii) Immunosuppressed chickens. The immunosuppressed, infected chickens characteristically showed a decreased perivascular and perineural inflammatory cell reaction. Where this was observed, however, it resembled a glial cell reaction. Neuronal cell degeneration and necrosis were more widespread in immunosuppressed infected chickens, with this being particularly prominent in the cerebellum and hind brain. Purkinje cell degeneration and necrosis, sometimes accompanied by necrosis and dissolution of the granular cell layer of the cerebellum was also common in these chickens. Endothelial cell hypertrophy and proliferation was also observed in small blood vessels throughout the brain. The lesions observed in the virus infected immunosuppressed chickens tended to be degenerative, whereas those in the normal infected birds were characterized by inflammatory cell reactions (see Figure 8).

(b) Visceral lesions. No lesions were detected in groups of chickens uninfected with IAEV.

(i) Normal chickens. Changes indicative of IAE were observed in the musculature of the proventriculus,

Figure 7

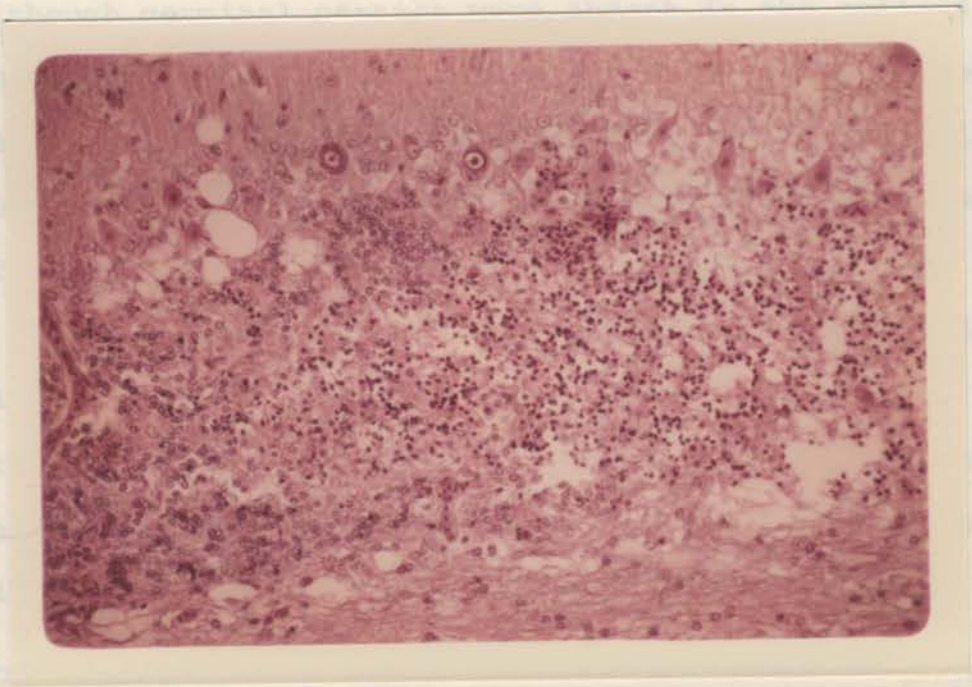


Lesions induced in the CNS of chickens infected with IAEV per os

Stain - haematoxylin and eosin

Magnification - 400X

Figure 8



Lesions in the cerebellum in IAEV affected,
immunosuppressed chickens
Stain - haematoxylin and eosin
Magnification - 400X

ventriculus and atrium of the heart. These lesions consisted of a diffuse lymphoid cell reaction, with some plasma cells also observed (see Figure 9). The spleen showed an increase in the number of germinal centres (see Table 39). The diffuse lymphoid tissue in the white pulp of the spleen appeared unchanged in infected normal chickens.

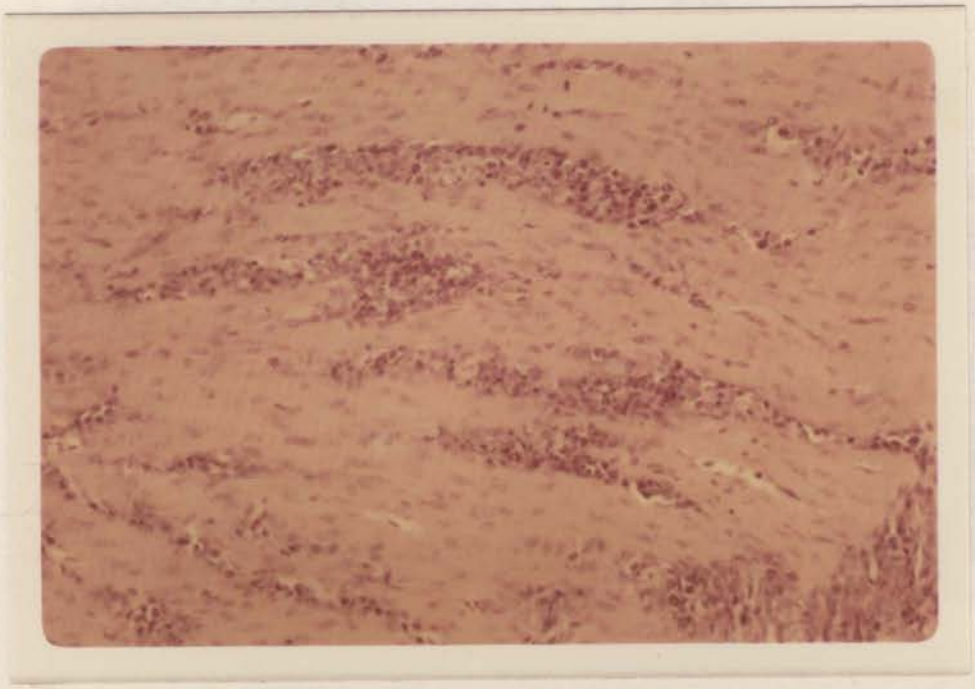
(ii) Immunosuppressed chickens. No changes were observed in the viscera of IAEV infected immunosuppressed chickens although germinal centres were absent in the spleens of the younger chickens. There was a regeneration of some germinal centres in the older birds (see Table 39). The cellular component of these germinal centres appeared the same as that of the normal chickens.

There was also an obvious depletion of the cells in the diffuse lymphoid tissue surrounding the central and penicillar arteries, and the sheathed capillaries in the spleen. This was particularly obvious in the one day old chickens treated with cyclophosphamide.

(c) Lesions in the bursa of Fabricius

(i) Normal chickens. The lymphoid follicles were large and could be clearly differentiated into a cortex of densely packed lymphocytes supported by a reticular cell network, a thin layer of epithelial cells, and a medulla of reticulo-epithelial cells and loosely packed lymphocytes. The connective tissue between the follicles was not prominent, and the mucosa of the plicae was lined by a columnar or pseudo-stratified epithelium. There was no detectable difference between the bursa of IAEV infected and uninfected, non-immunosuppressed chickens.

Figure 9

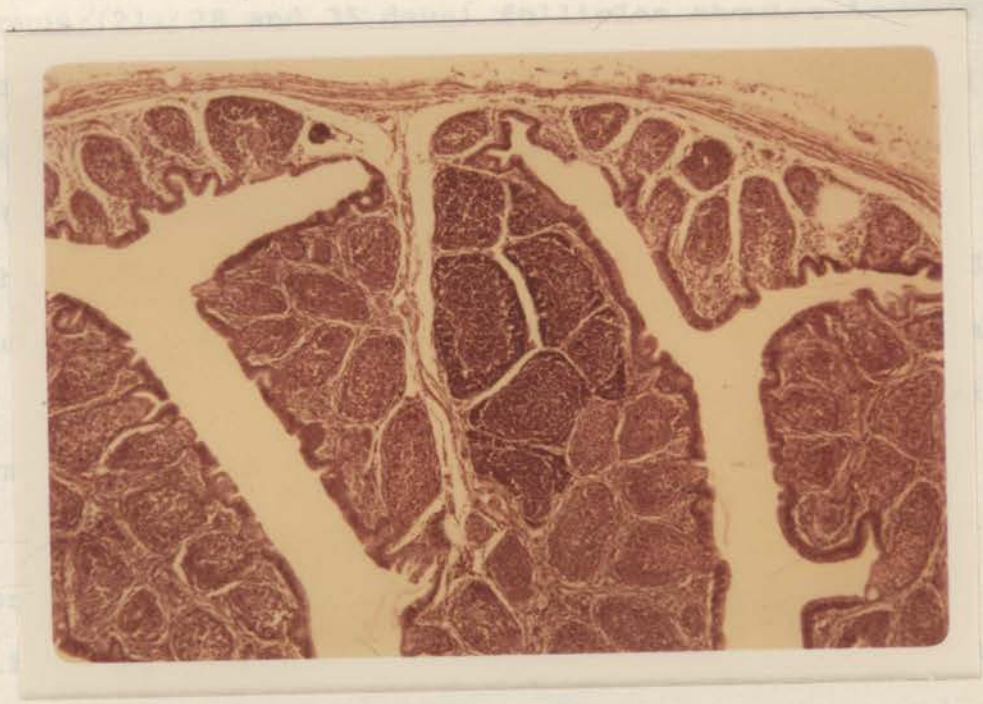


Lymphocyte infiltration of the musculature of the ventriculus of chickens infected with IAEV per os

Stain - haematoxylin and eosin

Magnification - 400X

Figure 10



Regeneration of the lymphoid follicles in the bursa of Fabricius after treatment with cyclophosphamide

Magnification - 100X

(ii) Immunosuppressed chickens.

Cyclophosphamide treated. There was a marked degeneration of the follicles in the bursa and an increase in the interfollicular connective tissue. The remnants of the follicles exhibited a metaplasia of the epithelial reticulum or showed acinar structures lined by columnar or pseudo-stratified epithelium. The mucosal surface of the shrunken plicae appeared normal. In the older age groups (21, 28 and 35 days) follicles showing lymphocytes were seen. These follicles commonly showed lymphocytic regeneration around the periphery of the metaplastic epithelial reticulum, though in the 35 day group, odd follicles the exhibited normal bursal lymphocytes throughout were detected (see Figure 10). However, even in these chickens, a majority of follicles had degenerated, and follicles that exhibited lymphocytic regeneration were smaller than normal.

Testosterone treated. Treatment with this hormone resulted in complete inhibition of lymphoid differentiation and follicle development was non-existent. The bursa remained in this condition throughout and exhibited no tendency toward regeneration.

(d) Lesions in the Thymus

(i) Normal chickens. The thymus could be divided into lobes, with each lobe consisting of a number of lobules partially separated by connective tissue. The lobules could be clearly divided into the cortex of densely packed small lymphocytes and scattered reticulum cells, and a medulla of less densely packed lymphocytes, reticulum cells and islands of epithelial cells or Hassall's corpuscles.

(ii) Immunosuppressed chickens.

Cyclophosphamide treated. The thymus appeared normal in all age groups except for the one day group. In this group, depletion of the lymphocytes in both the cortex and medulla of some lobules was observed. A variable degree of haemorrhages was observed in some medullas. Even thymus glands that exhibited a marked decrease in weight showed normal morphology.

Testosterone treated. The thymus appeared normal in all age groups studied, except in the one day group in which depletion of the lymphocytes in the cortex of most lobules in two chickens was observed.

Simonsen Assay

Pocks were not detected on the CAM's of embryos inoculated with blood obtained from the one and seven day old, CY treated chickens. The pock count of the 14 day old, CY treated birds was also low. All of the other counts in the various age groups and treatments were of the same order (see Table 42).

Table 42

The effect of immunosuppressant treatment on cell mediated immune response

Age of chickens (days)	Mean pock count per 0.1 ml. blood		
	Normal birds	CY treated	T treated
1	29.6±5.2	0	36.6±6.1
7	41.5±8.6	0	31.7±7.2
14	30.0±4.4	18.7±4.7	46.4±6.3
21	49.1±6.2	32.6±3.8	51.7±4.8
28	62.7±9.2	44.4±6.2	56.8±3.4
35	55.0±6.7	58.6±7.8	50.4±6.8

CY - cyclophosphamide T - testosterone

These results indicated the cyclophosphamide treatment had a temporary immunosuppressant effect on cell mediated immune responses.

Serology

Specific neutralizing antibody was not found in the sera of uninfected chickens. This indicated that chickens had been maintained free of infection throughout the test period.

IAEV neutralizing antibody was detected in all of the infected, normal chickens. There were statistically significant differences in the mean NI of the sera obtained from the different age groups tested. The mean NI of the sera from the 21, 28 and 35 day age groups were greater ($P<0.01$) than the mean NI of the one, seven and 14 day groups. The mean NI of the 14 day group was also greater ($P<0.05$) than the mean NI of the one day group.

Specific neutralizing antibody was not detected in the sera of the immunosuppressed chickens that had been infected with IAEV. The mean NI of the sera from the uninfected normal and immunosuppressed chickens were not significantly different (Table 43).

Protective effect of immunoglobulin inoculated IP against IAE

The anti-VR immunoglobulin protected the immunosuppressed and the normal chickens against the development of the clinical disease. Immunoglobulin inoculated at the time of challenge also protected the chickens against the development of histological lesions. Anti-VR immunoglobulin given IP 48 hours after virus challenge allowed the development of microscopic changes of IAE in two of six chickens in the one day group.

Table 43

The mean neutralization indices of normal and immunosuppressed chickens of different ages infected with the virus

Age of chicken (days)	Virus dose (CID_{50})	Chicken treatment	Number of chickens	Mean NI ± SD
1	180	Normal	8	2.09 ± 0.57
	180	CY	8	0.39 ± 0.40
		T	7	0.31 ± 0.30
	Nil	Normal	8	0.38 ± 0.27
		CY	7	0.31 ± 0.16
		T	8	0.40 ± 0.18
7	260	Normal	8	2.35 ± 0.57
		CY	8	0.48 ± 0.27
		T	7	0.46 ± 0.22
		Corn oil	5	2.20 ± 0.56
		Distilled water	5	2.31 ± 0.26
	Nil	Normal	8	0.24 ± 0.24
		CY	7	0.26 ± 0.35
		T	6	0.23 ± 0.16
		Corn oil	5	0.23 ± 0.13
		Distilled water	5	0.20 ± 0.14
14	306	Normal	8	2.63 ± 0.19
		CY	7	0.48 ± 0.34
		T	6	0.45 ± 0.33
	Nil	Normal	8	0.43 ± 0.25
		CY	6	0.37 ± 0.24
		T	7	0.34 ± 0.26

NI - neutralization index
CY - cyclophosphamide
T - testosterone
CID_{50} - chicken infective doses

Cont.

Table 43 (Cont.)

Age of chicken (days)	Virus dose (CID_{50})	Chicken treatment	Number of chickens	Mean NI ± SD
21	160	Normal	8	3.21 ± 0.35
		CY	6	0.30 ± 0.30
		T	4	0.25 ± 0.22
		Corn oil	5	3.40 ± 0.26
		Distilled water	5	3.26 ± 0.21
	Nil	Normal	8	0.23 ± 0.18
		CY	8	0.33 ± 0.31
		T	8	0.54 ± 0.33
		Corn oil	5	0.10 ± 0.17
		Distilled water	5	0.22 ± 0.13
28	280	Normal	7	3.39 ± 0.28
		CY	6	0.33 ± 0.33
		T	6	0.40 ± 0.28
	Nil	Normal	7	0.41 ± 0.33
		CY	5	0.26 ± 0.18
		T	5	0.16 ± 0.15
35	280	Normal	8	3.39 ± 0.37
		CY	5	0.30 ± 0.35
		T	4	0.53 ± 0.39
		Corn oil	5	3.32 ± 0.85
		Distilled water	5	2.96 ± 0.48
	Nil	Normal	6	0.28 ± 0.16
		CY	5	0.16 ± 0.20
		T	5	0.18 ± 0.08
		Corn oil	5	0.16 ± 0.16
		Distilled water	5	0.30 ± 0.34

NI - neutralization index, CY - cyclophosphamide, T - testosterone

The immunoglobulin from normal chickens failed to protect the normal and immunosuppressed chickens against the development of the disease. The normal chickens inoculated with normal immunoglobulin showed a decreased susceptibility to the development of the disease the older they were at the time of infection (Table 44).

The results of SN tests undertaken with sera obtained up to 28 days after virus challenge of chickens inoculated IP with immunoglobulin are summarized in Table 45. No neutralizing capacity was detected in the sera of CY treated chickens inoculated IP with normal immunoglobulin. In contrast the sera obtained from the non-immunosuppressed chickens that were inoculated IP with immunoglobulin showed neutralizing antibody to IAEV. This resulted from a serological response of these chickens to the virus. IAEV neutralizing antibody was not detected in chickens inoculated IP with anti-VR immunoglobulin. This passive immunoglobulin probably prevented infection of the chickens with the virus.

The persistence of anti-VR immunoglobulin in chickens

Neutralizing antibody was detected in the sera of chickens seven and 14 days after inoculation, but not at 21 days. No IAEV neutralizing antibody was found in the sera of the chickens inoculated with IAEV negative immunoglobulin (Table 46).

Table 44

Development of IAE after inoculation of virus and immunoglobulin

Age of chicken (days)	Virus dose (CID_{50})	Time after infection immuno-globulin inoculated (hours)	Chicken treatment							
			Bursectomized				Normal			
			anti-VR Ig		normal Ig		anti-VR Ig		normal Ig	
			Proportion affected*				Proportion affected*			
			Clinic	Histo.	Clinic	Histo.	Clinic.	Histo.	Clinic	Histo.
1	260	0	0/6	0/6	6/6	6/6	0/6	0/6	6/6	6/6
1	260	48	0/6	2/6	6/6	6/6	0/6	0/6	6/6	6/6
7	180	0	0/6	0/6	6/6	6/6	0/6	0/6	6/6	6/6
7	180	48	0/6	0/6	5/5	5/5	0/6	0/6	6/6	6/6
14	300	0	0/6	0/6	6/6	6/6	0/6	0/6	1/6	5/6
14	300	48	0/6	0/6	6/6	6/6	0/6	0/6	2/6	6/6

Ig - immunoglobulin

* Number affected/Number of chickens

Table 45

Mean NI of the sera of chickens inoculated IP with immunoglobulin (Ig)

Age of chicken (days)	Time after infection Ig given (hours)	Immunosuppressant treatment			
		Cyclophosphamide		Nil	
		Anti-VR Ig	Normal Ig	Anti-VR Ig	Normal Ig
1	0	0.60±0.45	0.41±0.27	0.88±0.80	1.42±0.56
1	48	0.86±0.21	0.64±0.39	0.40±0.39	1.35±0.50
7	0	0.74±0.22	0.27±0.22	0.74±0.22	1.61±0.48
7	48	0.91±0 58	0.28±0.42	0.45±0.29	1.55±0.38
14	0	0.81±0.33	0.31±0.28	0.21±0.48	2.51±0.60
14	48	0.76±0.21	0.35±0.21	0.11±0.33	2.40±0.41

Table 46

Persistence of IAEV immunoglobulin (Ig) in sera of chickens inoculated IP at one day of age

Type of Ig inoculated	Mean NI of sera		
	Time after inoculation sera obtained		
	7 days	14 days	21 days
anti-VR immunoglobulin	1.84±0.39	1.10±0.48	0.46±0.21
normal immunoglobulin	0.16±0.22	0.36±0.39	0.70±0.56

NI - neutralization index

Discussion

The results obtained in this chapter confirmed the influence of age on the development of IAE. Thus chickens became less susceptible to the development of clinical disease as they aged and by 21 days or older they failed to develop the disease after an oral challenge with the virus. However, there was histological evidence that the virus invaded the brain of some infected chickens in the 21, 28 and 35 day age group.

By contrast, chickens of all age groups that had been treated with either cyclophosphamide or testosterone succumbed to clinical IAE after a similar challenge with the virus. This suggested that the immune system had a role in the pathogenesis of IAE, as both cyclophosphamide and testosterone have been demonstrated to disrupt the immune mechanism and particularly the humoral immune system of the fowl (Meyer et al., 1959; Warner et al., 1969; Linna et al., 1972; Toivanen et al., 1972; Rouse and Szenberg, 1974).

The treatment of the chickens with the immuno-suppressants had an effect on hatchability and liveability. The hatchability declined approximately 25 percent following testosterone treatment of the eggs. Subsequently about 15 percent of testosterone treated chickens died from anatomical malformations of the cloaca and septicaemia. The mortality rate in the cyclophosphamide treated chickens was approximately 35 percent of all chickens treated. Most of this mortality occurred within seven days of the first cyclophosphamide inoculation. Although the total losses of chickens were similar in each group, testosterone treatment was used preferentially in the preparation of bursectomized chickens.

Testosterone appeared to have a less severe effect on morphology and function of the thymus and thymic dependent lymphoid system as judged by histological examination and the Simonsen assay.

Cyclophosphamide and testosterone had no detectable effect on the weight of the body, brain, and thymus of uninfected chickens. They also induced no detectable morphological change in the brain. However, both had a marked effect on the bursa of Fabricius, and caused morphological changes in the spleen and to a lesser extent in the thymus.

Chickens that had been treated with the immunosuppressants were unable to produce detectable levels of serum neutralizing antibody to IAEV within the test period. Disruption of the humoral responses by these compounds observed in this study is in agreement with the findings of Meyer et al. (1959), Warner and Burnet (1961),Glick and Sadler (1961), Svenberg and Warner (1962a), Warner et al. (1969), Seto (1970), Lerman and Weidanz (1970), Linna et al. (1972) and Toivanen et al. (1972).

Cyclophosphamide also had a temporary effect on cell mediated immune response. This was detected using the Simonsen assay on one, seven and 14 day old cyclophosphamide treated chickens. A temporary effect on the cellular immune responses of cyclophosphamide treated chickens have been described by Linna et al. (1972), Toivanen et al. (1972) and Rouse and Szenberg (1974). No such effect was caused by testosterone, although morphological changes in the thymus of testosterone treated chickens have been described (Warner et al., 1962; Pierce et al., 1966). However cellular immune responses remain virtually unimpaired in most testosterone treated chickens (Szenberg and Warner, 1962a).

Chickens that were treated with cyclophosphamide or testosterone and infected orally with IAEV developed the disease regardless of their age at the time of challenge and frequently developed the paralytic form of the disease. Paralysis caused the significant reduction in weight gain in these groups. This form of the disease was only observed in the youngest group of immunocompetent chickens. The other groups either developed mild disease or failed to show signs of it. Thus chickens of any age that had been treated with the immunosuppressants were as susceptible to the development of the disease as one day old normal chickens.

However, neither of the immunosuppressive techniques used were sufficiently selective to give unequivocal information on the role of the humoral immune system in the pathogenesis of IAE, particularly in the one, seven and 14 day age groups. To study the effect of a component of the immune system in the pathogenesis of a disease, it is useful to examine the selective restoration of part of the immune response in immunosuppressed animals. This can be undertaken by the inoculation of immunoglobulins, of immunoglobulin producing cells or the transfer of lymphoid cells of the thymic dependent immune system (Allison, 1972).

The intra-peritoneal inoculation of one ml. of an IAEV immunoglobulin preparation, that had a NI of 4.20 in its normal form,resulted in a neutralizing capacity persisting in the sera of chickens for at least 14 days after inoculation (see Table 46). This duplicated the persistence of IAEV maternal antibody (Calnek et al., 1961a; Matsukura, 1970). IAEV immunoglobulin inoculated in this way, into normal and immunosuppressed chickens, protected them against the development of IAE when challenged at

one, seven and 14 days of age. Further, this protective effect was induced in chickens inoculated IP with anti-VR immunoglobulin at the time of challenge with the virus or 48 hours afterwards. Histological evidence, however, suggested that the virus had invaded the brain of chickens treated with cyclophosphamide at one day of age and inoculated with the immunoglobulin 48 hours afterwards. Microscopic lesions were not found in the brain of any of the other chickens that received IAEV immunoglobulin, but were regularly found in the chickens that were administered normal immunoglobulin. These results suggested that IAEV immunoglobulin had an important role in protection against IAE. The serum neutralizing antibody not only prevented the invasion of the brain by the virus, but also appeared to have a dampening effect on the virus in the brain.

Evidence for the protective effect of neutralizing antibody is also implicit in earlier studies into IAE. Thus Sumner et al. (1957b) and Calnek and Jehnich (1959a) demonstrated the significance of circulating antibody to the virus in epidemiology of the disease. This explained the reason for the inconsistency of many of the earlier attempts in the transmission of the disease (Olitsky, 1939; van Roekel et al., 1939; Jungherr and Minard, 1942).

Circulating specific antibody has been demonstrated to be of significance in the pathogenesis of some other animal diseases. Zisman et al. (1971) showed the importance of antibody in protecting mice against yellow fever virus. Similarly antibody has been demonstrated to be of significance in systemic encephalomyocarditis, and Coxsackie B virus infections of mice (Murphy and Glasgow, 1967, 1968; Zisman and

Allison, 1971). Children with severe hypogammaglobulinaemia, but with intact cell mediated immunity, have also been shown to be more liable to develop paralytic poliomyelitis after exposure to vaccine strains than normal children (Schur et al., 1970).

In these infections it has been suggested that the neutralizing antibody protects by affecting the duration of viraemia and therefore the seeding and multiplication of the virus in the target organs (Glasgow, 1970; Allison, 1972).

If IAEV neutralizing antibody is important in the development of immunity to IAE, then the ability to produce this antibody must be important in the outcome of a challenge with the virus. Significant differences in the mean neutralization indices of the sera produced by chickens challenged at different ages were recorded in this study. The mean NI of the 21, 28 and 35 day old chickens were greater ($P<0.01$) than those of the one, seven and 14 day age group. Even though all of the normal age groups developed positive neutralization indices within the observation period, the speed of serological response could be of critical importance. In these experiments clinical IAE developed in the one, seven and 14 day old normal chickens, but not in the older birds, even though histological lesions of IAE were apparent in these birds. The sequential development of IAEV serum neutralizing antibody and its relation to the titre of the virus in the brains of normal and immunosuppressed chickens of various ages are investigated in the next chapter.

The germinal centre is a component of the bursal dependent lymphoid system (Payne, 1971), and therefore involved in humoral responsiveness. The challenge of chickens with IAEV resulted in a significant increase in the number of germinal

centres in cross sections of the spleen of normal chickens, except in the one day group. The high susceptibility of one day old chickens to IAE was associated with their relative inability to increase the number of germinal centres in the spleen after infection. This poor response may be related to an immaturity in the immunological system of the baby chicken. This immaturity would result in a poor antibody response to the virus and this permits seeding and multiplication of IAEV in the brain resulting in clinical disease. Further information in the antibody producing ability of young chickens could be obtained by qualitative and quantitative determinations of serum concentrations of IgM and IgG (Carey and Warner, 1964; Pierce et al., 1966; Warner et al., 1969; Linna et al., 1972) following challenge by specific antigens. Studies in the ontogeny of the immune system have so far shown that although chickens have some antibody producing capacity in the first week of life, full maturation of the immune system may not be attained until the second or third week of life (Solomon, 1971; Toivanen et al., 1972).

The role of local antibody in the gastro-intestinal tract in the pathogenesis of IAE was not studied. Orlans and Rose (1972) and Lebacq-Verheyden et al., (1972) have demonstrated a class of immunoglobulins analagous to mammalian IgA in chicken mucosal secretions. Parry and Aitken (1973) have demonstrated that the production of local antibody to NDV may be disrupted in chickens treated with cyclophosphamide. Local antibodies have been demonstrated to be significant in poliovirus infections (Keller and Dwyer, 1968) and their importance in IAE therefore requires clarification.

Similarly, the role of the reticulo-endothelial system was not investigated. If the functional efficiency of this system is depressed, impaired clearance of virus and lethal infections may result (Zisman et al., 1971). The role of the reticulo-endothelial system in the pathogenesis of IAE is an aspect of the disease that requires further investigation.

Summary

Chickens one, seven and 14 days of age developed IAE following an oral challenge with NSW-1 virus. Chickens 21, 28 and 35 days failed to develop clinical IAE, although some were affected sub-clinically. Chickens in all these age groups that had been treated with either cyclophosphamide or testosterone succumbed and developed severe IAE following a similar oral challenge.

Normal and immunosuppressed chickens that were inoculated intraperitoneally with an IAEV immunoglobulin preparation were protected against the development of the disease. This protection was conferred if the immunoglobulin was administered either at the time of challenge with the virus, or 48 hours following infection. Passive inoculation of immunoglobulin resulted in neutralizing antibody in the serum that persisted for at least 14 days.

These results suggested that the humoral immune system had a significant role in the pathogenesis of IAE. Differences in the ability to produce serum neutralizing antibody to the virus were detected between the different age groups. Normal chickens in the one, seven and 14 days age groups were inferior to the older chickens.

Chapter 3

VIRAEMIA, THE MULTIPLICATION OF THE VIRUS IN THE BRAIN AND THE DEVELOPMENT OF SERUM NEUTRALIZING ANTIBODY TO IAEV

Introduction

The results of work described in previous chapters demonstrated that serum antibody to IAEV had a significant protective effect in normal and in immunosuppressed chickens. These results suggested that the development of IAEV serum neutralizing antibodies was important in functional host immunity to the disease. In addition, young normal chickens appeared to be inferior in their capacity to produce antibody against IAEV, and this slower antibody response may have allowed the virus to invade and multiply in the central nervous system sufficiently to cause the disease.

In a number of viral infections of animals it has been observed that serum antibodies to a virus appear about the time that the titres of the virus are waning in the blood or target organs of the virus (Nathanson and Cole, 1971). The relationship between circulating neutralizing antibody to IAEV and the multiplication of the virus in the central nervous system have not been determined. Although sequential observations of this type in normal animals allow only weak inferences regarding the causal relationships, additional support can be provided by the investigation of these relationships in immunosuppressed animals (Allison, 1972).

The current concept of the pathogenesis of IAE is that the initial site of multiplication of the virus is in the gastrointestinal tract (Calnek et al., 1961b; Springer and Schmittle, 1968; van der Heide, 1970; Braune and Gentry, 1971b), but the

route(s) of invasion of the various target organs have not been determined. Viraemia was thought to occur (Olitsky, 1943) but has not been demonstrated (Olitsky, 1939; Braune and Gentry, 1971b). Lesions of IAE have not been observed in the peripheral nervous system and this could indicate that transmission along peripheral nerves does not occur (Luginbuhl and Helmboldt, 1972; Mohanty and West, 1973).

The aims of the investigations described in this chapter were to determine the relationship between the development of the disease, virus titre in the brain and production of IAEV serum neutralizing antibody. In addition, tests to detect viraemia were undertaken in an attempt to determine the significance of it in the pathogenesis of IAE.

Materials and Methods

Chickens. The chickens were hatched from WL/C flock.

Fertile eggs. The fertile eggs used in the virus-serum neutralization tests were obtained from WL flock B and C.

Virus. Van Roekel isolate of the virus was used. The titre of this preparation was $10^{6.1}$ EID_{50} per ml. Chickens were challenged intra-muscularly with an estimated 100 EID_{50} of the virus. The actual dose of virus used was subsequently titrated.

Virus titration. This was performed according to the technique described in the General Materials and Methods for viruses adapted to grow in the chicken embryo.

Virus-serum neutralization tests. These were undertaken by the method described in the General Materials and Methods. The van Roekel virus was used as the antigen in these tests.

Testosterone treatment. Chicken embryos were treated with testosterone as described previously (Part 2, Chapter 2). The efficiency of bursectomy in individual chickens was determined by histological examination of the bursa of Fabricius at post mortem, and by determination of the NI for IAEV. Control chickens were also prepared in the way previously described.

Regional distribution of the virus in the brain. Fourteen susceptible chickens were challenged IM at one day of age with an estimated 100 EID_{50} of VR virus, and an additional 20 chickens of the same source and age were challenged with a ten percent brain suspension from non IAEV infected chicken embryos. Two chickens from each group were killed at 48 hour intervals after challenge and the brain removed using a sterile technique. The brain of each chicken was then divided into three portions - (1) cerebellum, (2) forebrain, (3) hind brain. The relevant portions of the brains of both chickens were pooled and a 10 percent suspension (w/v) was prepared. The suspension was then stored at $-30^{o}C$. The virus content was later determined by titration in chicken embryos.

Viraemia, titration of the virus in the brain and estimation of serum neutralizing antibody to IAEV. The birds and treatments used to determine the distribution of virus after infection are shown in Table 47. Serial serum samples were taken from these birds and tested for neutralizing antibody to IAEV.

Fifty of the testosterone treated chickens in the 21 day age group were inoculated intra-peritoneally with immunoglobulin preparations at the time of challenge with the virus. Twenty five were inoculated with the IAEV immunoglobulin and 25 with

the immunoglobulin preparation containing no IAEV neutralizing antibody (see Part 2, Chapter 2).

Table 47

Number and age of chickens challenged, and treatments applied

Age at challenge (days)	Number of chickens challenged		
	Virus		Control
	Normal	Bursectomized	
1	37	40	37
7	33	-	33
21	33	90	33

Chickens in each group were culled for virological and serological examination on alternate days. Four chickens in each of the groups challenged at one day of age were killed for the first seven days. Subsequently only three were killed. Three chickens in the seven and 21 day old groups, except the birds inoculated with immunoglobulins, were killed on alternate days. In the groups inoculated intra-peritoneally with immunoglobulin, only two chickens were killed from each group.

A serum sample was obtained from each chicken by decapitation. The NI of the individual serum samples was determined and the mean NI computed from the results. Pre-inoculation serum samples were only obtained from the 21 day age group. Serum samples from chickens of the same age, and the same immuno-competence were used to provide a pool of IAEV negative serum for use in the serum neutralization tests for the one and seven day age groups.

The brain of each chicken was extruded through the foramen

magnum and a ten percent suspension (w/v) of the pooled brains of the chickens of each test group prepared. These suspensions were stored at -30°C until it was used in a titration of the virus infectivity.

The serum samples from the individual chickens were also used for virus isolation procedures. 0.1 ml. of serum was inoculated into the yolk sac of five embryonating eggs seven days of age. The eggs were incubated at 37°C for 11 days, and then opened and examined for lesions of IAE.

Results

Regional distribution of the virus in the brain

The virus was present in all portions of the brain and at similar concentrations within 5 to 7 days of challenge (Table 48). The whole brain was therefore used in subsequent tests on the invasion of the brain by the virus.

Table 48

Distribution of virus in chickens brains after infection with IAEV*

Days after challenge	Virus content $\log_{10}$ EID_{50} per ml.		
	Forebrain	Cerebellum	Hindbrain
1	0.0	0.0	0.0
3	0.0	0.0	0.0
5	2.55±0.18	2.45±0.43	1.10±0.35
7	3.60±0.17	2.98±0.24	3.45±0.30
9	4.73±0.31	4.05±0.35	4.17±0.22
11	5.06±0.22	4.01±0.16	4.78±0.26
13	3.14±0.24	3.17±0.40	3.43±0.34

* No virus was isolated from uninfected, control chickens

Titre of the virus in the brain of chickens of different ages

Clinical IAE was seen on the ninth day after infection in the normal chickens challenged at one and seven days of age. The 21 day old normal chickens failed to develop the disease.

The testosterone treated chickens developed IAE on the tenth and eighth day after infection in the one and 21 day age group respectively. The testosterone treated group that was inoculated IP with anti-VR immunoglobulin did not develop clinical signs of IAE. Clinical IAE developed in the testosterone group inoculated IP with normal immunoglobulin on the tenth day after infection.

The clinical disease consisted initially of inco-ordination and wing drooping. These signs progressed to paralysis in the one day old infected chickens and in the testosterone treated chickens of one and 21 days of age.

IAEV was recovered from the brain suspensions of all the groups challenged with the virus, except those that were inoculated IP with anti-VR immunoglobulin. However, the virus was not recovered until the fifth day after infection.

The older the normal chickens were at the time of challenge with the virus, the lower was the titre detected in their brain suspensions. The virus was also recovered for a shorter time from the tissues of the 21 day, than the one and seven day groups.

The testosterone treated chickens did not exhibit this pattern. The titre and persistence of the virus in the brain suspensions was similar in the one and 21 day age groups throughout the test period.

Virus was isolated from the brain of the chickens inoculated with the normal immunoglobulin. The titre and persistence of virus in the brain suspensions of these chickens was similar to that of the 21 day old testosterone treated group.

The titre of virus in the brain suspensions obtained during this study are recorded in Table 49.

Viraemia. The virus was detected in the sera of all the groups challenged with the virus, except the chickens inoculated IP with IAEV immunoglobulin.

In the normal infected chickens, viraemia persisted longer in the one day old group. This group also showed the severest clinical disease. The period during which viraemia was detectable decreased in the groups challenged at seven and 21 days of age.

Viraemia persisted longer in the testosterone treated groups but virus was not detected in the sera of the testosterone treated chickens that were inoculated IP with anti-VR immunoglobulin. The viraemia in the testosterone treated group inoculated IP with normal immunoglobulin persisted throughout the test period (Table 50).

Development of neutralizing antibody to IAEV

The mean neutralization indices (NI) of the sera from the different age group is recorded in Table 51.

IAEV serum neutralizing antibody was not detected in control uninfected chickens, nor in the infected chickens treated with testosterone.

Specific neutralizing antibodies were detected in the sera

Table 49

Titre of virus in the brain of the chickens challenged at different ages with the van Roekel virus

Days after challenge	Virus titre in the brain suspension									
	Age at challenge									
	Day 1			Day 7		Day 21				
	Normal	Bursect.	Control	Normal	Control	Normal	Bursect.	Bursect. + anti-VR Ig	Bursect + normal Ig	Control
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	2.90±0.24	2.40±0.28	0.00	2.94±0.50	0.00	2.85±0.44	2.56±0.54	0.00	1.95±0.62	0.00
7	3.35±0.24	2.96±0.44	0.00	3.36±0.28	0.00	2.97±0.74	3.20±0.38	0.00	2.85±0.44	0.00
9	4.00±0.34	3.72±0.41	0.00	3.94±0.35	0.00	1.30±0.44	3.62±0.52	0.00	3.50±0.36	0.00
11	4.64±0.41	5.10±0.36	0.00	1.90±0.74	0.00	0.00	4.40±0.76	0.00	4.85±0.41	0.00
13	3.37±0.24	5.30±0.20	0.00	2.64±0.41	0.00	0.00	4.62±0.48	0.00	5.28±0.27	0.00
15	2.10±0.27	5.14±0.38	0.00	1.85±0.27	0.00	0.00	4.75±0.54	0.00	4.98±0.36	NT
17	1.76±0.64	5.55±0.44	0.00	0.00	0.00	0.00	4.16±0.38	0.00	5.25±0.74	NT
19	0.97±0.52	6.34±0.27	0.00	NT	0.00	NT	5.00±0.36	0.00	5.80±0.34	NT
21	0.65±0.35	5.15±0.40	0.00	NT	0.00	NT	NT	0.00	5.64±0.38	NT

Bursect. - Bursectomized, Ig - Immunoglobulin, NT - Not tested

* $\log_{10}$ EID_{50} per ml. SD

Virus dose Day 1 - 180 EID_{50}

Day 7 - 245 EID_{50}

Day 21 - 320 EID_{50}

Table 50

Proportion of chickens developing viraemia

Age at challenge	Chicken treatment	Proportion of chickens with viraemia										
		Days after challenge										
		1	3	5	7	9	11	13	15	17	19	21
One day	Nil	2/4*	4/4	4/4	4/4	2/3	1/3	0/3	0/3	NT	NT	NT
	Bursectomy	3/4	4/4	4/4	4/4	NT	3/3	3/3	NT	3/3	NT	NT
	Control	0/4	0/4	0/4	0/4	0/3	0/3	0/3	NT	NT	NT	NT
Seven days	Nil	2/3	3/3	3/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	Control	0/3	0/3	0/3	0/3	0/3	0/3	0/3	NT	NT	NT	NT
21 days	Nil	1/3	3/3	2/3	0/3	0/3	0/3	0/3	0/3	NT	3/3	NT
	Bursectomy	2/3	3/3	3/3	3/3	3/3	3/3	NT	3/3	NT	3/3	NT
	Bursectomy + anti-VR Ig	0/3	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	Bursectomy + normal Ig	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
	Control	0/3	0/3	0/3	0/3	0/3	0/3	NT	NT	NT	NT	NT

NT - not tested

* - Number sera virus isolated from/No. examined

Bursectomy - Testosterone in ovo

Table 51

Development of IAEV serum neutralizing antibody in chickens challenged at different ages

Days after challenge	Age of chickens at time of challenge									
	One day			Seven days		21 days				
	Normal	Bursect.	Control	Normal	Control	Normal	Bursect.	Bursect. + anti-VR Ig	Bursect. + normal Ig	Control
1	0.80*	0.10	0.30	0.40	1.00	0.00	0.10	1.20	0.20	0.26
3	0.60	0.00	0.50	0.60	0.40	0.40	0.40	1.40	0.10	0.31
5	0.30	0.40	0.00	0.40	0.40	0.40	0.50	1.84	0.30	0.48
7	0.80	0.30	0.10	1.00	0.30	1.40	0.00	2.10	0.40	0.10
9	0.40	NT	0.20	0.80	0.10	1.60	0.30	1.40	0.10	0.00
11	1.10	0.70	0.40	1.40	0.20	1.80	0.80	0.80	0.60	0.16
13	1.80	0.40	0.30	1.00	0.00	2.80	NT	0.75	0.20	NT
15	2.00	NT	NT	1.90	NT	3.00	0.50	0.80	0.35	NT
17	NT	0.00	NT	1.80	NT	NT	NT	0.44	0.28	NT
19	NT	NT	NT	2.80	NT	NT	0.20	0.92	0.14	NT
21	NT	NT	NT	2.40	NT	NT	NT	0.42	0.00	NT

Bursect - bursectomy with testosterone

Bursect + anti-VR Ig - bursectomy and IAEV immunoglobulin IP

Bursect + normal Ig - bursectomy and normal immunoglobulin IP

NT - not tested

*NI - Neutralization index - $\log_{10}x$.

of all the normal infected groups of chickens. These were first detected on the eleventh day following challenge in the day one group, on the eleventh day in the seven day group and on the seventh day after infection in the 21 day group.

Serum neutralizing antibody to the virus was also detected for up to nine days after the IP inoculation of anti-VR immunoglobulin in testosterone treated chickens. No neutralizing capacity was found in the sera of the control chickens of this group.

The relationship between the titre of the virus in the brain, viraemia and the development of IAEV serum neutralizing antibody is shown in Table 52.

Table 52

Relationship between virus titre in the brain, viraemia and IAEV serum neutralizing antibody

Factors examined	Age at challenge		
	1	7	21
Time after challenge at first decrease of virus in brain	13*	11	9
Duration of detectable viraemia	11	7	5
Time after challenge, antibody first detected	11	11	7

* days after challenge

Discussion

The association between the age of the chicken at the time of infection with the virus and its susceptibility to the development of clinical IAE was again demonstrated. One day old normal chickens developed the paralytic form of IAE, whereas

the 21 day old birds failed to develop clinical signs of the disease.

The development of the clinical disease was associated with the isolation of the virus at a higher titre, and for longer periods, from the brain of the infected chickens. The virus was first isolated five days after infection in both the one and 21 day old groups. It was subsequently isolated for 10 more days in the one day group, but for only four further days in the 21 day old birds. Thus the shorter period of recovery was related to an earlier inhibition of the multiplication of the virus in the brain of the older birds.

The cessation of detectable viraemia also occurred earlier in the older birds. The virus was isolated from the sera of the one day old birds for 11 days after infection, but for only five days in the 21 day old group.

These results indicated that there was an association between the age of the chicken at the time of infection, the development of clinical IAE, the duration of viraemia, the titre the virus attained and the persistence of IAEV in the brain.

This pattern was not however seen in the testosterone treated chickens. Both the one and 21 day old testosterone treated chickens developed the paralytic form of the disease. The virus was isolated from the brain of the infected birds throughout the test period, and there was no decline in the titre of the virus in the brain. Viraemia was also detectable throughout the test period. The treatment of chickens with testosterone removed the age effect observed in chickens on the multiplication of the virus in the brain, the persistence

of viraemia and the development of clinical IAE.

Testosterone treatment appeared not to affect the early stages of infection of the disease, but rather the later stages, when inhibition of virus multiplication occurred. Virus titres in the brain of the normal and testosterone treated chickens were similar for the first seven to nine days. In the normal chickens there was a decline in virus titres after this time, whereas the titre of virus continued to rise or persisted at this level in the testosterone treated birds. This suggested that testosterone treated did not affect the initial virus - host interaction.

In all the normal chicken groups, the waning of virus in the brain and the termination of viraemia coincided with the development of serum neutralizing antibodies to the virus. These antibodies were detected earlier in the 21 day age group, the group which showed the shortest period of detectable viraemia and the earliest decline in virus titre in the brain. The significance of the association of antibody, virus multiplication in the brain and viraemia was demonstrated in the testosterone treated chickens. These chickens failed to produce antibody to the virus, and as a result there was no inhibition of virus multiplication. When IAEV immunoglobulin was inoculated however, the testosterone treated chickens failed to develop the disease, and the virus could not be isolated from the brain or sera. Specific neutralizing antibody to IAEV was detected in the sera of chickens inoculated IP with IAEV immunoglobulin for up to nine days after inoculation. Normal immunoglobulin inoculated in the same way neither protected chickens against the disease, nor prevented virus isolation from the brain or sera. The

titres of virus attained, the persistence of virus in the brain, as well as the duration of viraemia in testosterone treated chickens inoculated IP with normal immunoglobulin resembled those obtained in the chickens treated with testosterone only.

These results suggested that circulating neutralizing antibody to the virus were an important component in functional host immunity to IAE. The development of IAEV neutralizing antibody may also be important in the pathogenesis of the disease. The significant role of circulating antibody in the pathogenesis and immunity to a disease has been demonstrated in a number of diseases. Protection against the development of the disease in immunosuppressed mice inoculated with specific neutralizing antibody has been described for encephalomyocarditis (Murphy and Glasgow, 1967, 1968), Coxsackic B (Zisman and Allison, 1971) and in yellow fever virus infections (Zisman et al., 1971). Evidence for the role of antibody in the pathogenesis of poliomyelitis infection of man has been provided by Schur et al. (1970).

The varying humoral responses of several age groups of chickens that have been described in previous chapters suggest that antibody responses are important in the pathogenesis of IAE. In this chapter specific serum neutralizing antibody to IAEV was detected four days earlier in the 21 day age group than in the one or seven day group. This slower antibody response in younger chickens may allow the virus to invade and multiply in the brain sufficiently to cause disease. The concept that an acute primary viral infection can be conceived as a race between the virus and the host's defences is an old one (Lennette and Koprowski, 1944) but is still considered

useful in discussing the pathogenesis of some diseases (Nathanson and Cole, 1971). The poor humoral responsiveness of young chickens has been demonstrated in a number of studies. Paterson et al. (1962) found that the amount of γ globulin synthesis was low during the first two weeks after hatching, but that the rate of synthesis increased by the end of the first month of life, as shown by a rise in serum γ globulin levels. Results emphasising the inferior antibody production of young chickens have also been recorded by Wolfe and Dilks (1948), Keeble and Wade (1963) and Toivanen et al. (1972).

The importance of serum neutralizing antibody in protection against IAEV may be attributed to the occurrence of viraemia during the disease. It is generally believed that the initial site of multiplication of the virus in the chicken is in the alimentary tract (Calnek et al., 1961b), and that invasion of the central nervous system occurs subsequently (Springer and Schmittle, 1968; van der Heide, 1970; Braune and Gentry, 1971b),but the exact method of this invasion is unknown. The viraemia found in this study may be important in the dissemination of the virus to secondary or tertiary sites of virus multiplication. Viraemia has not been previously described in IAE. Although the viraemia was induced in this study following an intra-muscular challenge with van Roekel virus, it is also likely that it occurs in natural outbreaks of the disease. The significant protective effect of serum neutralizing antibody in chickens challenged by the oral route with the NSW-1, demonstrated in previous chapters would suggest that viraemia also occurred with this isolate. The failure to demonstrate viraemia by some other workers (Olitsky, 1939) may have been related to the timing

of the attempt. The appearance of the serum antibody response is such, that evidence of viraemia must be sought prior to the development of the clinical diesease. Similarly in the isolation of the virus, chickens that have just developed clinical signs of the disease should be used. In the one day old chickens, the clinical disease developed nine days after infection and the virus titre declined on the 13th day. In the seven day old group, IAE again developed on the ninth day after infection and the virus titre commenced to fall on the 11th day.

The viraemia detected in this study may not be the only route of dissemination of the virus within the bird. Experimental work has demonstrated that polioviruses are capable of spreading along nerve fibres in the peripheral as well as the central nervous system of monkeys. This occurred following intra-nasal, intra-muscular and intra-sciatic inoculation (Fairbrother and Hurst, 1930; Bodian and Howe, 1940). Such spread however has not been conclusively demonstrated from the alimentary tract (Bodian and Horstmann, 1965). An examination of whether transmission of IAEV along peripheral nerves to the central nervous system, is important in the pathogenesis of the disease would provide a further insight into IAE.

Summary

Sequential studies into the development of IAE demonstrated an association between the clinical disease, the titre and the persistence of the virus in the brain, the duration of viraemia, the detection of serum neutralizing antibody and the age of the chicken at the time of infection.

The older the chicken at the time of challenge with the virus, the milder the disease, the lower the virus titre in the brain, the shorter the viraemia, and the earlier the detection of serum neutralizing antibody.

Treatment with testosterone prevented the development of this decreased susceptibility in the older chickens, by preventing antibody production. The effect of testosterone could be overcome by the intraperitoneal inoculation of IAEV immunoglobulin.

These results suggested that serum neutralizing was important in functional host immunity to IAE and in the pathogenesis of the disease. The higher susceptibility of young chickens to the development of the clinical disease could be related to an immaturity in the humoral immune system of the neonatal chicken. This immaturity results in a slower serological response to the virus infection and the development of IAE.

Chapter 4

THE EFFECT OF IAEV MATERNAL ANTIBODY ON THE DEVELOPMENT OF IAE

Introduction

Many of the early attempts to transmit IAE were only partially successful (Jones, 1934; Jungherr and Minard, 1942). Similarly many attempts to cultivate the virus in embryonating eggs were unsuccessful (van Roekel et al., 1938; van Roekel et al., 1939; Feibel et al., 1952). One of the reasons for these sporadic results may have been the interference caused by maternal antibodies to IAEV. Sumner et al. (1957a, 1957b) were the first workers to recognize this important effect. Subsequently it became essential to use chicken embryos and chickens that were free of neutralizing antibody to the virus in the study of the disease.

However, most chickens hatched from commercial hatcheries possess maternal antibodies to the virus, as IAEV infection of breeding flocks is common. Chickens under ten weeks of age that receive maternal antibodies show an inhibited or delayed serological response to challenge with the virus. This occurs even though serum antibodies may not be detectable after four weeks of age (Calnek et al., 1961a). This factor has resulted in the vaccination of chickens for IAE being delayed until the chickens are 12 to 14 weeks of age. Chickens with maternal antibody to IAEV have also shown diminished secretion of the virus in the faeces (Calnek et al., 1960), and the effectiveness of oral vaccination for IAE depends, in part, on the natural transmission of the virus in the faeces.

Maternal antibody to IAEV is, therefore, of importance in the

prevention of the disease in the susceptible neonate, and has practical implications in the development of vaccination programmes using an orally administered vaccine.

The aim of the work described in this chapter was to study the persistence of maternal antibody in chickens and of passive immunity to IAE and to relate this to the subsequent development of active immunity.

Materials and Methods

Chickens. The susceptible chickens used were of the WLxA breed.

The immune chickens were obtained as one day old chickens from a poultry farm known to be infected with IAEV. These chickens were of the WRxNH breed.

Eggs. The fertile eggs used in the virus-serum neutralization tests were from WL flock, B, C and D.

Virus. NSW-1 isolate of IAEV was used in the oral challenge of chickens. The virus was prepared as a 10 percent suspension (w/v) of brain from the second passage of the virus in chicken embryos.

Van Roekel isolate was used in the virus-serum neutralization tests, and in the intra-cerebral challenge of birds.

Virus-serum neutralization test. This test was performed using the variable virus constant serum technique described in the General Materials and Methods.

Persistence of IAEV maternal antibody. Eighty susceptible and 120 immune one day old chickens were identified

by wing banding and then reared together. Five chickens from both the susceptible and the immune groups were bled at weekly intervals. The chickens in the groups bled at one day and seven days of age were exsanguinated, while those in the 14, 21, 28, 35 and 42 day age groups were bled from the brachial vein. The neutralization indices of the individual sera were determined and the result of the age group expressed as the mean neutralization index. If insufficient serum was obtained to perform a neutralization test, the serum was diluted two fold.

Ten chickens from the immune group, and five susceptible chickens of the same age as those from which the serum sample was obtained were then challenged intra-cerebrally with an estimated 100 EID_{50} of van Roekel virus. The actual dose used was subsequently titrated.

The challenged chickens were kept separated from the unchallenged birds and were observed for 28 days. These chickens were examined daily for the onset of clinical IAE, both when resting and when agitated by the observer.

Serological response of chickens at various ages. Ten susceptible and 10 immune chickens were challenged per os with an estimated 100 CID_{50} of NSW-1 virus at 14, 28, 42 and 56 days of age. The actual virus dose used was titrated using the technique described for viruses not adapted to grow in the chicken embryo.

Pre-challenge serum samples were obtained from all chickens by bleeding from the brachial vein. The serological response of the chickens was assessed five weeks after challenge.

Excretion of IAEV in the faeces of chickens of various ages

Ten immune and five susceptible chickens were challenged per os with an estimated 100 CID_{50} of the NSW-1 virus. Chickens were challenged at one, seven, 14 and 21 days of age. The dose of virus used was titrated. The chickens were kept until they were comatose, or for 42 days after challenge. They were then bled. The various age groups, and the immune and susceptible groups were kept on different premises.

The chickens were kept in wire cages, and the faeces collected for 21 days after challenge. The faeces fell onto fresh, clean paper placed under the cages. The faeces were harvested on alternate days. A ten percent suspension (w/v) of a random portion of the faeces was prepared, labelled and then stored at $-30^{o}C$.

Each faecal preparation was used to dose five susceptible one day old chickens per os. These birds were examined daily for 28 days after challenge for the development of IAE. The groups of chickens challenged were kept isolated from one another. The equipment used to house birds was disinfected between different tests.

Development of IAE in bursectomized chickens of different ages

Immune and susceptible chickens were bursectomized by treatment with testosterone as described previously (Part 2, Chapter 2). Control chickens were treated with corn oil. Ten immune testosterone and corn oil treated chickens were challenged with an estimated 100 CID_{50} of the NSW-1 virus per os. Ten immune testosterone and corn oil treated chickens were challenged with a normal chicken embryo brain suspension. Chickens were challenged at one, seven, 14, 21, 28, 35 and 42 days of age.

Five susceptible, testosterone treated chickens were challenged at seven, 14, 21, 28 and 42 days of age. Five corn oil treated, susceptible chickens were challenged at one, seven, 14, 21, 28 and 35 days of age. The control chickens were similarly challenged with a non virus containing suspension.

The chickens were observed for 28 days after challenge for the development of IAE. A pre-inoculation blood sample was obtained from 21, 28, 35 and 42 day old birds. The chickens were bled and killed at the end of the test period. The bursa of Fabricius of each chicken was fixed in 12.5 percent formal saline and then examined histologically.

Results

Persistence of maternal antibody to IAEV. The NI of the sera of the susceptible chickens were consistently negative (ie. NI<1.10). Positive group neutralization indices were detected in the one and seven day old immune groups. Positive NI were found in the sera of some of the birds in the 14 and 21 day age groups, although the mean NI of these two groups was negative. IAEV maternal antibody was not detected in any birds after 21 days of age.

All of the susceptible groups of chickens reacted to the intra-cerebral virus challenge, although not all birds in each group succumbed to clinical disease. By contrast, the immune chickens showed more resistance, particularly up to 21 days of age. After this age, these chickens became more susceptible to the disease. The increased susceptibility to virus challenge associated with the decline in the mean NI (Table 53). The high incidence of clinical disease in the IC challenged birds

Table 53

Persistence of maternal antibody to IAEV

Age of chicken (days)	Mean NI		VR virus challenge dose (EID_{50})	Proportion with clinical IAE	
	Immune group	Susceptible group		Immune group	Susceptible group
1	1.95 ± 0.30	0.30 ± 0.35	98	1/9*	4/5
7	1.60 ± 0.34	0.15 ± 0.25	120	1/10	5/5
14	1.08 ± 0.57	0.36 ± 0.24	300	3/10	4/4
21	0.60 ± 0.48	0.18 ± 0.14	88	5/10	5/5
28	0.46 ± 0.38	0.18 ± 0.26	200	9/10	5/5
35	0.16 ± 0.40	0.24 ± 0.21	150	8/10	4/5
42	0.16 ± 0.26	0.22 ± 0.20	380	7/10	4/5

NI - Neutralization index

EID_{50} - Embryo infective dose

* - Number sick/number of birds

was probably related to the use of WLxA and WRxNH chickens. Heavy birds are more susceptible to IAE (Feibel et al., 1952).

Serological response of chickens at various ages

The mean NI of the sera from chickens that had maternal antibody was lower ($P<0.01$) than the mean NI of the susceptible chickens. The neutralization indices of the sera obtained from the susceptible chickens challenged at 28, 42 and 56 days of age were greater than that of the 14 day old susceptible group. The mean NI of the 56 day old immune group was greater ($P<0.05$) than the 42 day group, the 28 day group ($P<0.01$) and the 14 day group ($P<0.01$). The mean NI of the 42 day group was greater than that of the 14 day old birds ($P<0.01$). The mean of the 28 day group was greater than the mean NI of the 14 day old chickens ($P<0.05$) (Table 54).

Table 54

The NI of the sera from immune and susceptible chickens challenged with the virus at various ages

Age at challenge (days)	Immune status	Challenge dose (CID_{50})	Mean NI
14	Susceptible	300	1.54 ± 0.47
	Immune		0.72 ± 0.39
28	Susceptible	260	2.41 ± 0.21
	Immune		1.02 ± 0.56
42	Susceptible	180	2.49 ± 0.39
	Immune		1.38 ± 0.21
56	Susceptible	350	2.93 ± 0.34
	Immune		1.79 ± 0.29

CID_{50} - chicken infective dose

NI - neutralization index, measured 35 days after challenge

Excretion of IAEV in the faeces of chickens

The number of chickens in the birds challenged with the NSW-1 virus that developed IAE is recorded in Table 55. The mean neutralization indices of the chickens is also recorded.

Table 55

Responses of immune and susceptible chickens to *per os* challenge with NSW-1 virus

Virus challenge dose (CID_{50})	Age at time of challenge (days)	Proportion of chickens with IAE		Mean NI (42 d. post challenge)	
		Immune Status			
		Immune	Susceptible	Immune	Susceptible
280	1	$0/_{10}$*	$5/_{5}$	0.20±0.42	1.48±0.20
350	7	$0/_{10}$	$5/_{5}$	0.48±0.21	1.60±0.54
200	14	$0/_{10}$	$3/_{5}$	0.85±0.48	1.80±0.42
390	21	$0/_{10}$	$0/_{5}$	1.76±0.38	2.01±0.36

CID_{50} - chicken infective doses

* - number sick/number of birds

The chickens challenged with the faecal suspensions collected from the one and seven day old immune groups failed to develop the disease.

By contrast, chickens challenged with the faecal suspensions from the susceptible birds of the same age developed IAE. Thus, the disease developed in the chickens challenged with the faeces collected from three to 17 days after challenge in the day one group, and three to 13 days after infection in the seven day group.

IAE developed in the chickens challenged with the faeces collected from both immune and susceptible chickens in the 14 and 21 day old age groups. The disease was, however, detected

for a shorter period in the groups challenged with the faecal suspensions from the immune chickens (Table 56).

Table 56

Infectivity of the faeces of chickens dosed per os with IAEV

Age at time of challenge (days)	Immune status	Duration of virus excretion after challenge
1	I	NIL 0
	S	Day 3 to Day 17 = 15 days
7	I	NIL 0
	S	Day 3 to Day 13 = 11 days
14	I	Day 5 to Day 9 = 5 days
	S	Day 3 to Day 9 = 7 days
21	I	Day 7 to Day 9 = 3 days
	S	Day 3 to Day 7 = 5 days

I - chickens with maternal immunity

S - chickens free of maternal immunity

Development of IAE in immune and susceptible, bursectomized chickens to challenge with NSW-1 virus

The number of chickens in each group that showed clinical IAE after an oral challenge with IAEV is recorded in Table 57.

The clinical disease was not observed in the corn oil treated, immune chickens challenged with the virus at any of the ages. By contrast, IAE developed in the corn oil treated susceptible birds, although only in the one, seven and 14 day age groups.

Table 57

Clinical and serological responses of chickens after per os challenge with the NSW-1 virus

Age of chickens at challenge (days)	Virus challenge dose (CID_{50})	Number of chickens with clinical IAE				Mean NI			
		Immune		Susceptible		Immune		Susceptible	
		Bursectomy	Normal	Bursectomy	Normal	B	N	B	N
1	500	$0/_{10}$ *	$0/_{10}$	NT	$5/_{5}$	0.42±0.11	0.24±0.36	NT	1.20±0.40
7	362	$0/_{10}$	$0/_{10}$	$4/_{5}$	$5/_{5}$	0.18±0.23	0.40±0.25	0.39±0.48	1.48±0.20
14	820	$1/_{10}$	$0/_{10}$	$5/_{5}$	$3/_{5}$	0.10±0.24	0.68±0.28	0.28±0.24	1.80±0.42
21	833	$3/_{10}$	$0/_{10}$	$5/_{5}$	$0/_{5}$	0.84±0.48	0.87±0.24	0.68±0.30	2.40±0.28
28	634	$4/_{10}$	$0/_{10}$	$4/_{5}$	$0/_{5}$	0.38±0.20	1.55±0.36	0.10±0.40	2.68±0.48
35	770	$6/_{10}$	$0/_{10}$	NT	$0/_{5}$	0.30±0.28	1.24±0.28	NT	3.10±0.56
42	764	$7/_{10}$	$0/_{10}$	$4/_{5}$	NT	0.24±0.48	1.36±0.21	0.68±0.40	NT

B - Bursectomy

N - Normal

NT - Not tested

CID_{50} - chicken infective dose

* Number sick/number challenged

Uninfected, control chickens did not develop IAE

The bursectomized, immune chickens developed clinical IAE in the 14, 21, 28, 35 and 42 day age groups, although not all of the birds in these groups succumbed. All of the groups of bursectomized susceptible chickens developed IAE.

Chickens in the bursectomized groups failed to develop serum neutralizing antibodies to IAEV. Chickens in the corn oil treated infected, groups usually developed these antibodies. The immune chickens in the one, seven, 14 and 21 day age groups failed to produce detectable levels of neutralizing antibody. All chickens in the uninfected, control groups failed to develop IAE or an antibody response to the virus.

Discussion

The findings of this work were in agreement with the observations of other workers (Calnek et al., 1961a; Matsukura, 1970) that IAEV maternal antibody provided protection against the disease. This protective effect was demonstrated by the IC challenge of chickens with the VR virus, and by the dosing of normal and bursectomized chickens with the NSW-1 virus. The protective effect however, waned with increasing age. The older chickens that received maternal antibody to IAEV were, at the time of IC challenge, the more that succumbed to clinical IAE (see Table 53). However chickens that were challenged orally with the virus did not exhibit this trend (see Table 55), probably because of the development of an age associated resistance in the normal chickens. The effect of testosterone in disrupting this resistance was demonstrated in both susceptible and immune chickens (see Table 57). The increase in the susceptibility of the older chickens to the development of IAE was associated with a decline in maternal antibody to the virus. This finding again

demonstrated the association between immunity to IAE and the presence of serum neutralizing antibody to the virus. Evidence for this relationship presented in this thesis includes (i) the immunity to IAE of chickens with maternal antibody to the virus, (ii) protection of birds inoculated intraperitoneally with specific immunoglobulin, (iii) the resistance of chickens capable of producing their own antibody and (iv) by the inability of bursectomized chickens to develop resistance.

Maternal antibody to the virus not only protected chickens against challenge with the virus, but also affected the excretion of the virus in the faeces and the serological responsiveness of chickens.

The presence of IAEV in faeces of chickens was demonstrated by the development of the disease in chickens given faeces per os. Thus, the virus was demonstrated in the faeces of chickens infected at one, seven, 14 and 21 days of age. The duration of the excretion of the virus, however, decreased as the chickens became older (see Table 56). Thus, the decreasing susceptibility of the chickens to the development of the disease, was also associated with a shorter period of detectable excretion of the virus in the faeces. A detailed study of the excretion of avian adenoviruses in the faeces (Clemmer, 1972) showed a similar reduction in faecal excretion between chickens one and 14 days of age. Kincade and Cooper (1971) and Clemmer (1972) have suggested that this reduction may be associated with maturation of immunoglobulin producing cells in the gut. The local production of antibody has been demonstrated in human poliovirus infections (Keller and Dwyer, 1968). The duration of excretion of the virus in the faeces in chickens ten to 15 weeks of age has not been described. This is of practical

significance in the vaccination of chicken flocks, particularly when using procedures that depend upon the natural transmission of the virus within the flock.

Maternal antibody to IAEV had a significant effect on the excretion of virus in the faeces. IAEV was not demonstrated in the faeces of the immune chickens challenged at one and seven days of age. It was detected, however, in the faeces of 14 and 21 day old immune chickens. Also the virus was recovered from the faeces of immune 14 and 21 day old chickens for a shorter period than from susceptible chickens of the same age. The shorter period of detection of virus excretion in the faeces in the maternally immune chickens may have resulted in less stimulation of the immune system, because the antibody response in these birds was lower.

The antibody response of the maternally immune chickens was demonstrated to be weaker than that of the susceptible chickens in the 14, 28, 42 and 56 day age groups. This effect persisted through to 56 days of age, even though IAEV maternal antibody were not found in any chicken after 21 days of age. Age groups of chickens in which maternal antibody was found, failed to produce detectable levels of IAEV neutralizing antibody (see Tables 55 and 57). Calnek et al. (1961a) also found that chickens with detectable levels of maternal antibody at the time of challenge failed to produce significant levels of IAEV serum neutralizing antibody. Similar poorer immunological responses in chickens that received maternal antibodies to other viruses have been described (Lancaster, 1964; Higgins, 1971). The persistence of this inferior response beyond the time that maternal antibody to a virus can be detected may be related to the sensitivity of the technique used to detect the antibody. In this study maternal antibody was not detected after 21 days

of age. Calnek et al. (1961a) demonstrated it four weeks after hatching, but not at six weeks. Matsukura (1970) was unable to detect maternal antibodies after 14 days of age. By contrast, Lawson and Gregg (1969) were able to detect IAEV maternal antibody for up to nine weeks after hatching. They used the constant virus-variable serum technique of the virus-serum neutralization test and this may have allowed a more sensitive determination of neutralizing antibody. This technique of the neutralization test has been recommended as the preferred method, particularly in systems in which the slope of the neutralization line of the virus was steep (Walker and Horsfall, 1950; Horsfall, 1957; Fasekas de St. Groth, 1962). Similarly the effect of chorio-allantoic route of inoculation of the virus-serum suspension may have allowed a more sensitive estimation of antibody. Comparative information on the sensitivity of the yolk sac and chorio-allantoic membrane route of inoculation in determinations of neutralization indices is not available. Calnek et al. (1961a) found that it was not until the tenth week after hatching that the serological response of chickens that received maternal antibody to IAEV was comparable to those that received none. In this study the response of the 56 day old immune chickens was still inferior to that of the susceptible chickens.

Differences in the antibody producing capacity of chickens of different ages was again demonstrated in this chapter. The mean NI of the susceptible chickens 28, 42 and 56 days of age was significantly greater ($P<0.01$) than that of the 14 day group. Differences were also found between the mean NI of the various age groups of immune chickens.

The value of maternal antibody in the control of IAE was

demonstrated in this study. Whereas susceptible chickens developed the disease after challenge per os with the virus at one, seven and 14 days of age, the immune chickens of the same age groups were found resistant to a similar challenge. Thus the maternal antibody persisted over that period in the chicken's life during which it was most susceptible to IAE.

Summary

IAEV maternal antibody was detected in the sera of chickens up to 21 days of age. This antibody protected the chickens against an intra-cerebral challenge with the van Roekel virus and an oral challenge with the NSW-1 isolate. Maternal antibody to the virus also reduced the excretion of the virus in the faeces and the ability of the chicken to produce specific serum neutralizing antibody. The affect on the serological responsiveness of the chicken persisted beyond the time maternal antibody was detected by virus-serum neutralization tests. The effect of maternal antibody on the faecal excretion of the virus waned between 14 and 21 days of age. The virus could not be detected in the faeces of the one and seven day old chickens with maternal antibody, and such chickens failed to develop serum neutralizing antibody.

An effect of the age of the chicken at the time of challenge with the virus on the excretion of IAEV in the faeces was demonstrated. This coincided with the resistance to the development of the disease that occurs as chickens become older.

CONCLUSIONS

An investigation of the pathogenesis of a disease comprises a study of a series of events and processes that combine to produce disease. Some of the factors involved in the development of IAE have been defined in this study.

One of the earliest recognized aspects of the pathogenesis of IAE was the significant interference produced by maternal antibody to the virus (Sumner et al., 1957a, 1957b). Maternal antibody to IAEV inhibited not only the disease in chickens, but also the growth of the virus in the embryonating chicken egg. This, combined with the demonstration of the relationship between the level of serum neutralizing antibody to the virus and resistance to intracerebral challenge with IAEV (Calnek and Jehnich, 1959a), provided early indications for the significance of the serological response in the pathogenesis of IAE.

The importance of circulating antibody in resistance to IAE was confirmed in this study by using both normal and immunosuppressed chickens challenged per os. This protective effect was demonstrated by - (a) the positive correlation between a positive neutralization index and resistance to disease, (b) the inability of bursectomized chickens to develop this resistance, (c) the protection provided to bursectomized chickens by the intra-peritoneal inoculation of IAEV immunoglobulin, (d) the strong protective effect of maternal antibody to the virus in normal and bursectomized chickens.

One of the reasons for the effectiveness of circulating antibody in protecting chickens against IAE may be due to the viraemia that occurs during the development of the disease. If this viraemia resulted in the dissemination of the virus

from the primary sites of multiplication to secondary or tertiary areas, and particularly the central nervous system, then antibody could effectively block this spread. However, IAEV serum neutralizing antibody was extraordinarily effective in preventing the disease in chickens challenged intra-cerebrally with the neurotropic VR virus. Therefore, it must also be inhibitory to virus multiplication. A correlation between the development of IAEV serum neutralizing antibody and a decline in the titre of the virus in the brain was demonstrated in this study. In addition, the administration of IAEV immunoglobulin as late as 48 hours after virus infection of bursectomized chickens resulted in protection. Histological studies of these chickens showed microscopic lesions of IAE in the brain of some of these chickens indicating that the disease process had commenced. This suggested that antibody was able to control the infection even at this stage.

As a result of the demonstration of the relationship between host immunity and IAEV serum neutralizing antibody, it was probable that there was an association between the development of clinical IAE and the ability to synthesise antibodies to the virus. The development of resistance to IAE as chickens aged, has been recognized since the first descriptions of the disease. This age associated resistance was demonstrated in this study in chickens challenged by different methods. This resistance could be effectively disrupted by bursectomy and was less well developed in chickens that were infected intra-cerebrally. Further studies into the relationship between the clinical disease, the invasion of the brain by the virus and the development of neutralizing antibody in chickens of different ages suggested a relationship between these factors. The sooner that antibody was produced,

the earlier the multiplication of the virus in the brain was inhibited and the less severe the disease. Young chickens were inferior in their antibody producing capacity, as judged by the NI of the sera for IAEV, and these groups suffered the severest clinical disease. The resistance to IAE that developed as the chickens aged could therefore be associated with maturation of the immune system,ie.the clinical disease developed in immunologically immature chickens.

The aspects of the pathogenesis of IAE studied in this section concentrated primarily on the reaction of the host to a virus. The development of the disease could also be markedly affected by known variations in the virulence of the virus (von Bulow, 1964; Willemart, 1965; Luginbuhl and Helmboldt, 1972).

Other factors in the response of host to the virus that may be of significance in understanding the pathogenesis of the disease are - the local production of antibody, both in the brain and gut, and the significance of the reticulo-endothelial system. Further studies are also warranted into the infection in adult birds, particularly into the mode and duration of the egg transmission of the virus from the individual hen.

PART 3

THE CONTROL OF IAE

Vaccination programmes using live or inactivated viruses have been successfully used in the control of IAE in many countries (Schaaf, 1958; Calnek et al., 196la; Brion et al., 1972; Deshumkh et al., 1973).

Control of the disease in Australia has been mainly attempted by the use of autogenous vaccines produced from the brains of chickens naturally or experimentally affected by the disease. This occurred because commercially produced IAE vaccines were not available. Although these autogenous vaccines were apparently successful in controlling the disease (Westbury, unpublished data), the reliance on such untested and unstandardized products was unacceptable to the poultry industry. The trend towards larger and more isolated breeding flocks, managed on the all in - all out system, underlined the need for a standardized and efficacious IAE vaccine.

Because of the increasing size of flocks it is also essential that mass means of vaccination be employed in the immunization of the birds. Administration of the vaccine in the drinking water of the chickens, is the most commonly used system of mass vaccination of birds in Australia. Consequently the development of any vaccine for IAE should be aimed at the production of products suitable for administration per os.

The results of this study, have confirmed the relationship between immunity to IAE and the development of specific serum

neutralizing antibody or to the presence of maternal antibody to the virus. The measurement of these antibodies provides an assessment of the immunity and the efficiency of a vaccination procedure.

The aim of the work described in this section was the development of a vaccine suitable for use in the control of the disease.

Chapter 1

THE DOSE OF THE VIRUS AND THE ROUTE OF ADMINISTRATION OF THE VACCINE, ON THE IMMUNE RESPONSE TO IAE

Introduction

Several studies on the dose-response of chickens to IAEV and the effect of various methods of administration of the vaccine, have been described (Schaaf, 1958; Calnek and Jehnich, 1959b; Calnek and Taylor, 1960; Butterfield et al., 1961; Calnek et al., 1961a; MacLeod, 1965; Bakos, 1966; Zamberg, 1966; Willemart, 1969). These studies have shown that chickens could be effectively immunized using the wing web, intramuscular and oral methods of administration of the vaccine,although there were differences in the dose of the virus required. Variations in the immunogenicity of the different isolates of the virus used were also demonstrated.

The results obtained in this thesis have indicated that the NSW-1 isolate was suitable for use in the production of an IAE vaccine. The NSW-1 isolate stimulated a serological and immune response in chickens challenged per os, was excreted in the faeces of infected chickens, could be assayed by titration in chicken embryos and propagated either in the chicken, chicken embryo or in tissue cultures.

The aim of the work described in this chapter was to study the relationship between the dose of the virus used, the route of administration of the vaccine and the production of IAEV serum neutralizing antibodies.

Materials and Methods

Chickens. These were of the WL x AO type.

Fertile eggs. These were obtained from WL flock C and D.

Virus. The NSW-1 isolate was used to vaccinate the chickens. It was used as a brain suspension of the second passage of the virus in the egg. The van Roekel isolate was used in the virus-serum neutralization tests.

Virus-serum neutralization test. This test was performed by the virus dilution - constant serum technique described in the General Materials and Methods.

Vaccination technique.

(a) Intra muscular (IM). The vaccine was injected into the thigh muscles. One ml. of the virus preparation was used - 0.5 ml. being inoculated into each leg.

(b) Wing Web (WW). A two pronged fowl pox vaccinating needle was used. The wing web between the humerus and the radius-ulna was the area of administration. This vaccinating needle delivered approximaely 0.01 ml. of the vaccine on each occasion used. Each chicken was vaccinated 10 times.

(c) Eye drop (ED). 0.1 ml. of the virus preparation was dropped into the left eye of each chicken.

(d) Oral (O). The required dose of the vaccine was administered per os to each chicken through an oesophageal tube. One ml. of each virus dilution was given.

Eight week old IAEV susceptible chickens were used. Pre-vaccination serum samples were obtained from all chickens by bleeding from the brachial vein.

One hundred and twenty chickens were divided into 22 groups of five birds, and were vaccinated by four different routes

using six different doses of virus. The doses used were approximately 0; 1; 10; 100; 1,000 and 10,000 CID_{50} of the virus. Chickens in the WW and ED groups were not vaccinated with the 10,000 CID_{50} range because of an inability to give this dose by these routes.

The chickens were maintained in cages on separate premises for each virus dose. Because of the limitation on the availability of accommodation, only one method of vaccination was tested at the one time.

Post-vaccination serum samples were obtained five weeks after vaccination. These serum samples were inactivated at 56°C for 30 minutes and then stored at -30°C.

The actual dose of the virus used in the vaccination was titrated after the completion of vaccination of the birds.

The control chickens were challenged with a 50 percent brain suspension from non IAEV infected chickens.

Excretion of the virus in the faeces of vaccinated chickens

The faeces of the group vaccinated orally with 100 CID_{50} of the virus were collected daily by allowing them to fall onto fresh clean paper. The faeces were weighed and a 10 percent (w/v) suspension of a random portion was prepared. This suspension was labelled with the date of collection, stored at -30°C and eventually given per os to five, one day old chickens.

As chickens developed the disease they were removed, bled, killed and their brain removed for histological examination. All other chickens were bled, killed and examined at 28 days post challenge. The serum samples were used in virus-serum neutralization tests.

The facilities used to house the groups infected with the faecal suspensions were cleaned and disinfected between test groups. The cages and rooms were scrubbed with a detergent disinfectant solution, steam cleaned and then allowed to stand two days before chickens were again introduced. On two occasions the effectiveness of this procedure was assessed by placing susceptible chickens in the cages after cleaning for 14 days. These chickens were then removed from the cages and placed under observation in another area for a further 28 days. They were then bled, killed and their brains removed for histological examination. The sera were inactivated and then tested for neutralizing activity to IAEV.

Results

Method of vaccination

A serological response was detected in chickens vaccinated by the IM, ED and O methods. Chickens in the WW vaccinated group failed to develop a group mean neutralizing antibody response (see Table 58). However, four individual chickens developed positive neutralization indices following WW vaccination.

Dose of virus administered

The dose of the virus administered to the different groups varied as only one method was tested at one time. A serological response failed to develop in the groups vaccinated by the WW method. Mean positive neutralization indices

Table 58

Serological response of 56 day old chickens vaccinated with the NSW-1 virus

Dose range (CID_{50})	Method of vaccination							
	Intra-muscular		Wing Web		Eye drop		Oral	
	Virus dose (CID_{50})	Mean NI*	Virus dose (CID_{50})	Mean NI	Virus dose (CID_{50})	Mean NI	Virus dose (CID_{50})	Mean NI
>10,000	30,000	2.40±0.54	NT	NT	NT	NT	70,000	2.00±0.16
1,000 to 10,000	3,000	1.95±0.40	6,530	0.88±0.26	8,750	1.70±0.52	7,000	1.78±0.19
100 to 1,000	300	1.54±0.33	653	0.96±0.65	875	1.02±0.18	700	1.36±0.22
10 to 100	30	0.90±0.46	65	0.24±0.11	88	0.64±0.27	70	1.28±0.19
1 to 10	3	0.46±0.22	7	0.36±0.15	9	0.26±0.18	7	0.96±0.25
Control	0	0.30±0.15	0	0.24±0.24	0	0.24±0.16	0	0.32±0.24

CID_{50} - chicken infective doses

NI - Neutralization index

* - Five chickens per group

developed in all the other groups vaccinated with virus in the dose ranges >10,000 and 1,000 to 10,000 CID_{50} of the virus. Similarly positive responses were found in the IM and O groups vaccinated with between 100 to 1,000 CID_{50} of the NSW-1 isolate of IAEV. Only the O vaccinated group developed a positive mean neutralization index in the group given 10 to 100 CID_{50} (see Table 58).

IAEV neutralizing antibody was not detected in the unvaccinated, control chickens in each group.

Recovery of virus in the faeces of immunized birds

Chickens developed IAE after challenge with a faecal suspension collected from the birds vaccinated with 700 CID_{50} of the virus per os (Table 59).

The period of excretion of the virus varied depending on the technique used to demonstrate the virus. Overt IAE developed in chickens challenged with faeces collected from three to nine days after vaccination. However, sub-clinical infections were also detected in these age groups and also in chickens challenged with faeces collected ten days after vaccination. Chickens in the group challenged with faeces collected on the 10th day after vaccination also developed positive neutralization indices but no clinical signs of infection.

Table 59

Excretion of IAEV in faeces of chickens vaccinated orally with the NSW-1 virus

Days after vaccination	Proportion of chickens with clinical IAE	Proportion of chickens with histo lesions	Mean NI (up to 28 days PV)
1	$0/_5$*	$0/_5$*	0.16 ± 0.14
2	$0/_5$	$0/_5$	0.42 ± 0.24
3	$1/_5$	$3/_5$	1.86 ± 0.29
5	$4/_5$	$5/_5$	2.20 ± 0.30
7	$5/_5$	$5/_5$	2.74 ± 0.31
9	$2/_5$	$3/_5$	2.08 ± 0.29
10	$0/_5$	$2/_5$	1.50 ± 0.48
11	$0/_5$	$0/_5$	0.56 ± 0.24
12 to 19	0	0	<0.50

* Number affected/number challenged

NI ($\log_{10}x$). Neutralization index

Virus infection was not detected in control chickens

Discussion

The lack of a suitable number of isolation facilities meant that only one route of vaccination could be tested at the one time. This lead to unwanted variation in the dose of the virus used to vaccinate the chickens, but some definite trends were apparent in the results obtained. This dose variation may have resulted from a poor repeatability of the end point in titrations undertaken by the Hoekstra technique. A positive serological response was induced in some chickens vaccinated by each of the routes tested. However there was considerable variation in the dose of the virus required to induce this response in the different vaccination methods tested.

The most satisfactory method of vaccination in terms of safety and the production of a serological response with the smallest dose of virus was vaccination *per os*. Mean positive neutralization indices were detected in all the virus dose groups tested, except in the lowest dose of 7 CID_{50}. In this dose group however, one bird showed a positive NI. In addition, clinical IAE did not develop. A similar result was obtained by Calnek *et al*. (1961a). Earlier, however, Calnek and Jehnich (1959b) had shown that at least 10^6 EID_{50} or more of an isolate adapted to grow in the chicken embryo was required to induce a serological response after vaccination by the oral route. This was far in excess of the dose required to induce an antibody response in chickens vaccinated IM or WW with this virus. These results emphasized the essential requirement for a virus to be suitable for use as an oral vaccine for IAE, namely that the virus could reproduce the disease in chickens challenged *per os*, that it was excreted in the faeces and spread by contact transmission, and that it induced a serological response in

vaccinated birds. The NSW-1 isolate used in this study conformed with these standards and therefore could be used as an oral vaccine.

The virus also immunized chickens vaccinated by other methods. The intra-muscular vaccination of chickens resulted in mean positive neutralization indices in the groups inoculated with at least 100 to 1,000 CID_{50} of the virus. However, the clinical disease developed in one chicken vaccinated with 30,000 CID_{50} of the virus. This method was therefore not as safe as the oral administration of the vaccine. The development of overt IAE in chickens vaccinated IM against the disease has also been recorded by Schaaf (1958) and Calnek and Jehnich (1959b).

The eye drop technique resulted in a positive serological response in the group vaccinated with 1,000 to 10,000 CID_{50} of the virus and in two chickens vaccinated with 100 to 1,000 CID_{50} of the NSW-1 isolate. This result indicated that this method could be used in field vaccination programmes, provided that at least 8,750 CID_{50} of the virus was used. Field experience obtained using the eye drop procedure for the vaccination of approximately 500,000 chickens against IAE has confirmed the usefulness of this technique (Westbury, unpublished data).

A positive serological response was not produced in the groups vaccinated by the wing web method. Two chickens in the 1,000 to 10,000 CID_{50}, and two in the 100 to 1,000 CID_{50} group however, developed positive neutralization indices. This suggested that WW vaccination with this virus was less efficient than the other techniques tested. This is in marked contrast to the findings of Schaaf and Lamoreux (1955), Zander (1959), Calnek and Jehnich(1959b) and Lawson and Gregg(1969). They found the WW method to be very effective in inducing an immune response

in all tested chickens. However much of the data obtained by these workers resulted from studies using IAEV isolates that had been serially passed in the brain of chickens. This passaging may have affected the immunogenicity of the virus compared to an isolate that had been propagated in the chicken embryo. In addition, the technique of WW vaccination used in this study may have been different. The technique used, however, was identical to that used to successfully vaccinate chickens against fowl pox. The response of two chickens in two of the dose groups indicated that higher dose rates may have resulted in a better serological response in the chickens vaccinated.

The recovery of the virus in the faeces of the chickens vaccinated with 700 CID_{50} of the virus was demonstrated by the development of the disease, histological lesions and serum neutralizing antibodies to IAEV in chickens challenged with the faeces. This excretion occurred from the third to the 10th day after vaccination. The development of histological lesions or of serum neutralizing antibody to the virus were more sensitive in detecting virus in challenged chickens than assessment based on the development of clinical IAE. Calnek et al. (1960) demonstrated the excretion of the virus in the faeces of experimentally infected chickens on the fifth, ninth and 12th days after challenge. The recovery of the virus in the faeces of the chickens vaccinated with the NSW-1 virus in this chapter confirmed the results of earlier studies.

The results of this study suggested that IAEV susceptible chickens should be vaccinated with at least 100 CID_{50} of the NSW-1 isolate by the oral route. The virus was secreted in the faeces of the vaccinated birds and it is therefore likely that natural spread of the virus would occur in vaccinated flocks.

Previous studies have indicated that it is necessary to vaccinate only one to five percent of the birds in a flock as the remainder become infected by natural means (Calnek et al., 1961a). Further studies would be required to demonstrate if this was applicable to flocks vaccinated with the NSW-1 virus.

Summary

A positive serological response was detected in at least some chickens in groups vaccinated with the NSW-1 virus by either the intra-muscular, wing-web, eye drop or oral methods of vaccination. The oral route was the most sensitive in terms of inducing a serological response with the lowest dose of virus. The wing web method was the least sensitive. The intra-muscular method was the most dangerous because of the development of clinical IAE. The virus was detected in the faeces of chickens vaccinated with 700 CID_{50} of the virus per os. This persisted from the third to the tenth day after vaccination.

The vaccination of chickens per os with at least 100 CID_{50} of the NSW-1 isolate in the second passage in the chicken embryo is suggested as the most suitable method of immunization of flocks against IAE.

CONCLUSIONS

The NSW-1 virus conformed to the basic requirements for an isolate of IAEV to be suitable for use as an oral vaccine for IAE control. The virus exhibited, (1) a satisfactory immunogenicity as determined by evidence of an antibody response; (2) excretion in the faeces of vaccinated chickens; (3) freedom from harmful effects on chickens 28 days of age or older after administration *per os* and; (4) antigenic relationship to the van Roekel virus. In addition, the virus could be propagated in the chicken embryo and satisfactorily quantitated. The virus also induced immunity in chickens vaccinated by the eye drop, intra-muscular and wing web methods of vaccination, although these techniques were less sensitive and safe.

There is a need for a standardization and simplification of the methods used in the assessment of immunity to IAE. Four techniques have been used, (1) the challenge of chickens with a known dose of virus some time after vaccination; (2) an estimation of the NI of the serum; (3) the embryo susceptibility test and; (4) the challenge of the progeny of vaccinated flocks. The methods used in each of the procedures have also varied. Alternative techniques such as agar precipitin tests or virus serum neutralization tests in tissue culture systems would simplify the testing procedures.

The vaccination of chickens of three to four months of age is usually recommended (Anon, 1973). In this study harmful effects resulting from the challenge of chickens 28 days or older with the virus *per os* were not detected. The antibody response of chickens that received maternal antibody to IAEV

was inferior to that of chickens deprived of this maternal antibody. This effect abated at approximately ten weeks of age (Calnek et al., 1961a). This reduced responsiveness, and the possibility of inducing the disease in young chickens, are the basis for the common recommendation on the age of the chicken for vaccination. There could be practical advantages in the earlier vaccination of chickens. The chickens could be vaccinated and then tested for immunity. If immunity had not been produced the flocks could be then re-vaccinated. By delaying vaccination until three months, the possibility of testing and re-vaccination of flocks in the case of an initial failure are reduced, as flocks should not be vaccinated within four weeks of the onset of egg production. The results of these studies suggest that flocks could be vaccinated as early as six weeks of age. Although the antibody response in chickens that received maternal antibody to IAEV may be reduced, a positive serological response nevertheless develops. Vaccination as early as six weeks may give some advantage by providing earlier evidence of satisfactory immunity in the fertile egg flock.

GENERAL DISCUSSION

The similarities between the neurohistological lesions of IAE and poliomyelitis of humans, have prompted the suggestion that the disease should be called poliomyelitis of newborn chicks (Yamagiwa et al., 1969).

The pathogenesis of IAE also resemble that of poliomyelitis. In poliovirus infections, the primary site of multiplication of the virus is in the alimentary tract. The virus may then invade the lymphatics, bloodstream and other tissues, and perhaps also the central nervous system. Although poliovirus can spread along nerve fibres in the peripheral as well as the CNS (Fairbrother and Hurst, 1930; Bodian and Howe, 1940; Sabin, 1956) the haemal spread of the virus is considered of prime importance in its dissemination through the body (Nathanson and Bodian, 1962). The significance of viraemia in the pathogenesis of the disease is emphasised by the success of killed virus vaccines in the prevention of paralytic poliomyelitis (Anon, 1964). The occurrence of a viraemic phase of the infection presumably allows serum neutralizing antibody to block the invasion of the brain and spinal cord by the virus.

An association between serum neutralizing antibody and immunity to IAE has been recognized for many years (Calnek and Jehnich, 1959a). However a viraemic stage in the development of the disease has not been described. It is clear from the results of this study, that viraemia is of considerable significance in the pathogenesis of IAE. This viraemia occurred primarily in the pre-clinical disease period of the infection, and was only detectable while serum neutralizing antibody to IAEV was not apparent. The length of the viraemia was

associated with the severity of the clinical disease. Where there was a short viraemia, the clinical disease was mild or absent. When the viraemia persisted for some time, as in one day old or bursectomized chickens, the disease was severe and the chickens became paralyzed. When IAEV immunoglobulin was inoculated IP into highly susceptible one day old, or bursectomized chickens at the time of challenge with the virus, the overt disease failed to develop. The same applies to one day old chickens with maternal antibody to IAEV.

However serum neutralizing antibody to IAEV not only acted by preventing the spread of the virus to the CNS, but also was associated with a reduction in virus multiplication in the chicken. Chickens with IAEV serum neutralizing antibody resisted an intra-cerebral challenge with a neurotropic isolate of the virus. This occurred in chickens with either active or passively acquired antibody to the virus. The virus could not be detected in the faeces of one or seven day old chickens that were challenged per os with IAEV when they received maternal antibody to the virus. IAEV neutralizing antibody also apparently controlled the disease even when inoculated 48 hours after bursectomized chickens had been challenged with the virus. The relationship between virus and antibody was also demonstrated in the decline of the multiplication of the virus in the brain as the level of serum neutralizing antibody increased. Where this antibody failed to develop, as in bursectomized chickens, or was slow in developing, as in baby chickens, virus multiplication continued and caused paralytic disease. Thus serum neutralizing antibody had a dampening effect on the multiplication of the virus, as well as inhibiting invasion of the CNS. The decline in viral infectivity of poliovirus in

nervous tissue is due to inhibiting forces unrelated to the development of serum or local neutralizing antibody. In animals inoculated intra-cerebrally with poliovirus, no detectable serum neutralizing antibody response occurs but the reduction of viral concentration is the same as in natural or experimental infections by other routes (Bodian and Horstmann, 1965). A similar effect was not demonstrated in IAE. Chickens challenged intra-cerebrally with the virus developed serum neutralizing antibody to IAEV. In bursectomized chickens, the multiplication of the virus in the brain continued unabated throughout the test period. However the techniques used in bursectomy were not selective enough to allow definite decisions on the role of circulating antibody in such a response. Parry and Aitken (1973) have demonstrated that local antibody production to Newcastle disease virus was affected by treatment with cyclophosphamide. An effect of cyclophosphamide on cell mediated immune responses has been demonstrated in this study and in others (Seto, 1970; Toivanen et al., 1972; Rouse and Szenberg,1974). Morphological changes have been described in the thymus of chickens treated with testosterone (Warner et al., 1962; Pierce et al., 1966).

It is evident from the results of this study, that circulating antibody to the virus is relatively important in preventing the spread and multiplication of IAEV in the chicken. The respective role of cell mediated immunity, the reticulo-endothelial system and of the production of local antibody in both the alimentary tract and CNS require further investigation.

Another factor important in the pathogenesis of IAE was the age of the chicken at the time of infection with the virus.

Chickens became less susceptible to the development of clinical IAE as they aged. This was demonstrated in chickens infected IM, IP and per os, and to a lesser extent in chickens challenged intra-cerebrally. The development of this age resistance was disrupted by bursectomy. It was also associated with the earlier detection of serum neutralizing antibody to the virus in the older age groups.

The full range and intensity of immunological responses characteristic of the adult chicken are only developed gradually. Wolfe and Dilks (1948) found that precipitin antibody production to bovine serum increased dramatically between one and 35 days of age. Keeble and Wade (1963) demonstrated that chickens up to eight days of age were not fully immunocompetent, as judged by the ability to produce haemagglutination inhibiting antibodies. Natural agglutinin levels were found to be low in chicken embryos and in chickens up to two weeks of age (Seto and Henderson, 1969). Inferior antibody production to sheep red blood cells and to Brucella abortus was demonstrated in four and eleven day old chickens (Toivanen et al., 1972). Paterson et al. (1962) found the half-life of gamma-globulin in the blood of newly hatched chickens was 72 hours, as compared to 35 hours in the adult. This indicated a sparing of gammaglobulin in the young chicken. They also found that the rate of gammaglobulin synthesis was low during the first two weeks after hatching and then improved. However, this test was performed in chickens not subjected to antigenic challenge. Since exposure to antigenic stimuli is important in determining the amount of gammaglobulin synthesised, it would be better to look at synthesis studies in animals that had been subjected to known antigenic stimuli.

Poorer antibody production in young chickens was also demonstrated in this study. The ability of the chicken to synthesize, and the intensity of production of serum neutralizing antibody could be of considerable importance in the outcome of infection with IAEV. This is especially so in IAE, considering the significance of circulating antibody in the pathogenesis of the disease. Thus a delayed or inferior production of IAEV neutralizing antibody in the baby chicken permits the continued multiplication of the virus in the CNS and the development of clinical IAE.

Another factor that could be involved in this age resistance is the vascular penetrability of the CNS. The permeability of the blood-brain barrier, as judged by the passage of trypan blue dye into the CNS, has been demonstrated to decrease with age (Vadlamudi and Hanson, 1966). They also found the resistance to the lethal effects of the Roakin strain of NDV inoculated sub-cutaneously, paralleled the development of this decreased permeability. However, these may be independent aspects of associated events as the transgression of the barrier by neuro-invasive strains of NDV failed to alter the dye permeability. In addition, the main factor involved in the development of resistance was the earlier curtailment of virus multiplication in the older age groups of chickens. The initial virus-host interaction was similar in all the age groups studied. This indicated that the virus was capable of invading the brain, regardless of the age of the bird. The response of the host to the virus was the critical factor involved in developing age resistance. A similar result was demonstrated in these studies on IAE.

An aspect of the pathogenesis of IAE that requires further

investigation is the mechanism of congenital infection. Because infected hens excrete the virus in their faeces during the transmission of the virus via eggs (Jungherr and Minard, 1942; Calnek et al., 1960), the possibility of external contamination has been considered (Calnek et al., 1961b). However, IAEV has also been recovered from the ovary of infected birds (Jungherr and Minard, 1942) and the fluorescent antibody test has demonstrated IAEV antigen in ovarian tissue (Braune and Gentry, 1971b). The possibility of transovarian transmission therefore exists.

The concept advanced in this thesis of IAE as an acute viral infection terminated by a rapid immunological response would suggest that egg transmission from an individual hen would be of short duration.

Another aspect of considerable interest in the epidemiology of the disease is the reaction of congenitally infected chickens to the virus. In some diseases of chickens in which vertical transmission takes place, birds may develop an immunological tolerance to the causative organism. Rubin et al. (1962) found that chickens congenitally infected with an avian leucosis virus developed a permanent viraemia and regularly produced leucosis virus infected progeny. Specific investigations of IAE in naturally congenitally infected chickens have not been reported. However, chicken embryos inoculated with the virus at five to seven days of age by the yolk sac route, develop IAE, and produce specific serum neutralizing antibody.

The investigation of some of these features depends to a great extent, upon the development of more efficient and practical techniques for the assay of the virus. Techniques currently used depend upon a plentiful supply of IAEV

susceptible fertile eggs. In many places these are not available. Although isolates of IAEV such as the van Roekel virus can be adequately assayed, the precision of the estimation of the end point of infectivity is not high. In this study the virus was found in chicken embryos not showing complete paralysis, muscle atrophy or over extended toes. In addition, these types of virus are not suitable for study of some aspects of the pathogenesis of IAE. The assay of "wild" viruses requires not only good isolation facilities and an abundant supply of time and patience, but also lacks precision as the clinical disease may not develop in some infected chickens. The virus will only be efficiently assayed when it can be grown and titrated in tissue culture. The inability to quantitate the virus in tissue cultures makes the use of plaque assays unavailable. Their high degree of accuracy (Cooper, 1961) would be desirable in investigations into IAE. The demonstration of the propagation of both the van Roekel and NSW-1 viruses in various tissue cultures indicates that further investigations into the cell culture propagation are warranted. This could involve a further search for cell lines sensitive to the virus, or for other IAEV isolates that are cytopathogenic. In addition,further examinations of modifications to the cell culture media to assist the cytopathogenicity of the virus should be undertaken. The presence of naturally occurring inhibitors in serum have been a problem with many types of viruses (Takemoto and Habel, 1959; Pagano, 1965; Thomsson et al., 1966; Lukert, 1973). In addition the finding that simple sugars inhibited the haemagglutination of a number of enteroviruses (Lerner et al., 1965, 1966; Kunin, 1967) indicated that these compounds were active in the virus-cell union, and may be

important here.

Similarly, new procedures for the detection and estimation of the serological response to the virus are needed. The agar gel precipitin (Lukert and Davis, 1971) and indirect fluorescent antibody test (Choi and Muira, 1972) require further evaluation as to their reliability and correlation with the results of virus serum neutralization tests. These techniques would, if efficient, greatly simplify the monitoring of flocks for infection with IAEV. They would also considerably assist the testing of the efficacy of IAE vaccines.

The NSW-1 isolate was shown to be suitable for use as vaccine. It could be administered either orally, by eye drop or intra-muscularly and induce a satisfactory immune response. The excretion of the virus in the faeces of vaccinated birds also indicated that it could be used in vaccination procedures relying on the natural transmission of the virus within a vaccinated flock (Calnek et al., 1961a). Although this technique has been widely used in the vaccination of flocks against IAE (Brion et al., 1972) it could lead to transmission of the virus to unvaccinated, susceptible flocks. As oral live vaccines for IAE must use a virus that has retained its invasiveness for the intestinal tract (Calnek et al., 1961a), inadvertent infection of flocks could occur. The use of inactivated vaccines would overcome this problem, but these are impractical in large flocks. Consequently additional features that could be considered as criteria for a vaccine virus are - (1) a restricted capacity to spread to incontacts and; (2) reduced ability to invade tissues remote from the alimentary tract. However such isolates must be able to induce a serum antibody response, as the aim of IAE vaccination

is to prevent infection of the parent flock, and also to produce maternal antibody to IAEV, so that the progeny are protected during the time they are most susceptible to the development of paralytic IAE. The suitability of these types of isolates as vaccines would also depend upon their ability to stimulate local antibody to the virus, and the importance of local antibody in immunity to IAE. An association of secretory antibody and protection against re-infection with poliovirus has been demonstrated (Ogra and Karzon, 1969a, 1969b). However evidence strongly suggests that poliovirus secretory antibody production in the nasopharynx and alimentary tract is an entirely local phenomenon, without any significant contribution from serum components (Ogra and Karzon, 1971). This requires further investigation in the chicken vaccinated for IAE.

An understanding of the protective role of serum antibody has led to the development of successful vaccines for the prevention of many viral infections. Further knowledge of the protective role of secretory antibodies could lead to the development of vaccines which not only produce protection at the humoral level, but offer a more important means of resistance at the portal of entry.

APPENDIX

Media and Solutions used for Cell Cultures

1.1 Growth medium for CEN cell cultures

	ml.
Hank's balanced salt solution (1)	8.0
Foetal calf serum (2)	20.0
Lactalbumin hydrolysate	0.5
Yeast extract	0.5
Sodium bicarbonate (2.8 percent) (3)	2.0
Crystamycin (4)	0.2
Amphotericin B(5)	0.1
Distilled water to	100.0

Maintenance medium for CEN cell cultures

Hank's balanced salt solution	9.0
Foetal calf serum	10.0
Yeast extract	0.5
Sodium bicarbonate (2.8 percent)	3.0
Crystamycin	0.2
Amphotericin B	0.1
Distilled water to	100.0

(1) Commonwealth Serum Laboratories, Parkville, Vic.

(2) Supplied by Australian Laboratory Services.

(3) Commonwealth Serum Laboratories, Parkville, Vic.

(4) Glaxo Australian Pty.Ltd. Boronia, Vic.

(5) Fungizone, E.R. Squibb and Sons, New York, U.S.A.

1.2 Growth medium for CEF cells

	ml.
Medium 199 (10 times) (1)	9.0
Non-inhibitory calf serum	5.0
Sodium bicarbonate (2.8 percent)	3.0
Crystamycin	0.2
Amphotericin B	0.1
Distilled water to	100.0

Maintenance medium for CEF cells

Medium 199 (10 times)	10.0
Non-inhibitory calf serum	1.0
Sodium bicarbonate (2.8 percent)+	3.0
Crystamycin	0.2
Amphotericin B	0.1
Distilled water to	100.0

1.3 Growth medium for CEK cells

Hank's balanced salt solution (10 times)	8.0
Tryptose phosphate broth (2)	10.0
Foetal calf serum	5.0
Sodium bicarbonate (2.8 percent)	2.0
Crystamycin	0.2
Amphotericin B	0.1
Distilled water to	100.0

(1) Commonwealth Serum Laboratories, Parkville, Vic.

(2) Difco Laboratories, Detroit, Michigan, U.S.A.

1.3 Maintenance medium for CEK cells (Cont.)

	ml.
Hanks balanced salt solution (x10)	9.0
Foetal calf serum	2.0
Sodium bicarbonate (2.8 percent)	3.0
Crystamycin	0.2
Amphotericin B	0.1
Distilled water to	100.0

1.4 Growth medium for CK cells

Medium 199 (x10)	8.0
Foetal calf serum	10.0
Tryptose phosphate broth	10.0
Sodium bicarbonate (2.8 percent)	3.0
Crystamycin	0.2
Amphotericin B	0.1
Distilled water to	100.0

Maintenance medium for CK cells

Medium 199 (x10)	10.0
Foetal calf serum	1.0
Sodium bicarbonate (2.8 percent)	3.0
Crystamycin	0.2
Amphotericin B	0.1
Distilled water to	100.0

1.5 Medium for tracheal organ culture

	ml.
Hank's balanced salt solution	9.0
Lactalbumin hydrolysate	0.5
Non-inhibitory calf serum	10.0
Sodium bicarbonate	3.0
Crystamycin	0.2
Amphotericin B	0.2
Distilled water to	100.0

1.6 Nutrient agar overlay. This was prepared by dissolving 1 gm. of Agarose for electrophoresis (British Drug Houses) in 100 ml. of distilled water. The preparation was sterilized by autoclaving.

Overlay medium was prepared to the same formula as the growth medium of the particular cells, except that twice the concentration of all constituents was present. Agarose at 45°C was mixed with an equal volume of pre-warmed overlay medium. The mixture was pipetted in volumes of 5.0 ml. to cell cultures.

1.7 Trypsin solution. A 5.0 percent stock solution of trypsin was used in the preparation of the working solution for trypsinization of chicken kidneys and chicken embryos. A 1:300 trypsin powder (1) was dissolved in PBS., centrifuged at 600 g for ten minutes and sterilized by filtration. The solution was dispensed and frozen at -30°C.

(1) Commonwealth Serum Laboratories, Parkville, Vic.

1.8 Lactalbumin hydrolysate (1). Five gms. of lactalbumin hydrolysate was dissolved in 100 ml. of de-ionized water, heated to aid dissolution (no higher than 50°C), the pH adjusted to 7.0 and then sterilized by filtration through a millipore filter No. 45µ. The solution was stored at -30°C.

1.9 Yeast extract (1). A 5 percent solution was prepared in de-ionized water, the pH adjusted to 7 and the solution sterilized by filtration.

1.10 Phosphate buffered saline. This was prepared by dissolving the salts listed below individually in de-ionized water. The solution was sterilized by autoclaving and stored at 4°C.

Sodium chloride	8.0 gm
Potassium chloride	0.2 gm
Sodium di hydrogen orthophosphate ($Na_2 HPO_4.2H_2O$)	1.15 gm
Potassium di hydrogen orthophosphate ($K H_2PO_4$)	0.2 gm
De-ionized water to	1000.0 ml.

(1) Commonwealth Serum Laboratories, Parkville, Vic.

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