THE AETIOLOGY AND PATHOGENESIS OF AVIAN INCLUSION BODY HEPATITIS

by

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ABSTRACT

Naturally occurring inclusion body hepatitis (IBH) in broiler flocks in New Zealand, and the experimental disease were characterized by a sudden onset of illness resulting in up to 30% mortality and severe liver damage associated with the formation of intranuclear inclusion bodies in the hepatocytes. Other features were anaemia and atrophy of the bursa and thymus associated with lymphoid depletion.

Serotype 8 avian adenoviruses (AAVs) were isolated from several affected broiler and one breeder flock. High titres of virus neutralizing (VN) antibodies were demonstrated in flocks which had recovered from the disease. By restriction endonuclease fingerprint analysis, two of the New Zealand isolates were found to be similar to each other and to the reference strain HVI, but markedly different from three Australian isolates of the same serotype.

Fatal disease resembling IBH was reproduced in 30% of broiler chickens following oral administration of one of the local isolates. Immunosuppression was demonstrated in both natural and experimental infections.

An enzyme-linked immunosorbent assay and an immunocytochemical technique were developed for the detection and quantification of adenoviral antigens in various chicken tissues. Both techniques detected less than 100 mean tissue culture infective doses per gram of infected tissue and a group-specific antigen common to the 12 serotypes of AAV.

A study of the pathogenesis of IBH infection was conducted following oral administration of AAV. Virus first multiplied to a high titre in the intestinal organs and passed into the blood by way of the lymphatics. Viral antigens were subsequently detected in phagocytic cells in the liver and then in the hepatocytes. Extensive replication resulted in severe liver damage, with release of virus into the blood stream and spread to other organs. Recovery was associated with the appearance of VN antibody from 7 days post inoculation.

Viral antigens were detected by ELISA directly in yolk and albumin of eggs derived from 50-60-week-old breeder flocks, although all birds had high titres of VN antibody in their blood.

The inclusion bodies found in hepatocytes were characterized antigenically and ultrastructurally.

STATEMENT

This thesis contains no material that has been used in whole or in part for the award of any other degree or diploma in any educational institution. The nature and extent of any assistance I have received is as stated in the Acknowledgements section of this thesis.

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PUBLICATIONS

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ABBREVIATIONS

AAV	Avian adenovirus
A-AV	Adenovirus-associated virus
ABC	Avidin-biotin peroxidase complex
AC	Allantoic cavity
AE	Avian encephalomyelitis
ATV	Antibiotic-trypsin-versene
BAV	Bovine adenovirus
BSA	Bovine serum albumin
CAA	Chicken anaemia agent
Ca-EDTA	Calcium-ethylenediamine tetraacetic acid
CAM	Chorioallantoic membrane
CELO	Chick embryo lethal orphan
CF	Complement fixation
CI	Challenge interference
CKC	Chicken kidney cells
CPE	Cytopathic effect
DAB	Diaminobenzidine
DEAE	Diamino ethane acetic acid
DNA	Deoxyribonucleic acid
EA	ELISA absorbance
EDS	Egg drop syndrome
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope (y)
ESA	ELISA specific absorbance
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAL	Gallus adeno-like
GC	Guanine plus cytosine
GD	Gel diffusion
GMT	Geometric mean titre
HA	Haemagglutination
HAD	Haemadsorption
HAd	Human adenovirus
HAI	Haemagglutination inhibition

ABBREVIATIONS (continued)

HE	Haematoxylin and eosin
HRP	Horseradish peroxidase
HUR	Hatchery utilization rate
HVT	Turkey herpes virus
IB	Infectious bronchitis
IBD	Infectious bursal disease
IBH	Inclusion body hepatitis
ICH	Infectious canine hepatitis
IF	Immunofluorescence
IM	Intramuscular (ly)
INIB	Intranuclear inclusion body
IP	Intraperitoneal (ly)
IP	Immunoperoxidase
LR	London Resin
MD	Marek's disease
MDCC-MSB1	Marek's disease lymphoma cell line
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
MSD	Marble spleen disease
MW	Molecular weight
ND	Newcastle disease
NE	Necrotic enteritis
NGS	Normal goat serum
OPD	Ortho-phenylenediamine
PA	Passive agglutination
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCV	Packed-cell volume
pi	Post inoculation/Post infection
PSK	Penicillin, streptomycin and kanamycin
PTA	Phosphotungstic acid
QB	Quail bronchitis
RPMI1640	Prefix derived from Roswell Park Memorial Institute
SAS	Saturated ammonium sulphate

ABBREVIATIONS (continued)

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SPF	Specific pathogen free
T	Tumour
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TCID50	Mean tissue culture infective dose
TE	Tris-EDTA
THE	Turkey haemorrhagic enteritis
UV	Ultraviolet
VN	Virus neutralization
WLH	White Leghorn

CHAPTER ONE

REVIEW OF THE LITERATURE

INTRODUCTION

The term adeno comes from the Greek word 'aden' meaning gland, and was used when the first adenovirus was discovered in uninoculated cultures of human adenoid tissue (Rowe et al., 1953). The adenovirus of infectious canine hepatitis (ICH) was isolated shortly afterwards (Cabasso et al., 1954) although this disease had been described and its possible viral aetiology suggested much earlier (Cowdry and Scott, 1930; Rubarth, 1947). The first avian adenovirus (AAV) was isolated from chicken embryos which had been inoculated with calf tissue during studies on the aetiology of lumpy skin disease (Van den Ende et al., 1949). Immediately after, the quail bronchitis (QB) virus was isolated from West Virginia quail suffering from an acute respiratory disease with high mortality (Olson, 1950). Subsequently, chick embryo lethal orphan (CELO) virus and gallus adeno-like (GAL) virus were isolated from chicken embryos and latently infected cell cultures by Yates and Fry (1957) and Burmester et al. (1960), respectively. Since then, isolation of CELO, QB and GAL viruses has frequently been reported from several countries (McFerran et al., 1977). To date, 12 avian serotypes plus the virus of egg drop syndrome (EDS) and the turkey haemorrhagic enteritis (THE) related viruses, 41 human serotypes and a number of types and strains of simian, canine, bovine, porcine, equine, murine and ovine origin have been reported throughout the world (McFerran, 1981a; Pettersson and Wadell, 1985).

The family Adenoviridae is subdivided into two genera, Mastadenovirus representing all mammalian adenoviruses and Aviadenovirus representing AAVs. Classification is on the basis of group-specific antigen, percentage DNA content, penton fibre antigen, sedimentation co-efficient of viral DNA and molecular weight (MW) of the structural proteins (Nermut, 1987).

AAVs are frequently isolated from apparently healthy birds and their aetiological role in specific clinical diseases is often regarded as insignificant (Cook, 1970; Yates et al., 1976; Winterfield, 1984). However, some AAV infections are associated with a variety of overt disease syndromes such as hepatitis, respiratory disease, drop in egg production, decline in egg shell quality, tenosynovitis, atrophy of the bursa and thymus, haemorrhages in muscles and viscera, splenomegaly, aplastic anaemia and nervous signs (Berry, 1969; Fadly and

Winterfield, 1973; Rosenberger et al., 1974; Tolin and Domermuth, 1975); Baxendale, 1978; McFerran et al., 1978b; Dhillon et al., 1982). The AAVs are divided into 3 immunologically distinct subgroups: the conventional or Group I AAV, Group II adenoviruses associated with THE, marble spleen disease (MSD) of pheasants and splenomegaly of chickens, and Group III adenoviruses closely related to EDS (Winterfield, 1984; Domermuth and Gross, 1984).

Inclusion body hepatitis (IBH) is an acute infectious disease predominantly affecting young chickens of 5-7 weeks of age caused by infection with any of several serotypes of Group I AAV. Natural outbreaks are characterized by a sudden onset of sharply increased flock mortality (up to 30%), a short clinical course, anaemia and necrotic hepatitis. Basophilic or eosinophilic intranuclear inclusion bodies (INIBs) are often observed in hepatocytes. More frequently, a mild form of IBH associated with similar serotypes of AAV has been reported in many countries. Variable mortality has also been demonstrated experimentally following administration of different isolates of a single serotype of AAV under similar conditions (Cook, 1983). It is likely that differences in virulence between viral strains may contribute to the variation in severity of the disease in addition to any environmental or host factors.

Differences between viral strains, revealed by growth in cell culture or in avian embryos, appear to be modified by passage history and titre of the inoculum (Izuchi and Hasagowa, 1982). Although the effects of AAV on embryos are more closely related to serotype than virulence of the virus, repeated passage in embryonating eggs has been shown to lower pathogenicity for susceptible chickens (Fadly and Winterfield, 1975).

Conventional serological techniques such as gel diffusion (GD), immunofluorescence (IF) and virus neutralization (VN) tests have not proven useful for pathotyping strains of AAV. Extensive cross-reaction between different serotypes of AAV, irrespective of their virulence, has been demonstrated by enzyme-linked immunosorbent assay (ELISA) (Calnek et al., 1982).

Analysis of the deoxyribonucleic acid (DNA) of AAV has been performed by electrophoresis of fragments produced by digestion with restriction endonucleases (Zsak and Kisary, 1984). Apart from grouping the different serotypes, no attempt has been made to pathotype the AAVs by analysing their genomes with restriction enzymes or other biochemical techniques.

The structure of human adenoviruses (HAds) has been thoroughly studied by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Maizel et al., 1968; Maizel, 1971) but information about the protein structure of AAVs is very limited.

Despite considerable investigations on the epidemiology of IBH throughout the world, very little is known of the pathogenesis of infection following exposure to virus by a natural route (Cook, 1983).

Most of the literature review presented in this chapter is related to the general features of adenoviruses including those of human and animal origin but with a major emphasis on the AAV. A limited review of the technical procedures relevant to the particular experiments undertaken is presented in the introduction to each respective chapter.

Classification

The family Adenoviridae comprises a large number of serotypes both of human and animal origin including those from birds. Most members of this family share common group-specific antigenic determinants, which, together with the capsid morphology, are used as criteria for inclusion in the family Adenoviridae (Alestrom et al., 1982). AAVs do not share the common group-specific adenovirus antigen (Norrby et al., 1976), and the inclusion of these viruses in the family is primarily based on morphological criteria (Alestrom et al., 1982; Bagust, 1982). Among the mammalian adenoviruses, the members of subgroup 2 bovine adenovirus (BAV) do not contain the common group-specific complement fixing (CF) antigen (Bartha, 1969).

The type-specific antigens that define the adenoviral serotypes are localized on the hexons and measured by VN tests. On the basis of these type-specific antigens, 41 human, 24 nonhuman primate, 9 bovine, 4 porcine, 5 ovine, 2 equine, 2 canine, 1 caprine, 2 murine, 1 peromyscus and 1 opossum serotypes comprising the genus Mastadenovirus and 12 chicken, 2 turkey, 3 goose, 1 pheasant, 1 duck and 1 frog serotypes plus EDS and THE related viruses comprising the genus Aviadenovirus, are recognized (Pettersson and Wadell, 1985). Adenoviruses isolated from different avian species are divided into three groups designated I, II and III on the basis of their subgroup-specific antigens. Group I represents the conventional 19 serotypes isolated from fowl, turkey, goose, pheasant, duck and other avian species. Group II represents viruses closely related to THE, MSD of pheasants and splenomegaly of chickens while the viruses associated with EDS of chickens form Group III (Wyand et al., 1972; Iltis et al., 1975; Van Eck et al., 1976; Domermuth et al., 1979; 1980; Winterfield, 1984; Domermuth and Gross, 1984; Zhang and Nagaraja, 1989). In addition to the recognized serotypes, serological intermediates are frequently encountered which indicates that wide genetic variations may be expressed within each group (Khanna, 1966b; Wigand and Fliedner, 1968; Cowen et al., 1977; Grimes and King, 1977a; Wadell et al., 1980).

The HAds have been studied most intensively. The 41 serotypes have been divided into six subgenera based on their genome composition (Green et al., 1979; Wadell et al., 1980), into four groups based on a different type-specific haemagglutination (HA) antigen located on the tip of the penton fibres (Rosen, 1960) and into highly oncogenic (A), weakly oncogenic (B) and nononcogenic viruses on the basis of their oncogenicity for newborn hamsters (Huebner, 1967). The nononcogenic HAds can, however, transform rodent cells in vitro and they have been divided into subgroups C and D based on the antigenic reactivity of their specific tumour (T) antigen (McAllister et al., 1969; Gerna et al., 1982). The virion polypeptides, studied by SDS-PAGE, have also been used as a more discriminative criterion for the classification of human (Wadell, 1979; Wadell et al., 1980) and simian (Tikchonenko, 1984) adenoviruses.

Information on homology between the DNAs of various HAds has been obtained by filter and liquid hybridization and by the use of restriction endonucleases (Green, 1970; Green et al., 1979). Genetic variability within each serotype can be studied by analysing the restriction enzyme digestion patterns of the DNA and, within the HAds, distinct viral genome types have been identified (Wadell et al., 1981).

The AAVs in chickens have been classified by a number of workers on the basis of their type-specific antigens as determined by VN (Clemmer, 1964; Kawamura et al., 1964; Khanna, 1966b; Burke et al., 1968; McFerran et al., 1972; Calnek and Cowen, 1975; Grimes and King, 1977a; McFerran and Connor, 1977). Strains of broad antigenicity which bridge 2 or more groups have also been recognized (Khanna, 1966b; Cowen et al., 1977; Grimes and King, 1977a). A proposed classification of Group I AAVs in chickens is shown in Table 1.

In addition, attempts have been made to classify the AAVs on the basis of oncogenicity (Ishibashi et al., 1980), INIB formation (Adair, 1978), HA activity (Clemmer, 1964; Burke et 1968) and analysis of the endonuclease digestion patterns (Zsak and Kisary, 1984). Eleven serotypes (17 strains) of AAV have been divided into 5 groups (A-E) by analysing the electrophoretic patterns of their genomes following digestion with the restriction endonucleases BamHI and HindIII (Zsak and Kisary, 1984). Group A represented strains CELO (serotype 1) and 112 (serotype 1); Group B, strains 340 (serotype 5) and TR22 (serotype 5); Group C, strains KR5 (serotype 4) and C2B (serotype 11); Group D, strains 380 (serotype 12), 685 (serotype 2), SR48 (serotype 2), SR49 (serotype 3) and A2 (serotype 10) and Group E, strains YR36 (serotype 7), CR119 (serotype 6), X11 (serotype 7), H6 (serotype 8), TR59 (serotype 8) and 764 (serotype 9).

Ideally, adenoviruses, or members of any other family of viruses, should be classified according to sequence homologies since the evolution of a virus is the result of changes in the nucleotide sequence of the genome (Alestrom et al., 1982).

Table 1. **Classification of Group I chicken adenoviruses by cross-neutralization in cell culture.**

Proposed serotype	Proposed prototype strain	Original references (prototype strain)
1	CELO	Yates and Fry (1957)
2	GAL1	Burmester et al. (1960)
3	SR49	Kawamura et al. (1964)
4	KR5	Kawamura et al. (1964)
5	340	McFerran et al. (1972)
6	CR119	Kawamura et al. (1964)
7	YR36	Kawamura et al. (1964)
8	TR59	Kawamura et al. (1964)
9	764	McFerran et al. (1972)
10	A2	Calnek and Cowen (1975)
11	C2B	Calnek and Cowen (1975)
12	380	McFerran and Connor (1977)

Obtained from McFerran (1981a)

Morphology

Electron microscopic (EM) studies of several adenoviruses from human and animal sources indicate that they are medium-sized isometric particles with an icosahedral shape and 70-80 nm in diameter (Horne et al., 1959; Davies et al., 1961; Horne, 1962). The virion has no envelope and possesses a core of 60-65 nm (Horne et al., 1959; Dutta and Pomeroy, 1963). The adenovirion consists of two structural complexes: the capsid (outer icosahedral shell) and the core (internal body comprising the nucleocapsid and the core shell) (Nermut, 1987). The nucleocapsid consists of DNA and 3-4 closely associated proteins (Nermut, 1987). The icosahedral capsid is composed of 252 hollow capsomeres (7-9 nm in diameter) arranged in 5:3:2 rotational cubic symmetry with 12 vertices (corners), 20 triangular facets and 30 edges

(Horne et al., 1959). The facets are formed by 240 nonvertex capsomeres called hexons which are symmetrically arranged so that each hexon is surrounded by six neighbours (Ginsberg et al., 1966). Each of the vertices contains a single capsomer (penton) which is surrounded by five neighbours (Ginsberg et al., 1966). From each vertex capsomer or penton base an antenna-like projection (fibre) with a terminal knob extends outwards. This may vary in length (10-37 nm) depending on the serotype (Valentine and Pereira, 1965). AAVs possess similar morphology to mammalian adenoviruses (Dutta and Pomeroy, 1963; Fenner et al., 1974). All of the isolates made at the Stormont Laboratories, Belfast, Northern Ireland, and the prototype avian strains fall within the size range of 74-80 nm and differences have been described between mammalian and AAVs in the size and number of the penton fibres (McFerran, 1981a). Laver et al. (1971) reported two fibres on CELO virus (serotype 1), one long (42.5 nm) and the other short (8.5 nm). More recently, double fibres have been shown to be a common feature for all AAVs other than EDS virus (Gelderblom and Maichle-Lauppe, 1982). Variations in fibre length have also been reported between serotypes of AAV by the same authors. Serotype-specific differences in fibre lengths have been observed for several HAds (Norrby, 1969; Schlesinger, 1969) and for canine adenoviruses (Marusyk et al., 1970).

Biochemical and biophysical properties

The estimated MW of the mammalian and avian adenovirion is 170-175 X 10^6 daltons (Schlesinger, 1969; Laver et al., 1971). Although it is generally accepted that the mature mammalian virion has a density of 1.33 gm/ml and the AAV virion a density of 1.35 gm/ml in CsCl (Norrby et al., 1976), there is little evidence for differences between specific serotypes of mammalian or avian viruses. The value of 1.34 gm/ml in CsCl for BAV (serotype 3) has been reported by Niiyama et al. (1975) and densities between 1.32 gm/ml and 1.37 gm/ml in CsCl have been estimated for AAV isolates (Laver et al., 1971; Potter et al., 1971; Yasue and Ishibashi, 1977; Todd and McNulty, 1978). A value of 1.32 gm/ml was obtained for haemagglutinating adenovirus (EDS), strain 127 (Todd and McNulty, 1978) and 1.32 - 1.34 gm/ml for the THE and MSD viruses (Carlson et al., 1974; Iltis et al., 1975).

The HAds contain 87% protein and 13% DNA in contrast to 83% protein and 17% DNA for the AAVs. No lipid has been demonstrated in adenoviruses. The sedimentation coefficient of the adenovirus particles is 560S and that of the DNA is 29-32S for mammalian and 35S for avian viruses (Nermut, 1987).

a) Resistance to chemical agents

Stability to at least one of the organic solvents that is, ether, chloroform and sodium deoxycholate has been reported by many workers (Sharpless et al., 1961; Monreal and Ahmed, 1963; Petek et al., 1963; Clemmer, 1964; Burke et al., 1968; McFerran et al., 1972; Winterfield et al., 1973). Kawamura et al. (1964) demonstrated the stability of eight Japanese strains of AAV to chloroform, ether, sodium deoxycholate and trypsin.

The AAVs have also been reported to be resistant to saponin (Krauss, 1965), 2% phenol and 50% ethyl alcohol (Petek et al., 1963; Adlakha, 1966a), frigen 113 and pentane (Krauss, 1965) and merthiolate (Adlakha, 1966a). The AAVs were found to be sensitive to 100% ethyl alcohol (Petek et al., 1963). Clemmer (1964) found serotype 1 AAV (strain 93) to be resistant to several genetron (fluorocarbon) treatments, but sensitive to a 1:10,000 concentration of formaldehyde. Resistance of serotypes 2 (C74) and 8 (764) AAVs to chloroform has been reported by McFerran et al. (1972). An adenovirus causing IBH in chickens has been shown to be stable to chloroform and acid (pH 3.0) (Winterfield and Fadly, 1973).

b) Changes in pH

The AAVs are resistant to variations in pH (Adlakha, 1966a; Cho, 1971; Mustaffa-Babjee and Spradbrow, 1975). Yates (1960) found that the titre of CELO virus was not affected over a pH range of 2-9. Similarly a number of American strains of AAV have been shown to resist pH values as low as 2 (Burke et al., 1965; Adlakha, 1966a). A goose strain of AAV showed no loss of infectivity at pH 3.0 (Csontos, 1967).

c) Heat

Human and other mammalian adenoviruses are considered to be heat labile, but the AAVs so far tested have shown relative heat resistance (Aghakhan, 1974). The resistance of CELO (Phelps) virus to 56^{o}C for 90 min was first reported by Yates and Fry (1957). The CELO (EV89) virus has also been reported to survive at 56^{o}C for 22 hr (Burke et al., 1959). Two strains (65 and 74) of serotype 2 AAV have been found to resist 60^{o}C and 50^{o}C for 40 and 180 min, respectively (Cho, 1971).

Heat sensitivity is reduced in the presence of Na^{+} and increased in the presence of Mg^{++} and Ca^{++} ions (Wallis et al., 1962). All strains were found to be sensitive at 50^{o}C in the presence of 1M $CaCl_2$ or 1M $MgCl_2$ (divalent cations) but were unaffected in the presence of 1M NaCl or in maintenance medium, glycerine or in distilled water (Petek et al., 1963;

Burke et al., 1965). A decreased stability in the presence of divalent ions was demonstrated by McFerran et al. (1972) for AAV serotype 8 (strain 764). No significant drop in titre was found in AAV held at 4-5^{o}C for 17 weeks, and viability was maintained over seven years at -20^{o}C (Yates, 1960).

d) Light

CELO virus was shown to be relatively resistant to ultraviolet (UV) irradiation but sensitive to photodynamic inactivation (Petek et al., 1963). Resistance to UV light was also reported for the Delhi strain of CELO virus (Adlakha, 1966a).

Adenoviral genome

The genome consists of a single linear molecule of double stranded DNA with a MW of 20-30 x10^{6} daltons (Green et al., 1967; Van der Eb et al., 1969). Biochemical evidence suggests that the adenoviral DNA molecules are quite compact because of their close association with polypeptides V, VII and X (Van der Eb et al., 1969; Nermut, 1987). The adenoviral genome contains a terminal protein covalently bound to the 5' end of each strand of the DNA molecule (Robinson et al., 1973; Robinson and Bellett, 1974; Rekosh et al., 1977). Since adenoviral DNA replication is initiated at this terminus of the DNA molecule (Winnacker, 1978), it has been proposed that the terminal protein may serve as a primer for initiation of replication (Rekosh et al., 1977). The HAd genome (11-12 um long, MW 20-25 x 10^{6}) constitutes about 13% of the total virion mass corresponding to 35,000 - 40,000 base pairs (Nermut, 1987). The characteristics of the genome vary significantly between different serotypes of the viruses. CELO is the only AAV in which the genomic DNA has been characterized to some extent. General properties of the genome such as the linear double strandness and the presence of the inverted terminal repeat in the nucleotide sequence are essentially similar to those of HAds (Laver et al., 1971; Robinson et al., 1973; Robinson and Bellet, 1975; Denisova et al., 1979; Alestrom et al., 1982). The MW of CELO virus DNA is 30 x 10^{6} daltons which constitutes 17.3% of the virion mass (Laver et al., 1971) and is 10-30% larger than the DNAs of any mammalian adenoviruses so far reported (Shimada et al., 1983). The sedimentation coefficient of the DNA genome is 29-32S for mammalian and 35S for AAV. The percentage of guanine and cytosine (GC) content is 47-60 in human, 51-62 in simian and 54 in CELO virus and the GC content of HAd DNA appears to be correlated with the degree of oncogenicity (Pina and Green, 1965). The MWs of human oncogenic adenoviral DNAs were found to be slightly lower than the MWs of nononcogenic viruses (Studier, 1965). So far, no comparison between the DNA of CELO virus and that of

the nononcogenic AAVs has been reported.

In addition to the recognized serotypes of adenoviruses, serological intermediates are frequently encountered (Wigand and Fliedner, 1968). This indicates that a wide genetic variation is expressed within each group of adenoviruses (Wadell et al., 1980). Restriction endonuclease digestion patterns of DNA provide a means to analyse the genetic variation within each adenovirus serotype by identifying distinct viral entities and genome types (Wadell et al., 1980; 1981). Analysis of the HAd genomes with the endonuclease SmaI showed that 41 serotypes could be divided into six subgroups which were designated A - F. This subdivision agreed well with classification on the basis of the apparent MW of the internal polypeptides, DNA homology, oncogenicity, T antigen groups, and GC contents of viral DNA (Green et al., 1979; Wadell et al., 1980; Pettersson and Wadell, 1985). Eleven AAV serotypes (17 strains) have also been divided into five subgroups, designated A - E, on the basis of genome analysis with the endonucleases BamHI and HindIII (Zsak and Kisary, 1984). Extensive sequence homology studies have shown a remarkably high degree of sequence variation between the different subgroups of HAds (Lacy and Green, 1964; Green, 1970; Garon et al., 1973; Alestrom et al., 1982). As determined by DNA:DNA hybridization, the genomes of selected members within each group of HAds share homology as high as 100% (except group A), whereas DNA from different groups may contain as little as 10% sequence homology (Green, 1970; Garon et al., 1973).

Comparative hybridization studies suggest that the DNA of HAd serotype 2 and CELO virus contain two widely separated regions which have a high degree of homology. One is close to the coding sequence for the HAd terminal protein and the other is within the coding sequence for the HAd hexon (Alestrom et al., 1982).

BAV has been divided into subgroup 1 (serotypes 1, 2, 3, and 9) and subgroup 2 (serotypes 4, 5, 6, 7 and 8) on the basis of restriction patterns of their genomes (Benko et al., 1988). The nucleotide sequence recognized by EcoRI is less frequent (4-7) in the genomes of subgroup 1 than in that of subgroup 2 members (10-16). The genome of the subgroup 1 serotypes is larger (32.73-37.67 kb) than the genome of subgroup 2 serotypes (28.78-31.18 kb). It is noteworthy that there is no common restriction site for EcoRI, HindIII and BamHI between the genomes of BAV 7 and BAV 3 though they share common group-specific CF antigens (Hu et al., 1984).

Structural proteins

Protein constitutes 87% and 83%, respectively, of the total virion mass of the mammalian and avian adenoviruses (Nermut, 1987). SDS-PAGE analysis of HAds revealed a minimum of 10 structural polypeptides (II, III, IIIa, IV-X) (Maizel, 1971; Everitt et al., 1973). Five of them (II, III, IV, V, VII) are integral parts of hexons, pentons, fibres and cores (Maizel et al., 1968; Prage and Pettersson, 1971; Russell et al., 1971). In addition to II, polypeptides VI and VIII appeared to be associated with hexons (Everitt et al., 1973). Polypeptide IX seems to be the cementing polypeptide which is located between the hexons on the facets of the virion capsid. Pentons contain the major polypeptide III. The polypeptide IIIa may be closely associated with the pentons (Everitt et al., 1973). The fibre contains the polypeptide IV. The chemical composition of the HAd core has been studied intensively. The core contains viral DNA and 180 copies of polypeptide V and 1070 copies of VII (Laver, 1970; Prage and Pettersson, 1971; Everitt et al., 1973). Polypeptide VII is an alanine-arginine rich protein containing 18-19% of alanine and 21-23% arginine residues. Polypeptide VII comprises approximately 14% of the viral proteins. Everitt et al. (1973) have proposed that the core polypeptide V is associated both with the viral DNA and the penton bases, whereas polypeptide VII is tightly bound to the viral DNA. In addition to two major core polypeptides (V, VII), 125 copies of the fastest migrating polypeptide designated as u or X (MW 5000-6000) are recognized in the HAd core (Anderson et al., 1973; Hosakawa and Sung, 1976). The internal polypeptides XI and XII are identified by SDS-PAGE but these may be the cleavage fragments of higher MW proteins (Nermut, 1980).

At least 14 structural proteins with a range of MWs from 100Kd to 6Kd have been identified in CELO virus (Li et al., 1984a). The protein content of CELO virus was found to be 80.7% compared with 88.4-86.5% for HAd (Schlesinger, 1969; Laver et al., 1971). The CELO virus core contains polypeptides IX, XI and XII which are quite different from those in HAd (polypeptide V and VII) (Li et al., 1984b).

Tryptic and chymotryptic peptide maps, labelled with I^{125} demonstrated differences in the hexons and terminal proteins from CELO virus and HAd serotype 5 (Li et al., 1983). When ^{125}I and ^{35}S methionine-labelled terminal proteins were compared by SDS-PAGE, the CELO viral protein migrated slightly faster with a MW of 46000 compared with 52000 for the terminal protein of HAd serotype 5 (Stillman et al., 1981). Using protein markers with MW over 100000 (Jornvall et al., 1981) a MW of 101000 daltons was obtained for the CELO viral hexon (Yasue and Ishibashi, 1977) compared to 103000 (Bahr-Lindstrom et al., 1982) for HAd serotype 5 hexon. The proteolytic cleavage patterns showed that while the two hexons exhibited no detectable similarities, the terminal proteins showed similarities in the number

and sizes of the peptides generated by partial proteolysis (Li et al., 1983).

Viral antigens

Most of the information on the antigenic structure of adenoviruses has been derived from studies with a relatively small number of human serotypes. However, ultrastructural and biochemical studies have shown that, in structure and composition, avian and mammalian viruses are similar. Since the major antigens of the human viruses have been related to structural components of the intact virions, it is likely that the antigens of the avian viruses are similarly located.

An adenoviral infection, or growth of adenovirus in cell culture, is accompanied by the production of a large number of antigenically different, virus-specific proteins (Huebner et al., 1954; Pereira et al., 1959), each of which carries several epitopes (Pettersson and Wadell, 1985). Three types of antigen designated A, B and C, were demonstrated by immuno-electrophoresis (Pereira et al., 1959). The same antigens were separated by elution from DEAE-cellulose and were called E (early eluting), L (late eluting) and T (toxin) (Wilcox and Ginsberg, 1961). Ginsberg et al. (1966) proposed that these antigens be named hexon (antigen A or L), penton (antigen B or toxin) and fibre (antigen C or E).

Two major antigens, alpha and epsilon, are associated with hexons and are responsible for the group- and the type-specific reactions which form the basis of the adenoviral classification (Petersson and Wadell, 1985). The penton base has a weak group-reactive antigenic factor called beta and the toxic or cell-detachment factor. The penton fibre has both type- and subgroup-specific antigenic factors. The type antigen gamma, located on the terminal knob, is responsible for inducing type-specific haemagglutination inhibiting (HAI) antibodies. The subgroup-specificity depends on the shaft of the fibre. In addition, the fibres of some serotypes carry intersubgroup-specific determinants (Norrby, 1969; Wadell and Norrby, 1969; McFerran, 1981a). It has been suggested that the length of the fibre influences the antigenic complexity, with larger fibres tending to possess the intrasubgroup and sometimes the intersubgroup-specificity (Pettersson et al., 1968). Different antigens on each of the structural proteins may be detected by different procedures e.g. CF, VN, HAI, IF and GD using antisera to virions or purified capsid proteins (Green et al., 1979).

The AAVs isolated from fowl, turkeys, geese and other avian species have a common CF group-specific antigen located on the hexons and this antigen is distinct from that of the human and other mammalian adenoviruses (Kawamura et al., 1964; McFerran et al., 1975).

Three subgroups of AAV are recognized depending on their group-specific antigens (McFerran, 1981a). The conventional AAVs (Group I) which have been isolated from all avian species possess a common group antigen which is different from that of the other two groups (Kawamura et al., 1964; McFerran et al., 1975). Adair et al. (1980) demonstrated that antibody to serotype 1 AAV (Phelps strain) could be detected using any of the prototype strains of chicken (12 serotypes) as antigen in an IF test. Similar subgroup-specificity has also been demonstrated between 12 serotypes of AAV in chickens by ELISA (Calnek et al., 1982; Saifuddin and Wilks, 1990a). The type-specific antigens may be detected by VN tests and have permitted classification of Group I AAVs into 12 serotypes for chicken, 2 for turkey, 3 for goose, 1 for pheasant and 1 for duck. The type-specific HA (gamma) antigen located on the penton has been useful for classifying the HAds. HA activity has only been demonstrated with serotype 1 strains of Group I AAVs and EDS viruses from Group III. The conventional AAVs of chickens have also been isolated from turkeys, ducks, pigeons, quail, pheasants, guinea fowls and from several psittacine species (DuBose and Grumbles, 1959; Pascucci et al., 1973; Blalock et al., 1975; McFerran et al., 1976b; Scott et al., 1986). All of these isolates share a common Group I antigen. The EDS viruses do not share any serological relationship with the conventional adenoviruses (Group I) or THE related viruses (Group II) in IF or GD tests (Baxendale, 1978; McFerran et al., 1978a). However, birds which had been infected with Group I AAV, but no longer had detectable precipitating antibody, and were subsequently infected with EDS virus developed precipitating and HAI antibody to EDS virus as well as precipitating antibody to the AAV group antigen, indicating that EDS and AAV share an antigen (McFerran et al., 1978a; Darbyshire and Peters, 1980). This is further evidence for the inclusion of EDS viruses within the genus Aviadenovirus. The viruses of THE, MSD of pheasants and splenomegaly of chickens share a common subgroup antigen, detectable in GD tests, which is distinct from that of conventional AAVs, EDS, human or any other mammalian adenoviruses (Jakowski and Wyand, 1972; Iltis et al., 1975; Silim et al., 1978; Domermuth et al., 1979; Darbyshire, 1980;).

A class of specific early proteins known as T antigens is detectable by CF and IF tests with sera from hamsters bearing tumours induced by oncogenic AAVs or HAds (McAllister et al., 1969; Gerna et al., 1982). These T antigens are not viral structural proteins and presumably represent viral-coded polypeptides induced during early stages of infection (Huebner, 1967; Green et al., 1979).

Haemagglutination

Rosen first demonstrated the HA activity of an adenovirus in 1958. Subsequently it was

shown that almost all the HAd serotypes agglutinated various mammalian red blood cells (Rosen, 1960; De Jong et al., 1983). Rosen (1960) originally proposed four (I-IV) subgroups of HAds depending on their ability to agglutinate rat and rhesus monkey erythrocytes. Subgroup I agglutinated rhesus monkey but not rat erythrocytes. Subgroup II agglutinated rat but not rhesus monkey erythrocytes and subgroup III caused partial agglutination of rat but not rhesus monkey erythrocytes. Subgroup IV comprised those viruses causing no agglutination of rat or rhesus monkey erythrocytes. Hierholzer (1973) divided HAds into 10 subgroups based on their ability to agglutinate rhesus monkey, human and rat erythrocytes.

A number of Group I AAVs have been shown to agglutinate rat erythrocytes (Clemmer, 1964; Burke et al., 1968; Fadly and Winterfield, 1975). Fadly and Winterfield (1975) demonstrated the agglutination of sheep RBC by Indiana C strain (serotype 1) of AAV. Burke et al. (1968) demonstrated agglutination of rat erythrocytes but not sheep or avian erythrocytes by 5 of 7 American strains of AAV. No HA or haemadsorption of fowl erythrocytes was found with Japanese serotypes (Kawamura, et al., 1964). The EDS virus (strain 127) agglutinates fowl, duck and turkey but not mammalian erythrocytes (Adair et al., 1979b; McFerran, 1984).

Several other mammalian adenoviruses including simian, bovine, equine and canine have been reported to agglutinate erythrocytes of different species (Tyrrell et al., 1960; Rapoza, 1967; Hierholzer, 1973; Wilks and Studdert, 1973).

HA studies suggested that the penton fibre contains two immunological specificities, namely, type-specificity and subgroup-specificity (Pereira and de Figueiredo, 1962; Valentine and Pereira, 1965; Norrby and Skaaret, 1967). The type-specific determinant (Y) of the fibre interacts with the specific receptors on the red cell surface of certain animal species (Valentine and Pereira, 1965; Pettersson et al., 1968). A broad variability is seen in the reactivity of receptors on different erythrocytes (Pettersson and Wadell, 1985). The number of receptors for different adenovirus serotypes varies for different subgenera (Wadell et al., 1969; Inouye and Norrby, 1973). Antibodies directed against the Y specificity of the fibre can be assayed by HAI test using specific antisera (Pettersson and Wadell, 1985).

Virulence

Marked differences in virulence between isolates of the same and different serotypes of

AAV have been demonstrated (Winterfield, 1984). A variable mortality rate (30% and 9%) and different duration of viral persistence in tissues following oronasal administration of two strains (HV7 and GAL) of serotype 2 AAV to day-old SPF chickens under similar conditions has suggested that the virulence of AAVs is not necessarily serotype-dependent (Cook, 1983). Cook (1983) observed a mortality rate of less than 3% after oronasal administration to day-old SPF chicks of serotype 8 (strains TR59 and H6) AAV, whereas, Reece et al. (1987) demonstrated 30% mortality following oral inoculation of day-old SPF chicks with VRI33 strain of serotype 8 AAV. In recent years, IBH has been a significant problem in the Australian poultry industry and serotype 8 AAVs were commonly isolated from the affected flocks (Kefford and Borland, 1979; Kefford et al., 1980; Reece et al., 1986a). In earlier reports, most of the IBH outbreaks in Australia were associated with concurrent infection with infectious bursal disease (IBD) virus whereas in recent years, no evidence of IBD has been reported in some outbreaks of IBH infection (Reece and Beddome, 1983; Reece et al., 1986b). This suggests that the strains of AAV involved in the recent outbreaks are capable of acting as primary pathogens rather than requiring prior immunosuppression by an agent such as IBD virus.

It appears that there is an age-related resistance to infection. After 3-4 weeks of age, chickens are increasingly more resistant to the clinical effects of infection with isolates causing the IBH-anaemia syndrome, even though they lack VN antibodies (Winterfield, 1984). However, clinical IBH has also been reported in 9 weeks old (Bickford et al., 1973), 12 weeks old (Reece et al., 1986b) and older birds (Hoffmann et al., 1975; McFerran, 1981a).

Twelve serotypes of AAV have been recognized. Some of these can be readily isolated from both apparently healthy and diseased flocks and this has led to the conflicting views on their role as pathogens. Although many investigators (McDougall and Peters, 1974; MacPherson et al., 1974; Rosenberger et al., 1974; Grimes et al., 1977b; McCracken et al., 1976) have been able to reproduce the IBH syndrome following inoculation of virus by a parenteral route, clinical IBH following a natural route of exposure with any serotype or isolate has been infrequently reported (Cook, 1983; Reece et al., 1987). However, workers at Purdue University, were able to consistently reproduce a severe hepatitis in chickens using the Tipton (Fadly and Winterfield, 1973) and Indiana C (Gallina et al., 1973; Winterfield et al., 1973) isolates by both natural and parenteral routes. McDougall and Peters (1974) have been unsuccessful at inducing hepatitis by natural routes of infection with serotypes 1, 2, 5, and 8 but these isolates were capable of producing clinical hepatitis when they were inoculated parenterally.

It is clear that the aetiology of IBH is not confined to any serotype of AAV. Thus serotypes 1 (Winterfield et al., 1973), 2, 3 and 4 (McFerran et al., 1976b; Grimes and King, 1977a), 5 (Fadly and Winterfield, 1973; McFerran et al., 1976b), 8 (MacPherson et al., 1974; McFerran et al., 1976b; Grimes et al., 1977b), and 9 (Grimes et al., 1978) have all been isolated from clinical outbreaks of IBH. In addition, unclassified adenoviruses have also been found in association with clinical IBH (Di Franco et al., 1974; Wells and Harrigan, 1974; Kefford et al., 1980; Reece et al., 1986b).

Occurrence

The first recorded isolation of an AAV was from chicken embryos which had been inoculated with calf tissue (Van den Ende et al., 1949) in South Africa. In the same year Olson (1950) isolated QB virus from West Virginia bobwhite quail in the USA which were suffering from an acute respiratory disease with high mortality. This was considered as the first recorded clinical infection caused by an AAV. Subsequently the occurrence of CELO virus in chicken embryos (Yates and Fry, 1955; 1957) and GAL virus in cultures of chicken embryo cells (Burmester et al., 1960; Fontes et al., 1958; Sharpless et al., 1958). were reported in the USA. In Texas, a similar outbreak of respiratory disease was reported in quail (DuBose et al., 1958). In 1959, DuBose and Grumbles demonstrated that the QB virus was indistinguishable serologically from the CELO virus. Since then the isolation of several serotypes of AAV including CELO, GAL and QB viruses and serological evidence of their presence have been frequently reported in several countries including the USA (Ablashi, 1962; Taylor and Calnek, 1962; Clemmer, 1964; Winterfield et al., 1973), Ireland (McFerran et al., 1971; 1972; Campbell and O'Connor, 1973), Canada (Trewick and Lang, 1971), Japan (Kawamura et al., 1963; 1964), Germany (Woernle and Brunner, 1963; Luthgen et al., 1967), Sweden (Bakos, 1963), France (Sharpless, 1962), Hungary (Khanna, 1964; 1966a), Italy (Lombardi, 1967; Rinaldi et al., 1968), Yugoslavia (Mazija et al., 1972), Czechoslovakia (Forejtek and Jurajda, 1972), India (Adlakha, 1966b), Korea (Choi, 1973), Egypt (Ahmed et al., 1969), Australia (Cox, 1966; Wells and Harrigan, 1974) and New Zealand (Green et al., 1976; Christensen and Saifuddin, 1989).

Until 1970 there was limited serological evidence for the occurrence of AAV in Britain (MacPherson et al., 1961; Oxford and Potter, 1969). Since then serological evidence of CELO virus infection has been demonstrated in a high percentage of birds and QB was observed in several states in North America (Cook, 1968). VN antibodies to numerous serotypes of AAVs have been detected in birds throughout the world, confirming the widespread occurrence of adenoviral infections (Kawamura et al., 1964; Aghakhan, 1974;

Calnek and Cowen, 1975; McFerran et al., 1975).

IBH was first reported in 7-week-old chickens by Helmboldt and Frazier (1963) in the United States. Subsequently it has been reported in broiler flocks in Canada (Howell et al., 1970; Pettit and Carlson, 1972), USA (Fadly and Winterfield, 1973), England (Laursen-Jones, 1972), Ireland (Young et al., 1972; McFerran et al., 1976b), Japan (Nakamatsu et al., 1968; Yamanaka and Kita, 1974), Chile (Cubillos et al., 1986), Australia (Wells and Harrigan, 1974; Wells et al., 1977; Reece at al., 1986a) and New Zealand (Christensen and Saifuddin, 1989).

Host range

The adenoviruses are widely distributed in several species of animals (including man) and birds. So far, several serotypes of mammalian adenoviruses have been isolated from man, nonhuman primates, cattle, pigs, sheep, horses, dogs, goats, mice, opossums and deer (Pettersson and Wadell, 1985; McFerran, 1981a).

Chickens, turkeys, geese and ducks each have their own adenoviruses (Group I) and at least 12 different serotypes have been identified from chickens (McFerran and Connor, 1977), at least 2 from turkeys (Scott and McFerran, 1972; Sutjipto et al., 1977), 3 from geese (Csontos, 1967), 1 from pheasants and 1 from ducks (McFerran, 1981a). The turkey AAVs (Group I) studied share a group antigen with chicken adenoviruses, but are not neutralized by antisera to the prototype chicken strains (McFerran et al., 1975), and either do not grow or grow poorly in cells of chicken origin (Scott and McFerran, 1972).

The AAV serotypes isolated from chickens have also been reported in turkeys, ducks, geese, pheasants, quail, pigeons and various psittacine birds (McFerran et al., 1976a; Ahmed, 1971; Blalock et al. 1975; Cho, 1976). Olson (1950) first isolated chicken serotype 1 AAV from quail associated with severe bronchitis. Quail and chickens may both be naturally infected with the same strain of CELO or QB virus (AAV serotype 1). Serological findings indicate that CELO virus infection also occurs in turkeys (Ahmed et al., 1969), pheasants (Yates, 1960; Cakala, 1966), guinea fowls (Pascucci et al., 1973), goshawk (Stehle, 1965) and red wing blackbirds and swans (Yates, 1960). There has been no report of the isolation of GAL virus or the occurrence of specific antibodies to it in species other than chickens (Kohn, 1962; Sharpless, 1962). Other serotypes of chicken adenoviruses such as 2, 8 and 5 have been isolated from budgerigars, pigeons and budgerigars and mallard duck, respectively (McFerran et al., 1976a). The occurrence of AAVs in different

species of migratory birds has also been reported (Ahmed et al., 1968; Mathur et al., 1972). Adenovirus particles seen in the bursa of Fabricius in herring gulls may be related to AAV (Leighton, 1984). Frog adenovirus type 1, isolated from a granuloma-bearing kidney of a leopard frog (Rana pipiens) has been considered as a member of the genus Aviadenovirus because this virus does not possess the group specific, CF antigen of mammalian adenoviruses (Clark et al., 1973; Pettersson and Wadell, 1985).

IBH is usually a disease of young chickens caused by any one of several serotypes of AAV (Howell et al., 1970; MacPherson et al., 1974; Hoffmann et al., 1975). It has also been reported in quail (Jack et al., 1987), in turkeys (Guy et al., 1988), geese (Riddell, 1984), guinea fowls (Reece and Pass, 1986), pigeons (Coussement et al., 1984; Goryo et al., 1988), kestrels (Sileo et al., 1983) and various psittacines including parrots (Simpson and Hanley, 1977), cockatiels (Scott et al., 1986), love birds (Pass, 1987), budgerigars and parakeets (Mori et al., 1989).

The EDS viruses have only been reported in chickens (Van Eck et al., 1976; McCracken and McFerran, 1978) and the THE related viruses in turkeys, pheasants and chickens (Domermuth et al., 1979; 1980).

Transmission

Shedding in the faeces and to a lesser extent in the naso-oral secretions are the major factors in the spread of AAV which occurs through contaminated food, water and the environment (McFerran and Adair, 1977; McFerran, 1984). The high titre of AAV in the faeces during the acute phase of infection (Kohn, 1962; Kawamura et al. 1963; Clemmer, 1965; 1972) makes it likely that oral transmission occurs via contaminated water and food. Excretion of AAV in the faeces for 21 days post inoculation (pi), with peak titres at 4-7 days pi has been demonstrated (Kohn, 1962; Kawamura et al., 1963). Khanna (1966b) reported the excretion of virus in faeces for 2 months after infection. Suprisingly, Ahmed (1971) isolated the virus from gall bladder and intestines at 93 weeks after infection although it has been postulated that the virus present in the litter was reinfecting the birds (McFerran and Adair, 1977). It is unknown if some isolates, or serotypes, can be shed over a prolonged period or whether a carrier state may be established in infected chickens. Air borne transmission of AAV is not an efficient method for spread of virus (Kohn, 1962; Monreal, 1966; Cook, 1974a; Montgomery, 1974), although Yates (1960) and Winterfield and DuBose (1984) suggested that air borne infection may also be one of the principal routes of infection under certain circumstances.

Farm to farm transmission by contaminated litter, feed and other fomites, aided by the stability of the virus, is undoubtedly important. While wild birds for example, pigeons, are susceptible to infection with AAVs and could therefore aid the spread of virus, it is probable that they are not of major importance (McFerran and Adair, 1977).

Transmission of CELO virus through eggs was first suggested by Yates and Fry (1957) because they were occasionally able to isolate virus from chicken eggs. It has been postulated that vertical transmission is an important factor in the epidemiology of IBH (McFerran and Adair, 1977) and evidence of vertical transmission of IBH virus has been presented by Reece et al. (1985). Fadly and Winterfield (1973) also presented evidence of egg transmission of the pathogenic Tipton strain (serotype 5) since the progeny of infected breeders were infected and showed signs of an IBH-anaemia syndrome. Presumably, following vertical transmission, the virus becomes unmasked with the loss of maternal antibody at 2-4 weeks, and spreads slowly through the flock (McFerran, 1981b; Kohn, 1962). It appears that birds in flocks in USA start shedding AAV at about 4 weeks of age and this continues for up to 30 weeks or so, with the highest levels of shedding occurring between 5 and 14 weeks. This differs from the pattern in flocks in Northern Ireland where shedding of virus commences usually at 3 weeks and reaches a peak from 4 to 8 weeks (McFerran, 1981b).

Epidemiological evidence suggests that the EDS virus is usually transmitted vertically but otherwise has limited lateral spread (McFerran, 1981b). There is no evidence of vertical transmission of THE virus and it is considered that the infection spreads laterally due to virus which persists in the litter between batches of birds (Domermuth and Gross, 1984).

Pathogenesis

Very little is known about the pathogenesis of infection following exposure of birds by a natural route to AAVs associated with IBH.

AAVs have been isolated from various tissues including blood (Cho, 1970), liver and spleen (Bakos, 1963), kidney (McDougall and Peters, 1974), trachea (Kawamura et al., 1963), intestine and bursa (Mustaffa-Babjee, 1971) and faeces (Burke et al., 1959). Kawamura et al. (1963) infected day-old chickens orally with serotype 1 AAV (strain Ote) and demonstrated widespread dissemination of virus in the body, with maximum titres in trachea and faeces. A viraemia was indicated by the isolation of virus from the serum on the 4th day pi. Infected birds excreted virus in the faeces for up to 21 days pi with peak titres at 4 to

7 days pi (Kohn, 1962; Kawamura et al., 1963). Chickens infected at 1 day of age excreted virus for longer periods and at higher titres than birds which were infected at 21 days of age (Clemmer, 1972). Kohn (1962) also found a relatively widespread distribution of GAL virus following intratracheal inoculation at 2 days of age in spite of high levels of maternal VN antibody. He considered the alimentary rather than the respiratory tract to be the main site of viral replication. Cook (1974b) recovered virus from several organs, with the highest titre in liver and intestine, after intraperitoneal (IP) inoculation of day-old chicks with GAL virus. The presence of IBH virus in a variety of tissues except brain was demonstrated in cell and organ cultures at different intervals following oral and intranasal administration of 5 strains representing 3 serotypes (2, 3 and 8) of AAV (Cook, 1983). Besides demonstrating the duration of virus, this study did not determine the first appearance or the amount of virus in a particular tissue, so the information obtained was limited.

Pathology

Although Group I AAVs are often associated with inapparent infection in chickens (McFerran et al., 1971) they are also frequently involved in various disease syndromes including IBH, respiratory illness, depressed egg production, arthritis, bursal and thymic atrophy, depressed growth and enteritis (McFerran and Adair, 1977). In quail, the effects of AAV infections are usually pronounced, resulting in acute respiratory disease with high mortality (up to 100%) and the signs include rales, coughing, depression, nervous signs and, in some birds, conjunctivitis (Olson, 1950; DuBose et al., 1958; DuBose, 1967).

IBH is associated with a sudden increase in mortality usually in the absence of overt clinical signs, and is characterized by the presence of hepatic necrosis with prominent eosinophilic (Howell et al., 1970; MacPherson et al., 1974; McFerran et al., 1976b) and/or basophilic (Young et al., 1972; Itakura et al., 1974; Reece et al., 1986a) INIBs. Palor of the bone marrow, anaemia and haemmorrhages of the muscles have been observed in conjunction with IBH (Fadly and Winterfield, 1973; Hoffmann et al., 1975). Occasionally severe gizzard erosion with haemorrhages has been reported (Grimes et al., 1977b). Multiple serotypes of Group I AAV have been implicated as causes of IBH in chickens (McFerran, 1981a) and birds can be infected by several serotypes at one time (Grimes et al., 1977a).

McCracken et al. (1976) described different hepatopathies produced by different strains of AAV given intramuscularly (IM) to 2-week-old specific pathogen free (SPF) chickens. Thus a diffuse and generalized hepatitis, diffuse and patchy hepatitis, focal hepatitis or no pathological changes were observed under experimental conditions. They also suggested

that hepatitis can be reproduced by several adenoviral serotypes but a poor correlation between the field IBH syndrome and experimental adenoviral infection has been reported (MacPherson et al., 1974; McCracken et al., 1976). Whilst muscle haemorrhages and palor of the bone marrow are seen in field cases of IBH (McFerran et al., 1976a) neither features were observed in the experimental disease. In addition, the frequent occurrence of gross liver changes at the margins of the liver lobes has not been recorded in natural IBH (McDougall and Peters, 1974; McCracken et al., 1976). In the natural disease, the inclusion bodies are almost always brightly eosinophilic and surrounded by a distinct halo (Howell et al., 1970; MacPherson et al., 1974; Hoffmann et al., 1975; McFerran et al., 1976b) whereas McCracken et al. (1976), Kawamura and Horiuchi, (1964), McDougall and Peters, (1974), McFerran et al. (1976b) and MacPherson et al. (1974) demonstrated that the inclusion bodies in experimentally induced hepatitis are consistently basophilic. The basophilic inclusion bodies generally occupy the entire nucleus so that a distinct peripheral halo is not evident (Bickford et al., 1973; McDougall and Peters, 1974; McCracken et al., 1976). Basophilic inclusion bodies have only been infrequently described in the natural disease (Young et al., 1972; Itakura et al., 1974). Eosinophilic inclusions are composed of filamentous material and do not contain virus particles whereas basophilic inclusions often enlarge the nucleus and contain virus particles (Pass, 1987).

However, in most outbreaks of IBH in Victoria, Australia, the INIBs in hepatocytes were basophilic, not eosinophilic (Reece et al., 1986a). Fadly and Winterfield (1973), Gallina et al. (1973), Winterfield et al. (1973) and Cook (1974b) described the presence of eosinophilic INIB (Cowdry type A) in hepatocytes of experimentally infected birds.

Although hepatitis with INIB in the hepatocytes is characteristic of IBH (Howell et al., 1970; Pettit and Carlson, 1972) the involvement of lymphoid organs, particularly bursa, thymus, spleen and bone marrow has also been reported by several workers (MacPherson et al., 1974; Fadly et al., 1980). Even experimentally, some workers have been able to reproduce similar IBH syndromes involving lymphoid organs (Hoffmann et al., 1975; Grimes et al., 1977b; Fadly et al., 1980).

It has been suggested that prior infection with IBD virus enhances the virulence of AAV and the bursal lesions described in natural outbreaks of IBH may have been due to IBD virus infection (Rosenberger et al., 1975; Fadly et al., 1976). However, results from both natural outbreaks (MacPherson et al., 1974; Christensen and Saifuddin, 1989) and experimental infections (Grimes et al., 1977b; Fadly et al., 1980) suggest that the IBD virus or any other factor is not always necessary for the development of the IBH syndrome and AAV can also produce lymphoid depletion of bursa, thymus and spleen. According to the morphological

findings of Hoffmann et al. (1975), both the cellular and humoral systems are involved in IBH infection in chickens. The histopathological changes in the bursa include severe follicular reduction with a few damaged follicles surrounded by increased fibrous tissue. Sometimes scattered cysts were observed in the medullary areas of lymphoid follicles (Itakura et al., 1974). Decreased lymphoid cells in the germinal centres with proliferation of reticular cells in the spleen, multiple haemorrhages in the medulla of the thymus and a small cortex are often demonstrated in IBH infection (Hoffmann et al., 1975).

Guy et al. (1988) demonstrated IBH associated with a high mortality rate in day-old turkeys. At necropsy, the turkeys appeared anaemic and had pale yellow livers. Histopathological examination of liver tissue revealed diffuse hepatic degeneration and multifocal necrosis with approximately 70% of the hepatocytes containing large basophilic INIB. Affected nuclei were enlarged and distorted, varying from round to elongated or crescent-shaped, with marginated nuclear chromatin.

Mori et al. (1989) described two types of INIBs in the renal tubules of budgerigars and parakeets depending on the size (large and medium) and staining characteristics. The large INIB contained basophilic granules which, by ultrastructural examination, were shown to consist of virus particles and fine filamentous material. The eosinophilic, medium-sized INIB almost filled the entire nucleus, had a clear halo, and were composed of aggregates of fine granules.

Immune response to infection

Under normal conditions, birds acquire maternal antibodies from the yolk sac to a number of serotypes of AAV (McFerran, 1981b). Individual chickens may have both maternal antibody and still be carrying virus (McFerran, 1981b). The titre of maternal VN antibody is at a peak 24-36 hr after hatching and thereafter declines over a 2-4 weeks period. At this stage in broiler flocks, the excretion of virus commences and virus can be isolated from the faeces, nasal mucosa and pharynx (McFerran, 1981b). A comprehensive survey demonstrated that VN antibodies to 7 or 8 serotypes of AAV were present in the sera of birds in 14 of 16 normal chicken breeder flocks in Georgia (Grimes et al., 1977a).

After intratracheal exposure to a high titred inoculum of a serotype 1 AAV (strain 188/67), all the birds rapidly developed precipitating antibody. Birds kept in contact also developed VN antibody but only some of these birds developed precipitating antibody which could be detected for only a short time (Cook, 1974a). Yates et al. (1977) demonstrated VN

antibodies at 1 and 2 weeks after intranasal infection of SPF chickens with serotype 1 (Phelps) virus with peak titres (1:320) being reached at 3 weeks. No precipitins were detected after the primary infection, but a strong reaction was seen in GD tests after a second intranasal infection at 8 weeks. Clemmer (1965) found that birds were resistant to reinfection with the same serotype 45 days after the primary infection. High mortality rates were observed in chickens with maternal VN antibody titres <20, but no mortality occurred in those with higher titres of antibody to serotype 5 or 8 AAV when they were inoculated separately at 2-3 days of age (Fadly and Winterfield, 1973; Grimes and King, 1977b). Also, relatively minor lesions were observed in birds which had maternal antibody.

Different serotypes of AAV may vary in their ability to elicit antibody responses following exposure to virus under similar conditions. Neither precipitating nor VN antibody was detected at 21 days pi after oral and intranasal administration of KR5 strain of AAV (serotype 4) virus but high levels of VN antibody were produced following inoculation of other strains, HV7 and GAL (serotype 2), SR49 (serotype 3), and TR59 and H6 (serotype 8).

An active infection appears to provoke protection against reinfection with the same serotype for up to at least 6 weeks after recovery, but by 8 weeks birds could be reinfected (McFerran, 1981a). It has been suggested that, in humans, low levels of VN antibody are protective (Hilleman et al., 1958). But Yates et al. (1977) were able to reinfect birds which had relatively high levels of VN antibody and maternally derived antibody was not protective against a natural route of challenge (Clemmer, 1965). It may well be that local immunity, either cellular or humoral, is more important than circulating VN antibody in preventing the growth of virus at mucosal surfaces (McFerran, 1981b).

Satisfactory protection was obtained in 4 weeks old SPF birds which had been vaccinated subcutaneously or by eye-drop at 3 days of age with serotype 5 AAV (Tipton strain) which had been passaged 74 times through embryonating eggs. It was also found that chicks rapidly developed an age resistance to IBH virus following the decline of maternal immunity which provided protection against early infection with the virus. These facts suggest that the best approach for protection against IBH in chickens might be to immunize the breeder flocks (Fadly and Winterfield, 1975).

In the case of HAds, antibodies begin to appear in blood by 8-10 days pi, reaching maximal levels 14-21 days later. Both VN and CF antibodies rise simultaneously but, whereas the CF antibodies begin to decline at 2-4 months, the VN antibodies persist for many years with only a slight decrease in titre. Minor rises in heterotypic VN antibodies occur following

infection, especially if they are already present as a result of prior infection with another strain (Andiman and Robert, 1989). Antibodies to the early antigens of HAd appear about 5 days after the onset of clinical symptoms, reach a peak 15-30 days later and begin to decline after a few months (Gerna et al., 1981). The level of specific IgA in nasal secretions is inversely correlated with the severity of disease following natural infection with adenoviruses associated with acute respiratory disease (McCormick et al., 1972). During adenoviral conjunctivitis, a significant rise in the level of specific IgG in tears has been observed and this is accompanied by a decline in specific serum IgG levels (Gupta and Sarin, 1983).

Birds younger than 4 weeks have proved refractory to experimental infection with THE virus due to the presence of maternal antibody (Harris and Domermuth, 1977). An increased antibody response to THE virus in turkeys has been demonstrated from 13 to 19 weeks of age (McDougall and Stuart, 1980). Susceptible birds infected with EDS virus develop VN, HAI and precipitating antibodies within 6 days of infection. Peak HAI and GD titres were demonstrated at 21 days and VN titres were still rising at 28 days after infection. Chicks hatched from hens with antibody to EDS virus have maternal antibody with a half life of 3 days and, on the basis of a serological response to challenge, immunity lasts about 4 weeks (Darbyshire and Peters, 1980). Birds derived from infected eggs may carry latent virus but not develop antibody until clinical EDS develops (McFerran, 1981b).

Serological tests used for diagnosis

Detection of AAV infection currently depends on demonstration of antibody or on isolation of the virus either in cell cultures or in embryonating eggs. Detection of precipitating antibody by GD is the most commonly used serological test. Serological surveys of poultry flocks have usually been based on the qualitative detection of the common antigen of Group I AAV in GD tests and this has proven useful for monitoring SPF flocks for freedom from infection (Cook, 1970; Boyle, 1973; Campbell and O'Connor, 1973; McFerran, 1981b). Unfortunately the GD test has a low sensitivity and may not detect primary infection in chickens (McFerran et al., 1975; Grimes et al., 1977a; Yates et al., 1977; Cowen et al., 1978b).

The VN test is more sensitive than the GD test but can only be used for recognized serotypes. It is useful for serotyping new isolates using reference antisera. However, it is time consuming, tedious and expensive to use routinely for the 12 recognized serotypes (Grimes et al., 1977a).

The microtitre IF test also detects the AAV group antigen and is very sensitive (Adair et al., 1980). Antibodies to any of the Group I AAVs can be detected in serum using rabbit anti-chicken serum conjugated with fluorescein isothiocyanate (FITC) and a cell culture infected with a single serotype of AAV.

ELISA tests have been developed for detecting antibody to AAV (Dawson et al., 1980; Calnek et al., 1982). Significant cross-reactivity has been reported between 10 serotypes of AAV (Calnek et al., 1982). The ELISA test has been found to be very sensitive and would be the best choice for screening SPF flocks.

Other tests such as passive HA (Moreau, 1974) and single radial diffusion (Pereira et al., 1972; McFerran et al., 1975) have been used, but have not gained widespread acceptance.

At present the GD test, using spleens from infected turkeys as the source of antigen, is the only useful test available for the diagnosis of THE and MSD (Domermuth et al., 1973). The HAI test is commonly used for detecting antibodies to EDS virus. The VN test is useful for confirming HAI results, because occasionally a few sera are found with non-specific titres in the HAI test (McFerran, 1981b).

Cultivation

a) Cell culture

Primary monolayer cultures derived from chicken embryo kidney or liver cells or chicken kidney cells (CKC) from 1-4 week-old birds are usually considered to be more sensitive than chicken embryos for the isolation and propagation of AAV (McFerran, 1980). Duck kidney cells and chicken embryo fibroblast cells have also been used for AAV propagation but they have not been found as satisfactory as chicken kidney or liver cells (McFerran, 1980). In cell cultures inoculated with any of the prototype strains, the first noticeable change is swelling of cells within 18 hr pi. The swollen cells become round and begin to detach from the surface by 24-48 hr pi. In haematoxylin and eosin (HE) stained cultures, basophilic INIBs are found at 24 hr after inoculation (Kawamura et al., 1964). Often more than one blind passage of infected tissue suspension is necessary before a visible cytopathic effect (CPE) is apparent on monolayer cell culture (Green et al., 1976; Bagust, 1982; Cook, 1983).

Although virtually all of the serotypes of AAVs produce a characteristic CPE with INIBs, Adair (1978) indicated that not all AAV serotypes behave in cell cultures in the same way. Thus 13 AAV strains representing 8 serotypes (McFerran et al., 1975) fell into two

subgroups depending on the nuclear changes induced in cell culture. This is similar to the situation with the HAds. The subgroup 1 comprised AAV serotypes 1, 2, 4, 5 (strains 340 and Tipton) and 8 (strains HVI, 58, TR59). This group was characterized in the early stages by the production of refractile "pearl-like" inclusions. Eventually a central basophilic inclusion was formed which was surrounded by a clear halo. The subgroup 2 comprised serotypes 3, 5 (TR22), 7 and 8 (764). This group was characterized by the development of non-refractile, irregular, eosinophilic inclusions which increased in number and size to fill the entire nucleus. Kawamura et al. (1964) also found that the inclusion bodies produced in cell culture by eight serotypes of AAV isolated in Japan differed in morphology. Two types of inclusion bodies, produced by viruses isolated in Japan in cell culture, were described as reticular or aggregate types which corresponded to the subgroup 1 and 2 viruses respectively (Maeda et al., 1967). In addition, several other types including granular and aggregated forms of inclusion bodies have also been described in the nucleus of AAV infected cells (Adair et al., 1979a).

EM study has demonstrated major differences in the distribution and arrangement of virus particles and virus-associated inclusions between the two different subgroups of viruses (Adair et al., 1979a). With subgroup 1, as virus particles increased in number they tended to distribute peripherally, close to the nuclear membrane, leaving the virus associated inclusions in the centre of the nucleus. With subgroup 2, virus particles and associated inclusions become concentrated initially at the centre of the nucleus and gradually the intermixed virus particles and associated inclusions occupied the whole nucleus (Adair et al., 1979a).

There are no reports of the growth of AAVs in any established cell lines. CELO virus was shown to transform hamster embryo fibroblastic cells in vitro and a virus-specific intranuclear T antigen was detected by IF test in most cells. Cytopathic effects were never observed in hamster embryo fibroblast cells (Anderson et al., 1969).

b) Avian embryo

Although cell cultures appear to be the more sensitive and suitable media for the isolation and propagation of AAV (McFerran, 1980), the chicken embryo has also been successfully used in diagnostic laboratories for initial isolation and propagation of AAV (Winterfield et al., 1973). The highest yield of virus has been obtained in embryos which do not contain neutralizing antibodies to the homologous adenoviral serotype (Winterfield, 1984). Some isolates of AAV have been propagated successfully by the yolk sac and chorioallantoic membrane (CAM) routes of inoculation and death of embryos has been reported by 2-7

days after inoculation via CAM (Gallina et al., 1973). Embryos which die soon after infection are generally congested and haemorrhagic while, with the delayed death pattern, stunting and curling, similar to that seen in embryos infected with infectious bronchitis (IB) virus, has been observed. A hepatitis, characterized by varying degrees of mottling and necrosis was often detected. Histologically, eosinophilic INIBs were observed in hepatocytes (Gallina et al., 1973). Rinaldi et al. (1968) reported basophilic, granular INIBs in lesions on the CAM. Fadly and Winterfield (1973) observed death of embryos within 5-10 days following yolk sac inoculation using the Tipton strain (serotype 5). INIBs were seen in hepatocytes of embryos that died as late as 10 days pi.

Kawamura et al. (1964) observed that when a large amount of virus was inoculated onto the CAM of 9-day-old embryonating eggs, all of the prototype strains produced thickening of the CAM and killed the embryos by 3-11 days pi except for the SR49 strain (serotype 3). When 9-day-old embryos were inoculated with a large amount of virus into the allantoic cavity (AC), the strains Ote (serotype 1), TR22 (serotype 5) and CR119 (serotype 6) killed the embryos at 3-11 days pi. When a small amount of virus was inoculated, only the Ote strain killed the embryos.

Cowen, (1988) evaluated the CAM and yolk sac route of chicken embryo inoculation for the replication of 42 strains (11 serotypes) of Group I AAV which had been propagated in cell culture (Calnek and Cowen, 1975; Cowen et al., 1977; Cowen and Naqi, 1982). Commonly observed embryonic changes were death, stunting and curling, hepatitis, splenomegaly, congestion and haemorrhage and urate formation in the kidneys. Basophilic or eosinophilic INIBs were observed in hepatocytes. Higher titres of viruses and more uniform damage to the embryos (mortality and gross lesions) were obtained if the yolk sac route of inoculation was used.

Gallina et al. (1973) demonstrated stunting, dwarfing and hepatitis in embryos inoculated with AAV. Eosinophilic INIBs were detected in the hepatocytes by 2 days pi. Death of the embryos, accompanied by curling and stunting, was observed by Otsuki et al. (1976) after inoculation of AAV isolates via the yolk sac and AC.

Entry of adenoviruses into the cells and their replication

Adenovirus enters cells by the pathway of receptor-mediated endocytosis (Pastan et al., 1986). The adenovirus fibre binds to a receptor on the cell surface and the virus moves into a coated pit which is a depression in the plasma membrane. The virus is then transferred

into an endocytic vacuole (receptosome or endosome) within a few minutes (Dales, 1973; Pastan and Willingham, 1983; Goldstein et al., 1979; Maxfield et al., 1981). The endocytic vesicles appear to fuse with tubular membranous elements of the Golgi system. Soon after, virus escapes into the cytosol by disrupting the membrane of the vesicle and becomes associated with the nucleus after about 30 minutes (Fitzgerald et al., 1983).

After uncoating, the viral DNA is transcribed into messenger ribonucleic acid (mRNA) for early nonvirion polypeptides and late proteins. Virus-coded proteins (DNA polymerase, 72 KDa DNA-binding protein, 55 KDa terminal protein) as well as cellular factors (including topoisomerase) are required (Nermut, 1987). DNA synthesis occurs within the nucleoplasm by a semiconservative strand-displacement mechanism and reaches its maximum level at about 19 hr pi (Nermut, 1987). The transcription in the nucleus is followed by splicing into mRNAs. The mRNAs migrate into the cytoplasm where the structural polypeptides are synthesized. Assembly of the virus particles again takes place in the nucleus and release ensues by slow disintegration of the damaged cells. The viral infection efficiently shuts off host cell DNA, RNA and protein synthesis. Intranuclear inclusions may contain nucleic acid, viral proteins and virions in paracrystalline arrangement (Appel, 1987).

Replication of AAV follows a similar pattern to human and other animal adenoviruses and AAVs also multiply in the nucleus of infected cells (Kawamura et al., 1963). After an eclipse phase of 7-10 hr, extracellular virus particles were noticed (Kawamura et al., 1963; Burke et al., 1968). In general, maximum titres of virus have been obtained in 40-96 hr (Winterfield, 1984).

Oncogenicity

CELO virus is the only AAV whose oncogenicity in hamsters (Sarma et al., 1965; Stenback et al., 1973; Ishibashi et al., 1980) and transforming activity in various mammalian cultured cells (Anderson et al., 1969; Ishibashi et al., 1980) has been demonstrated positively. Initially, only a well differentiated fibrosarcoma was reported at the site of viral inoculation of newborn hamsters (Sarma et al., 1965), but subsequently hepatomas (Anderson et al., 1971), ependymomas (Mancini et al., 1969), adenocarcinomas and hepatomas (Stenback et al., 1973) and other types of sarcomas (Jones et al., 1970) have been described. The virus-induced fibrosarcomas usually develop after a lag of several months following subcutaneous inoculation (Ishibashi et al., 1980). Tumours seemed to be more frequent in female hamsters than in males as also appeared to be the case with human adenovirus-induced tumours (Yohn et al., 1965; Jones et al., 1970; Ishibashi et al., 1980). In addition to CELO

virus, four Japanese isolates of AAV [SR48 (serotype 2), SR49 (serotype 3), KR5 (serotype 4), CR119 (serotype 6)] are also reported to induce tumours in hamsters (Ishibashi et al., 1980). Serotype 1 (Indiana C), 2 (GAL) and 5 (Tipton) have failed to induce tumours in newborn hamsters (Sarma et al., 1965; Fadly et al., 1976).

The existence of viral DNA in induced tumours and transformed cells has been reported and the number of viral DNA molecules carried by these cells varied over a wide range (0.95 - 920) per diploid amount of host cell DNA (Ishibashi et al., 1980; Yasue et al., 1980). Most of the viral DNA in the tumour and in transformed cells appeared to be integrated into cellular DNA and mainly at the terminal regions (Yasue et al., 1980).

HAd serotype 12 and 18, the first two HAds to be isolated, were shown to induce tumours in newborn rodents in 1962. However, all HAds can transform rodent fibroblast cell cultures but they differ in their ability to cause tumours in rodents. Huebner (1967) suggested that HAds be divided into subgenera on the basis of their oncogenicity for newborn hamsters. The GC content of HAd DNA appears to be correlated with the degree of oncogenicity and with the type of tumour or neo-antigen induced (Pina and Green, 1965). So far, no comparison between CELO virus and nononcogenic AAV has been reported. Tumor-related antigens, which are distinct from virion antigens, have been observed in CELO virus-induced tumours by using IF (May et al., 1978) and immunoprecipitation (Ishibashi et al., 1980) tests.

Adenovirus-associated virus (A-AV)

A-AVs are small (20-24 nm) defective parvoviruses that require a host cell coinfected with a 'helper' adenovirus for productive infection. Dutta and Pomeroy (1967) first described the avian A-AV particles which they detected in association with QB virus. They believed that the particles observed were internal or core components of the mature adenovirion. Subsequently these A-AVs isolated from the Olson strain of QB virus were characterized as defective parvoviruses (Yates et al., 1973) which were resistant to heat, chloroform and low pH and did not agglutinate avian and mammalian erythrocytes (Yates et al., 1973).

The A-AVs are often found in conjunction with AAV infection (Yates et al., 1973; 1976). Yates et al. (1976) suggested that these viruses are contaminants of many AAV stocks. Although the extent of contamination is unknown, Mishad et al. (1975) demonstrated the presence of avian A-AV in more than 50% of avian strains obtained from Ireland and the United States. The high frequency of contamination could be due to increases in titre during

passage or to the presence of a latent avian A-AV infection in the tissue culture used (Yates et al., 1976). The avian A-AV has also been observed to be transmitted vertically with CELO virus infection (Dawson et al., 1979; 1981).

There is no evidence of disease in chickens caused by A-AV (McFerran, 1980) but their presence exerts an inhibitory effect on AAV replication in cell cultures, embryos and young chickens (Rhee, 1976; Pronovost et al., 1978). Pronovost et al. (1978) demonstrated significant inhibition of the virulence of Tipton (serotype 5) strain of AAV in 3-day-old chicks which had been coinfected with A-AV by IP injection. Pronovost et al. (1979) demonstrated both enhancement and inhibition of the replication of AAV in kidney cells depending on heavy or light co-infection with A-AV, respectively. The degree of enhancement or inhibition observed varied with the adenovirus serotypes used and with the differences observed in their growth rates. Of the AAVs, the CELO virus replicated the fastest and the Tipton (serotype 5) replicated the slowest in the presence of A-AV infection. Primate A-AV has been shown to inhibit the oncogenicity of HAd serotypes 12 and 31 in newborn hamsters (Kirschstein et al., 1968).

Richardson and Westphal (1983) observed that early adenovirus gene expression is necessary to provide the helper effect to A-AV. According to the replication studies of A-AV in chicken embryos and cell cultures coinfected with AAV, the two types of virus particles were not readily found in the same cell, demonstrating "mutual interference" (Mayor et al., 1967; Hoggan et al., 1966; Parks et al., 1967).

Both CF (Mishad et al., 1975; Yates et al., 1976) and GD (Yates et al., 1977) tests have been used to detect A-AV and it appears that the A-AV is a more efficient stimulator of antibody than the adenovirus (Yates et al., 1977).

A-AVs have also been found in association with adenoviruses of man (Hoggan et al., 1966), monkeys (Archetti et al., 1966), cattle (Luchsinger et al., 1970), dogs (Sugimura and Yanagawa, 1968) and turkeys (Scott and McFerran, 1972). The characteristics of avian A-AV and its mode of replication are essentially the same as those described for other species including man (Yates et al., 1973). The avian A-AVs are serologically distinct from primate and other mammalian types of A-AVs (Yates et al., 1973; Mishad et al., 1975).

AIMS AND SCOPE OF THE THESIS

To investigate the naturally occuring outbreaks of IBH in New Zealand broiler flocks, to establish the aetiology and to determine whether they were related to any predisposing immunosuppressive factors or agents.

To develop methods for the isolation and identification of adenoviruses associated with the outbreaks of IBH and to compare their antigenic relationships with the standard prototype strains.

To analyse and compare the restriction endonuclease digestion patterns of the DNA of adenovirus isolates from New Zealand and Australia and the prototype strains.

To determine whether any of the isolates of AAV from New Zealand broiler flocks were capable of inducing disease resembling IBH following experimental infection of conventional and SPF chickens.

To determine whether any of the New Zealand isolates of AAV caused suppression of the immune response in infected chickens.

To develop an ELISA and an immunocytochemical procedure to detect and quantify AAV antigens and to locate the site of viral replication in infected chicken tissues respectively.

To use the viral assay techniques to study the pathogenesis of infection and the persistence of virus in experimentally infected chickens.

CHAPTER TWO

INVESTIGATION OF THE FIELD OUTBREAKS OF INCLUSION BODY HEPATITIS IN CHICKENS IN NEW ZEALAND

INTRODUCTION

IBH has previously been described in New Zealand (Bains and Watson, 1977). The disease reported was similar to that originally described in the United States (Helmboldt and Frazier, 1963) but mild compared with reports from Australia (Wells et al., 1977), Europe (MacPherson et al., I974), and North America (Pettit and Carlson, 1972) (Table 2.1). AAVs have also been isolated from clinically normal chickens in New Zealand (Green et al., 1976) and two of the isolates were serologically related to strains isolated from cases of IBH elsewhere.

This chapter describes the occurrence of IBH in broiler flocks derived from one breeder flock in the South Island of New Zealand during June-August, 1987. The significance of the outbreak lies in its severity, ranking with the most severe outbreaks described in the literature, the severity of immune system damage, and the clear temporal association with declines in performance of six out of eight of the company's breeder flocks which were housed on two farms in the Christchurch area. A number of field and experimental reports (Rosenberger et al., 1975; Fadly et al., 1976; Dhillon et al., 1982; Winterfield, 1984) have emphasized the predisposing role probably played by IBD in the development of severe IBH infections. New Zealand's freedom from IBD is officially recognized (Government of New Zealand, 1984). This is based on extensive serological investigations of the poultry flocks in New Zealand (Howell et al., 1982; With, 1985; Jones, 1986; With and Christensen, 1987). It therefore seemed that this outbreak of IBH was a primary AAV infection or that it was related to other infectious, environmental or chemical immunosuppressive agents.

This chapter also describes the prevalence of antibodies to different serotypes of AAV in New Zealand chicken flocks.

Isolation of adenoviruses from embryonating eggs or latently infected cell cultures has been often reported (Yates and Fry, 1957; Burke et al., 1965; Khanna, 1966a; Cook, 1968). It has been suggested that vertical transmission is one of the most important factors in the dissemination of AAVs in chicken flocks (McFerran, 1981b). It has also been postulated

that vertical transmission is an important factor in the epidemiology of IBH (McFerran and Adair, 1977; Cowen et al., 1978b; Reece et al., 1985). To further the understanding of the epidemiology of IBH, the potential for transmission of virus through fertile eggs was investigated. Previous studies to determine the presence of AAV in eggs have relied on isolation in cell culture of infectious virus from eggs (Dawson et al., 1981) or from newly hatched chicks (Cowen et al., 1978a). This present report describes the use of an ELISA to detect viral antigens directly in the yolk and albumen of eggs laid by broiler breeder birds in New Zealand.

Table 2.1 **Relative severity of previously documented IBH outbreaks in broiler flocks.**

Reference	Location	Cumulative mortality (%)	Peak daily mortality(%) (day of peak)	PCV	Association with specific donor flocks
Bains and Watson (1977)	NZ	5.3	1.8 (43)	NR	NR
MacPherson et al. (1974)	UK	13.5	NR (42-45)	25-40	Yes
Helmboldt and Frazier (1963)	USA	0.1	NR (49) (anaemic)	NR	No
Pettit and Carlson (1972)	Canada	7.0	1.0 (35)	20-25	No
Wells et al. (1977)	Australia Victoria	5.3	0.2 (40-45)	NR	NR
	NSW	32.3	1-2 (35-40)	Low (jaundice)	Yes

NR = not recorded

MATERIALS AND METHODS

Broiler flocks

A high mortality rate was reported from 15 flocks on South Island broiler farms, in an area extending 550 km from Christchurch to Invercargill. The broiler chickens were delivered to the farms on the day of hatch or the following day and were housed in conventional, controlled-environment sheds on fresh litter. Flock size varied from approximately 2000 to 12000 birds, with a mean of 7500. Stocking density averaged 20 birds per square metre. At one day of age, 14 out of 46 flocks delivered to farms during the period of the outbreaks received a half dose (500 plaque forming units) of turkey herpesvirus (HVT) Marek's disease (MD) vaccine [MD-Vac CFL (lyophilized), Salsbury, Iowa, USA] by subcutaneous injection. Mortality figures were recorded daily or in some cases weekly.

Feed from at least five different compounders, including two home mixers, was fed to affected flocks.

Breeder flocks

The breeder chickens were housed on two multi-age breeder farms approximately 2 km apart. The breeder birds were reared until 18 weeks of age in a separate facility with separate staff. Each flock consisted of approximately 1750 females plus 140 (8%) males. Sheds were stocked with birds of the same age and birds were fed automatically.

Egg production, breeder mortality, feed consumption and time taken to eat the daily ration (because the birds were on a ration-restriction program), were monitored for each flock. The hatchery utilization rate (HUR) that is, number of eggs set per chick produced was monitored for the breeder operation as a whole. There were two hatches per week.

To reduce the effects of recorded variation in hatching percentage resulting from variation in incubator performance, the weekly average HUR was plotted as a 2-week moving average, with the current week's figure given twice the weight of the previous week, according to the formula:

$$\text{Weighted HUR} = \frac{2 \times \text{current week HUR} + \text{previous week HUR}}{3}$$

Where the HUR is the hatcheryman's measure of a breeder flock's hatchability, HUR expresses a flock's hatchability in terms of the number of eggs that has to be placed in the incubator to produce 100 saleable chicks (e.g., a HUR of 1.26 implies that 126 eggs need to be set for each 100 chicks ordered).

The primary breeders define a target performance for egg production and hatchability of breeder flocks at various ages under commercial conditions. As a number of flocks of different ages were in production during the period of this outbreak of IBH, the breeder's average target performance was calculated as the average of all flock's production expressed as a percentage of the targets set by the primary breeders. This average was adjusted to account for normally expected changes in egg production and hatchability as flocks aged and were replaced by new young flocks.

The breeder birds were fed on 16% protein breeder mash from mill A.

A small number of dead or moribund breeders was subjected to necropsy.

Feed trial

Because there was considerable evidence that breeders were finding the feed (breeder mash) from mill A unpalatable, tests were carried out on a particular flock of breeder and broiler birds as follows:

The unpalatable feed was removed, feeding equipment was cleaned, and feed was replaced by a new mix of breeder mash from mill A. The time taken for flocks to clear the new feed was noted. Also the new feed from mill A was delivered by hand into half of a track, and the other half was filled with equivalent feed (breeder mash) from mill B and the reaction of the birds to the feed from both mills was noted.

Two pens of 200 day-old broilers were fed for 7 days on breeder starter from mill B which had not been involved in any reported episodes of feed refusal. One of the pens, consisting of 100 week-old broilers, was then fed on the unpalatable breeder mash from mill A and the other pen on equivalent feed from mill B. Feed consumption, average body weight and weekly mortality were recorded. Birds that died after being fed on mill A feed were submitted for necropsy.

Analysis of feed for mycotoxin

Three feed samples each consisting of 200 gm of breeder mash from mill A were sent to Dawson Laboratories, Hamilton, for analysis of mycotoxin. An extract of each sample was tested by an indirect IF test using antisera to various types of mycotoxin.

Post mortem examination

Dead and moribund birds from affected broiler and breeder flocks were submitted for necropsy. Blood was collected from the moribund birds in tubes containing calcium-ethylenediamine tetra-acetic acid (Ca-EDTA) for measurement of packed-cell volume (PCV) and in plain tubes for serological examination. The birds were examined thoroughly during post mortem examination. Samples of liver, thymus, bursa, spleen, kidneys, proventriculus, gizzard, intestine and long bone (femur) were collected in 10% buffered formalin for histopathology. Liver samples were taken aseptically and frozen at -20^{o}C or -75^{o}C for virus isolation. Occasionally bursa and intestine were also collected for virus isolation. Smears of bone marrow and blood from some of the grossly affected birds were taken to examine the blood cell population following Giemsa's or Wright's stain.

Isolation and identification of AAV

A 10% suspension of pooled liver, bursa or intestinal tissues from each of the affected broiler and breeder flocks was prepared in Eagle's minimum essential medium (MEM) (Flow Laboratories, Irvine, Ayrshire UK), containing penicillin (200 units/ml), streptomycin (200 ug/ml) and kanamycin (200 units/ml). After 2 cycles of freezing and thawing the liver suspension was centrifuged at 1000 xg for 20 min and the supernatant was inoculated onto established monolayer cultures of primary CKC derived from SPF birds. Up to 3 blind passages were performed for each sample before it was considered as negative.

When a CPE was observed, it was allowed to progress, usually up to 3 days, to involve 80-90% of the cells in the monolayer which were then harvested after 2 cycles of freezing and thawing. The resulting suspensions were clarified by low speed centrifugation (1000 xg for 20 min) and stored at -75^{o}C in 1.0 ml aliquots until further use. Cover-slip cultures of CKC in Leighton tubes were inoculated with each viral isolate and, at 12 hr, 24 hr, 36 hr and 48 hr pi, the infected and control cells were fixed in Bouin's fluid and stained with HE for cytopathology.

Clarified supernatants from cultures showing greater than 80% CPE were subjected to ultracentrifugation at 200000 xg for 3 hr. The resulting pellet was stained with 2% phosphotungstic acid (PTA) and examined by EM (Philips 201C). Sensitivity of the viral isolates to chloroform was tested following the method of Feldman and Wang (1961).

Serological evidence of adenoviral infections in New Zealand poultry flocks

Serum samples were received from 7 broiler (2-7 weeks of age) and 34 breeder (11-60 weeks of age) flocks; most of them were from the South Island. Eight sera from each flock were inactivated at 56^{o}C for 30 min and tested against serotype 8 AAV (prototype strain HVI) in a VN test. Serum samples from any particular flock with a very low antibody titre against prototype HVI strain were similarly tested against the other 11 prototype strains (CELO, SR48, SR49, KR5, 340, CR119, YR36, 764, A2, C2B and 380). Fifty microlitres of two-fold serial dilutions (1:10 to 1:5120) of each serum were reacted with approximately 100 mean tissue culture infective doses ($TCID_{50}$) (50 ul) of virus for 1 hr at room temperature. One hundred and fifty microlitres of CKC suspension in growth medium were then added to each well. After 5 days of incubation at 37^{o}C, the plates were stained with crystal violet solution. The VN titre for each serum was calculated as the reciprocal of the highest dilution of that serum which completely inhibited the CPE caused by the particular virus. The neutralization test is described in detail in Chapter 3.

Five sera were obtained from each of 5 affected broiler and 10 breeder flocks and tested for antibodies to IBD virus in an agar gel precipitation test using a commercially available inactivated IBD virus antigen (Salisbury Laboratories, Charles City, Iowa, USA). Positive and negative sera supplied by the same company were used as controls.

Five sera were also obtained from each of 3 affected broiler and 4 affected breeder flocks and were examined by indirect IF test for antibodies to chicken anaemia agent (CAA) at the National Animal Health Laboratories, Tokyo, Japan.

Examination of the infected liver tissues for CAA

Ten percent (w/v) suspensions of liver tissue from 5 broiler flocks (2-5 weeks old) involved in IBH outbreaks were prepared in phosphate buffered saline (PBS, pH 7.2, 0.1 M), and heated at 70^{o}C for 5 min. After low speed centrifugation (1000 xg for 15 min), the

supernatant was stored at -75^{o}C as the inoculum.

a) Cell culture

The MD lymphoma (MDCC-MSB1) cell line received from the National Animal Health Laboratories, Tokyo, Japan was used for the attempted isolation of CAA. The cells were grown in RPMI 1640 (Sigma Chemical Co, St. Louis, USA) medium containing 10% foetal bovine serum (FBS) and antibiotics (penicillin 100 units/ml, streptomycin 100 ug/ml, kanamycin 100 units/ml) in cell culture tubes (2 ml each) in an atmosphere supplemented with 10% CO_2. The cell suspension was made to contain 5 x 10^5 cells per millilitre of growth medium. Each tube was inoculated with 200 ul of 10% liver suspension according to the method described by Yuasa et al. (1983). On alternate days, 1.0 ml of growth medium was removed from each tube and replaced with fresh growth medium. The cells were gently shaken once a day during the incubation period. Using an inverted microscope the tubes were examined each day for viral growth as evidenced by cellular death and by increase in the pH of the growth medium. After 5 days of incubation, the supernatant culture fluid was collected after low speed centrifugation and 200 ul of it was reinoculated into a new cell-culture tube with fresh MDCC-MSB1 cells.

b) IF test

After 7 passages, both control and inoculated cells were pelleted onto microscope slides for 10 min at 1300 rpm using a cytocentrifuge (Shandon Southern Products Ltd. Cheshire, England). After air-drying the cells were fixed in cold glutaraldehyde (25% aqueous solution) for 10 min. A 1:20 dilution of chicken anti-CAA (supplied by Dr. N. Yuasa) was added to the cells and incubated at room temperature for 30 min in a humid box. The slides were washed in PBS for 15 min (2 changes) in a Coplin jar with gentle stirring. Dilutions of rabbit anti-chicken serum conjugated with FITC (Gibco, Grand Island, USA) were made in PBS and added to the cells for 30 min at room temperature. After washing in PBS for 15 min (2 changes) the slides were mounted and examined by UV microscopy for evidence of specific fluorescence.

c) Challenge of birds

Twenty-five chicks, hatched from SPF eggs, were used at one day old for each sample. Chicks were weighed and blood samples were collected in heparinized micro-haematocrit tubes (Terumo Corporation, Tokyo, Japan; 2 tubes per bird) for measurement of PCV. Each of twenty birds was inoculated subcutaneously with 1.0 ml of 10% liver suspension and kept

in a positive-pressure bubble isolator (Fig. 2.1) and supplied with autoclaved feed and water *ad libitum*. The remaining 5 birds were kept as controls in isolation with similar feed and housing. The inoculated birds were closely observed each day for any signs of illness. The body weights and PCVs of all the birds were measured at 3, 6, 9, 12 and 15 days pi when the experiment was terminated.

Fig. 2.1 **Positive-pressure bubble isolator.**

d) Inoculation of avian embryos

Five SPF embryos (6 days old) were inoculated via the yolk sac route with 200 ul of each 10% liver suspension. The embryos were examined daily, until they were hatched, for lesions or mortality suggestive of CAA. Blood samples were collected from the chicks at 2, 5, 10 and 15 days of age in heparinized micro-haematocrit tubes for measurement of PCV.

Transovarian transmission

Ten eggs, from each of six different sheds (1 to 6) of a broiler breeder flock (Waihow GC), were tested for the presence of adenoviral antigen by an ELISA. The development and

standardization of this ELISA are described in detail in Chapter 5. Birds in each of the sheds had VN antibody titres against serotype 8 AAV ranging from 320-1280. Egg yolk and albumen samples were collected separately from each egg with a syringe by thoroughly cleaning the shell and puncturing a hole at the apex of the egg. Each sample was diluted 1:5 with PBS and tested for the presence of viral antigens by ELISA. Egg yolk and albumen samples collected from SPF eggs and cell culture virus (serotype 8) were similarly tested.

Floor litter, collected from sheds 5 and 6, was also tested for viral antigen by ELISA. About 10 gm of the litter was soaked in 10 ml of PBS for 24 hr at $4^{o}C$. After centrifugation at 1000 xg for 20 min, the supernatant was assayed by ELISA. The same litter was autoclaved for 30 min and similarly processed and tested by ELISA.

ESA values were determined for each sample by subtracting the mean absorbance value for four SPF yolk or albumen samples from the values obtained with the test samples. ESA values which were more than 3 standard deviations (SD) that is, >0.06 above the mean of the SPF values were considered postitive for specific viral antigens.

Where appropriate, data in this and subsequent chapters were analysed using the ANOVAR statistical package.

RESULTS

Clinical and necropsy findings in affected broilers

Growers reported that, from 3 days of age, affected flocks were quiet or "dopey", and increased mortality was usually noted from about day 17, often associated with necropsy findings of subcutaneous haemorrhage, particularly of the underside of the wings. Some affected flocks experienced mortality rates higher than normal (about 1.0%) at the first week of age. Mortality rates increased markedly up to 30% in the third or fourth week of age and began to drop by the end of week 5. Although birds often died suddenly without showing any signs of illness, some showed drowsiness, ruffled-feathers, decreased food intake and occasionally white pasty faeces for a few hours to 1 day before they died. After 7 weeks of age the mortality rates in the affected flocks decreased to the same level as noninfected flocks.

Figure 2.2 shows cumulative daily mortality in five affected broiler flocks. Flock 6, reared immediately after flock 1, showed only up to 2.8% mortality from the age of 1 to 49 days, which is the normally expected mortality pattern in average flocks.

Lesions seen in birds that died in the third week included aplasia of bone marrows, with replacement by fatty tissue. This lesion was evident from the age of 14 days onward. There was atrophy of the bursa of Fabricius and the thymus. Affected bursae were smaller than expected for their age and were flaccid. When incised, they had a matt appearance. Affected thymuses (Fig. 2.3) were small (1-2 mm diameter) and haemorrhagic. In some birds, there was proventricular haemorrhage and pale kidneys, and in others tracheitis and airsacculitis. Livers were enlarged and dark.

The later stages of the disease in a flock were characterized by the development of jaundice and enlarged livers with subcapsular ecchymotic haemorrhages, or focal areas of necrosis up to 2 cm in diameter.

Secondary bacterial infection was a feature of the outbreak. In flocks which were affected early in the outbreak, bruising, pericarditis and perihepatitis were observed together with jaundice and focal hepatic necrosis. In the later stages of the outbreak, necrotic enteritis (NE) was the chief immediate cause of mortality.

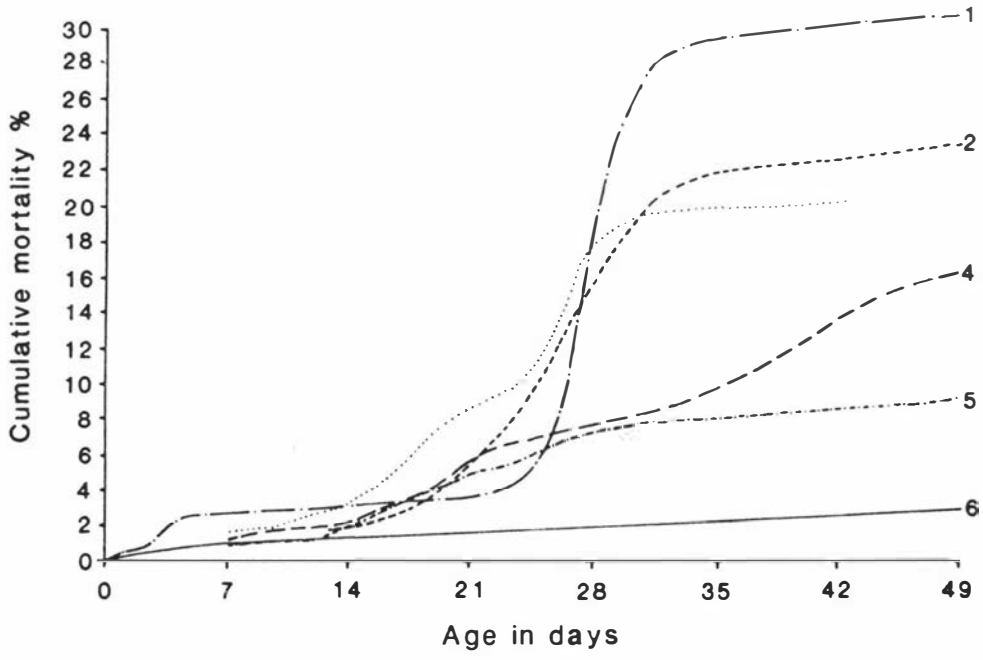

Fig. 2.2 **Cumulative daily mortality in five flocks affected by IBH and one unaffected flock.**

Fig. 2.3 **Comparison between thymuses of affected (right) and unaffected (left) birds. Field outbreak in 4-week-old broilers.**

Oxytetracycline, furaltadone, and furazolidone had no effect on the course of the disease in flocks in which the signs were of the haemorrhagic-anaemic type. All three drugs were effective in reducing mortality in flocks where deaths were due to NE.

The incidence of IBH in HVT-vaccinated and unvaccinated broiler flocks during the period June-August 1987 is shown in Table 2.2. There is no apparent effect of HVT vaccination on disease prevalence ($X2 = 0.96$, $p > 0.10$).

Table 2.2 **Relationship of turkey herpesvirus (Marek's disease vaccine) vaccination to the appearance of inclusion body hepatitis (IBH) in broiler flocks.**

Vaccination status	IBH		Total
	affected	unaffected	
HVT vaccinated	6*	8	14
HVT unvaccinated	9	23	32
Total	15	31	46

* Number of flocks

$X^2 = 0.96$, $p > 0.10$

Histopathology

The most significant histopathological sign was the presence of eosinophilic INIBs, with margination of chromatin in the hepatocytes of affected birds (Fig. 2.4).

Bursae of Fabricius showed atrophy of follicles, beginning with loss of medullary lymphoid cells (Fig. 2.5) and proceeding to a marked interlobular and intralobular fibrosis (Fig. 2.6). Atrophic thymuses were characterized by loss of lymphocytes and haemorrhage.

Gram-stained sections of intestine from moribund birds affected with NE revealed numerous Gram positive rods, with a morphology characteristic of *Clostridium sp.*, closely adherent to necrotic mucosal tissue.

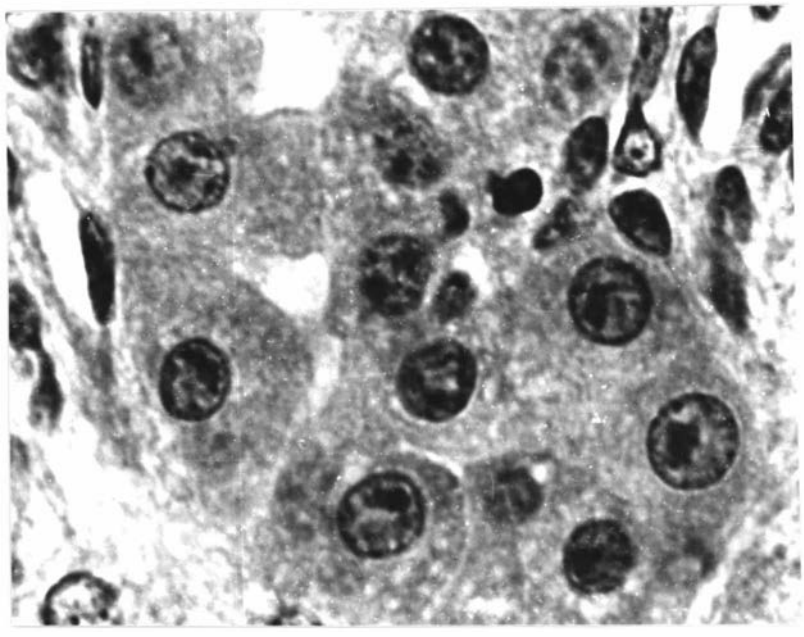

Fig. 2.4 **Eosinophilic intranuclear inclusion body in hepatocytes of 18-day-old broiler field case (haematoxylin and eosin, x40).**

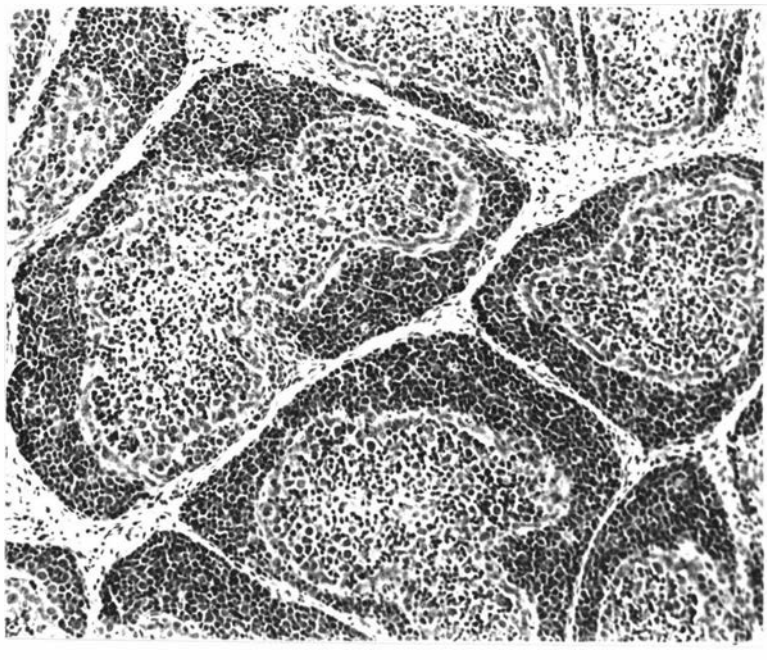

Fig. 2.5 **Early lesions in bursa characterized by depletion of medullary lymphocytes in 17-day-old broiler (haematoxylin and eosin, x20).**

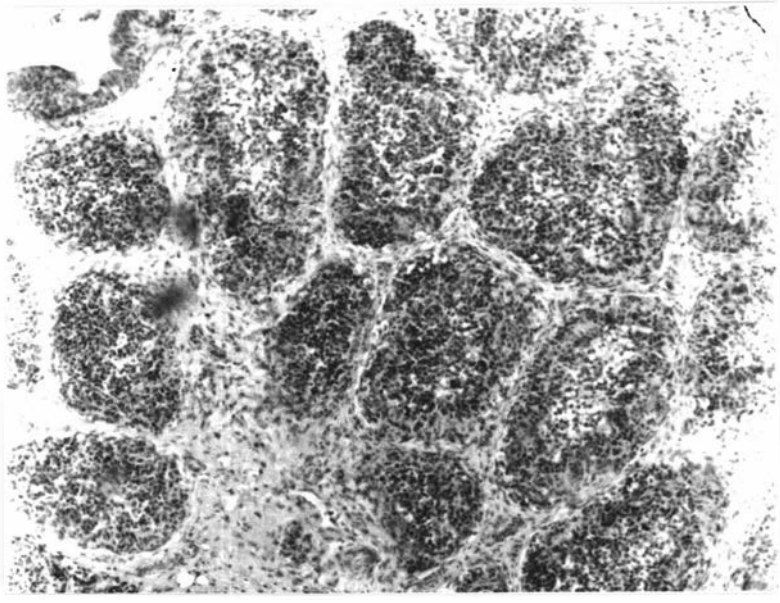

Fig. 2.6 **Advanced bursal atrophy. Field case in 24-day-old broiler (haematoxylin and eosin, x20).**

PCV

The PCVs of birds from affected flocks varied from 17% to 29%. Birds which were pallid gave the lowest PCV. In a number of cases, blood was still unclotted in a tube without anticoagulant 3 min after collection.

Breeder flocks

It was concluded on the basis of post mortem examination that birds had died of starvation. The vents of 10-15% of the birds were smeared with faeces. When the birds were consuming feed, they were taking 6-8 hr to consume the ration instead of the normal 2-3 hr.

Figure 2.7 shows the egg production of the breeder flocks from weeks 13 to 38; the graph shows a decline in production over weeks 15 to 26.

The expected normal mortality rate for these birds is 0.2% per week from housing. The hen mortality is shown in Figure 2.7, expressed as a percentage of this norm.

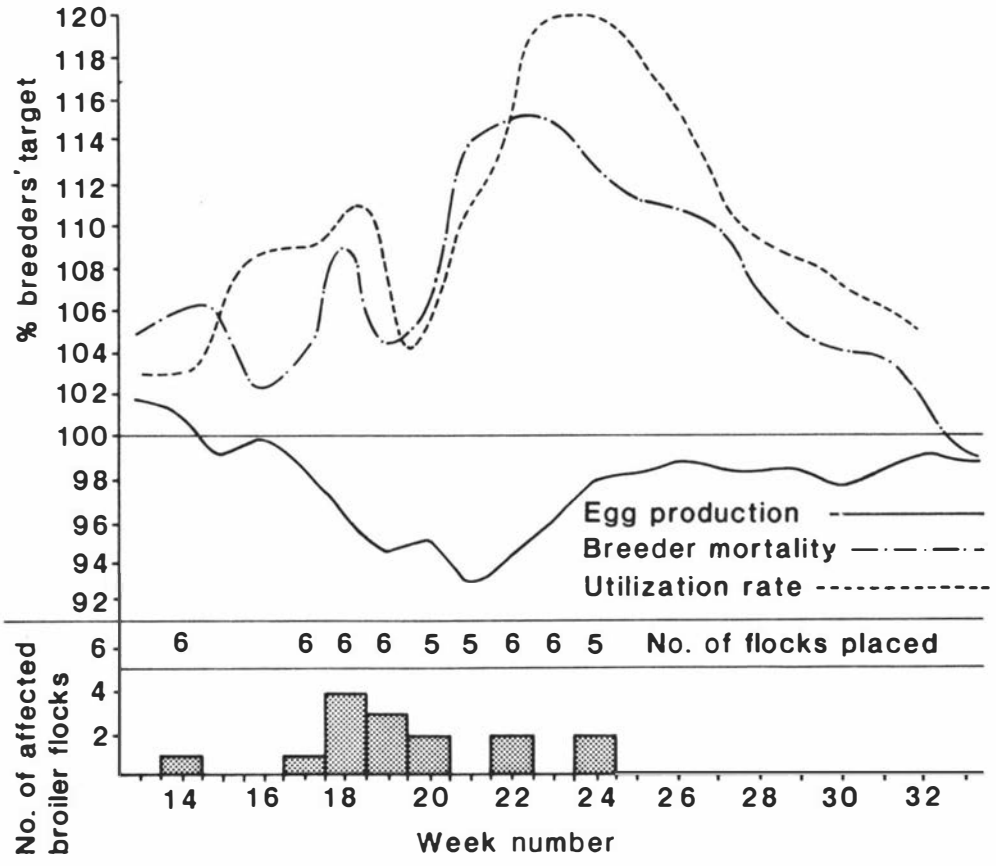

Fig. 2.7 **Breeder flock performance during IBH outbreak.**

Hatchery performance is shown in Figure 2.7 as a weekly HUR expressed as a percentage of the breeders' targets. This was excessive for eggs laid during the period of poor production, although part of the very poor results for eggs laid in weeks 24 and 25 (hatch weeks 28 and 29) was due to incubator failure.

Also shown in Figure 2.7 are the dates of production and hatch of the affected broiler placements. These correspond closely with the period of impaired breeder performance.

Feed trial

Table 2.3 shows growth rates and mortality of broilers which were fed with suspected breeder feed. The actual weights are not comparable with normal commercial broiler

growth rates because the birds were fed a breeder ration.

Table 2.3 **Growth and mortality rates of one-week-old broilers fed on suspected breeder mash (feed).**

Weeks	Feed from Mill A (suspected) *			Feed from Mill B *		
	Feed type	Mean body Wt.(gm)	Mortality (No. died)	Feed type	Mean body Wt.(gm)	Mortality (No. died)
2	breeder mash	255	0	breeder mash	235	0
3	"	482	4	"	455	0
4	"	550	44	"	638	2

* Each group consisted of 100 birds

Post mortem examination at week 4 of 11 birds from pen A showed a diphtheritic membrane in the intestine characteristic of NE. Two birds had haemorrhages in the proventriculus and/or gizzard. These two birds and two others had slightly pale bone marrow. There were no signs of coccidiosis.

Histopathological examination of tissues from birds from the feed trial that showed the most marked changes noted above, revealed very little pathological change in bursae and thymuses. There were no INIBs in any organs.

Analysis of mycotoxin in feed

Analysis of feed samples failed to show the presence of aflatoxin, ochratoxin A, or zearalenone. The extracts of the samples did show a complex and intense fluorescence. This fluorescence was separable into 12 distinct compounds, one of which was identified as sterigmatocystin. It was not possible to identify the remaining compounds within constraints imposed on the investigation (Dawson PJ, Dawson Laboratories, personal communication).

Culture of feed samples for mould revealed that there was a high level of *Penicillium sp.* and *Aspergillus sp.* spores.

Serological examination

The antibody titres to CAA and AAVs are shown in Tables 2.4 and 2.5, respectively. All the sera tested from broiler and breeder flocks were negative (less than 1:2) for IBD virus antibodies. Low levels of VN antibodies (GMT 2-12) were detected in the sera of 5 infected broiler flocks against serotype 8 AAV and less than 5 against other 11 serotypes. In two other normal broiler flocks, the geometric means of the antibody titres to serotype 8 were 538 and 320. High levels (320-1280) of VN antibodies to serotype 8 AAV were detected in most of the breeder flocks. Occasionally very low titre antibodies (<5-40) against serotype 8 were observed in a few breeder flocks. Two of the breeder flocks (Nos. 33 and 34) tested had high levels of VN antibody against both serotype 8 and 1 AAVs. Very low levels (titre 5-20) of antibody to CAA were seen in some of the broiler and breeder flocks.

Table 2.4 **Serum antibodies to chicken anaemia agent in New Zealand poultry flocks tested by indirect immunofluorescent test.**

Flock	Age	No. Positive/tested	Remarks
S6*	16 wk	0/20	Subsequent production good
B2	29 wk	14/40	Production unaffected during outbreaks. No evidence of IBH in both groups of progeny
S5	35 wk	19/30	
S4	40 wk	2/10	Worst affected breeder flock; progeny involved in high percentage of IBH outbreaks
S4**	1 day	0/16	Performed adequately
S4	42 days	0/19	6.5% mortality at 28-33 d/o
S4	49 days	16/20	7% mortality at 21-35 d/o

Antibody titres to CAA (*breeders = 1:5-1:20; **broilers = 1:5-1:10)

Type of flock (age in weeks)		Mean antibody titre*(range) to serotype 8 AAV	Mean antibody titre to other serotype(s)
Broiler			
Flock No.	1 (2)	5** (<5-40)	<5
	2 (2)	10 (<5-40)	<5
	3 (3)	12 (<5-80)	<5
	4 (5)	2 (<5-10)	<5
	5 (5)	8 (<5-40)	<5
	6 (6)	538 (320-640)	NT
	7 (7)	320 (160-640)	NT
Broiler breeder			
Flock No.	1 (11)	587 (320-1280)	NT
	2 (12)	538 (320-1280)	NT
	3 (12)	640 (320-1280)	NT
	4 (13)	415 (320-1280)	NT
	5 (17)	54 (10-160)	NT
	6 (17)	5 (<5-20)	<5
	7 (17)	77 (20-320)	NT
	8 (17)	104 (20-640)	NT
	9 (18)	67 (20-640)	NT
	10 (19)	44 (10-160)	NT
	11 (22)	48 (10-160)	NT
	12 (23)	20 (5-40)	<5
	13 (24)	113 (40-320)	NT
	14 (26)	281 (40-640)	NT
	15 (27)***	113 (20-640)	NT
	16 (29)	80 (40-320)	NT
	17 (29)	587 (320-1280)	NT
	18 (30)	538 (320-1280)	NT
	19 (30)	830 (640-1280)	NT
	20 (33)	10 (<5-40)	<5
	21 (33)	87 (40-320)	NT
	22 (37)	16 (<5-80)	<5
	23 (37)	698 (320-1280)	NT
	24 (37)	830 (640-1280)	NT
	25 (39)	60 (20-640)	NT
	26 (42)	698 (320-1280)	NT
	27 (43)	267 (40-640)	NT
	28 (44)	830 (320-1280)	NT
	29 (47)	26 (10-80)	<5
	30 (58)	830 (320-1280)	NT
	31 (59)	760 (320-1280)	NT
	32 (60)	587 (320-1280)	NT
	33 (?)	640 (320-1280)	293 (160-640) CELO
	34 (?)	830 (320-1280)	226 (80-320) CELO

Isolation and identification of virus

a) AAV

Adenovirus was isolated in CKC culture from samples of 7 livers, 1 bursa and 1 intestine from affected broiler flocks. Characteristic round-type CPE, indicative of adenovirus, was obtained usually on the second or third passage of inoculated CKC. Virus was isolated from one liver suspension derived from an affected breeder flock on the third passage in inoculated CKC. Cover-slip cultures of CKC, inoculated with each of the viral isolates and stained with HE, showed INIBs characteristic of adenovirus. Negatively stained icosahedral virus particles of 70-80 nm in diameter were observed under EM. All of the isolates retained infectivity after treatment with chloroform (details in Chapter 3).

b) CAA

Culture of liver tissue suspensions on MDCC-MSB1 cells failed to show the presence of CAA by indirect IF test. No signs of illness, lesions or changes indicative of CAA were observed in the chicks and embryos after inoculation of any of the suspected liver samples.

Detection of viral antigens in eggs

Viral antigens were detected in some eggs, either in egg yolk or both in egg yolk and albumen, from each of the 6 sheds of breeder chickens (Table 2.6).

Eggs which contained viral antigens in the albumen always contained them in the yolk whereas viral antigens were not detected in the albumen of every egg which had such antigens in the yolk. Viral antigens were detected in the egg yolk (16 of 60 samples) more frequently than in the albumen (5 of 60 samples) ($p < 0.01$). Also the amount of antigen detected in egg yolk (mean ESA 0.11) was usually higher than that in albumen (mean ESA 0.10) ($p = 0.1$). The maximum ESA obtained with egg yolk and albumen was 0.32 and 0.15, respectively.

Viral antigens were not detected in any of the floor litters tested from the above sheds by ELISA.

Table 2.6 **Detection by ELISA of AAV antigens in eggs derived from conventional breeder chickens (50-60 weeks of age).**

Chicken (breeder) sheds	Number of positive eggs (range of ESA* values) Egg yolk	Egg albumen
1	2** (0.07-0.11)	0
2	3 (0.07-0.12)	1 (0.09)
3	4 (0.08-0.13)	2 (0.07-0.09)
4	2 (0.08-0.09)	0
5	1 (0.32)	1 (0.15)
6	4 (0.07-0.10)	1 (0.08)
Total (mean ESA)	16***(0.11+)	5 (0.10)

* ESA = ELISA specific absorbance obtained by subtracting the absorbance wih SPF eggs from that obtained with test eggs (values >0.06 considered positive).

** 10 eggs were used from each shed of breeder chickens.

*** $p < 0.01$

\+ $p = 0.10$

DISCUSSION

This chapter has described the investigation of outbreaks of disease in broiler flocks which, on the basis of histological findings and virus isolation, are believed to be IBH. Studies to determine the pathogenicity of the AAVs which were isolated are described in Chapter four. The prevalence of VN antibodies to AAVs in broiler and breeder flocks and the demonstration of transovarian transmission of IBH virus in breeder flocks in New Zealand are also described.

Evidence from the field appearance of this condition pointed to a disease of much greater severity than any previously recognized in New Zealand. In fact, with the exception of an outbreak in New South Wales, Australia, which was of similar severity (Wells et al., 1977), the disease was more severe than any other outbreak of IBH reported previously. The field data imply a strong association between IBH outbreaks in broilers and the impaired performance of breeder flocks during the time when the eggs from which affected broilers hatched were laid. Although adenoviruses are hardy organisms (Winterfield, 1984) that persist in the environment, it is hard to imagine that such severe disease could break out spontaneously in such geographically separated broiler farms over such a short period. The common factor was the parent flocks. As the flocks were small, individual broiler placements all contained chicks from more than one parent flock, making it impossible to determine if one or more than one parent flock gave rise to infected broilers. Analysis of data for individual breeder flocks (not presented) showed that at least four individual breeder flocks underwent declines in performance, averaging 3 weeks in duration for each flock.

It is not known whether the breeders were always infected with the virus and an immunosuppressive agent caused an increased excretion of the virus, the flocks were newly infected with a hitherto unknown strain of adenovirus or whether the adenovirus gained entry to the eggs as a result of impaired egg hygiene due to the diarrhoea (evident as pasty

vents) in the breeders.

The presence of mycotoxins (sterigmatocystin and possibly others) in the breeder feed (Dawson PJ, personal communication), suggests that an immunodepressive episode affected breeder flocks. That the birds were under some feed stress was evident by their reluctance to consume the feed presented each day. Feeding took about three times longer than expected. Near-total feed refusal was noted on two occasions. Therefore, in addition to the feed stress, the birds were ingesting the mycotoxins present in the feed, but probably only after a period of hunger had overcome their reluctance to eat. Mycotoxins were excluded as a direct cause of the haemorrhagic anaemia condition in the broilers on epidemiological grounds. Affected broiler flocks were fed on feed from a large number of sources, and broiler flocks all showed signs of infection at closely similar ages. This pattern is not indicative of a feed-borne mycotoxicosis.

Of other possible agents of this outbreak of haemorrhagic disease, CAA was considered. No evidence for the presence of CAA could be found by cultural means or by immunofluorescence. Furthermore, while low levels of CAA antibodies were detected in sera from some flocks, their appearance was not consistent with criteria for the field development of CAA (Yuasa et al., 1983). The disease appeared in broiler flocks with and without CAA antibodies in sera collected at slaughter, that is, at 6 to 7 weeks of age and at least 10 days after peak mortality in affected flocks.

The results from breeder flocks were not easily interpreted with respect to CAA status, as flocks that were implicated in field outbreaks (flock S4, for example), and those that were not (B2, for example) were both positive for CAA antibodies when sampled.

The effect of MD as an immunodepressive agent was considered, because MD had been diagnosed 8 weeks earlier as a contributory cause of poor growth on one of the farms affected with IBH. The incidence of IBH in HVT-vaccinated and unvaccinated flocks is

shown in Table 2.2. HVT vaccination had no clear effect on the incidence of IBH. Therefore, it is concluded that MD did not play an important role in the appearance of IBH. Other authors (Rosenberger et al., 1975; Fadly et al., 1976; Dhillon et al., 1982) have noted the potentiating effect of IBD virus on the field appearance of IBH. The facts that New Zealand is free from IBD (Howell et al., 1982; Government of New Zealand, 1984; Jones, 1986; With and Christensen, 1987), and that the IBH affected broiler flocks were confirmed as being free from antibody to IBD virus, are important in establishing the AAV as a major pathogen in its own right, in the absence of IBD virus.

Sera from all of the clinically affected broiler flocks tested had very low levels of VN antibodies to AAVs, whereas sera from two normal broiler flocks tested later contained high levels of antibody to serotype 8 AAV. Although most of the breeder flocks had high levels of VN antibodies against serotype 8 AAV, very low titre antibodies were occasionally observed in a few flocks probably those with low performance during the outbreaks of IBH. In the first few weeks, protective levels of VN antibody to AAVs in broiler flocks are expected to have been derived from the parent flocks, containing higher levels, of antibody. After weaning, antibody titres either rise, as a result of the flock being exposed to the virus from the environment, or they drop to undetectable levels over the next few weeks. During this period, birds become susceptible to AAV and can readily be infected and show clinical signs of IBH. The broiler birds with very low levels of maternal antibody could also be infected with IBH virus during the first 2-3 weeks of age. Similarly, high titres of antibodies in breeder flocks may or may not be maintained depending on the extent of continuing exposure to viral antigens.

Presumably 10 years after the outbreak of IBH in New Zealand in 1977 (Table 2.1), the antibody titres against AAV dropped to very low levels in some of the strictly isolated breeder flocks. In 1987, severe outbreaks of IBH occurred in the progeny broiler flocks which originated from these parent flocks. Subsequently, a high level of antibody is built up in most of the breeder flocks and both they and their progeny flocks remain protected by

their high levels of antibody against IBH virus. Such an occurrence would account for the cyclical nature of IBH outbreaks in the absence of immunosuppressive factors or agents such as IBD virus.

Sera from two breeder flocks contained high titres of antibodies to both serotype 8 and 1 AAVs. Presumably some of the flocks containing high levels of antibodies to serotype 8 AAV may contain antibodies to several other serotypes which is similar to the findings of Grimes et al. (1977a).

Although transovarian transmission of strains of AAV associated with IBH has been previously suggested (Cowen et al., 1978a; Reece et al., 1985), there have been no reports of direct detection of virus in eggs. In this study, viral antigens were detected by ELISA in the egg yolk and egg albumen of about 25% and 10%, respectively, of the eggs derived from conventional broiler-breeder flocks. Although these birds had high levels (titre 320-1280) of VN antibodies, it was possible to detect viral antigens by ELISA whereas it might not have been possible to isolate infective virus in cell culture. Cowen et al. (1978a) isolated adenovirus in cell culture from 14% of cloacal swabs taken from newly hatched chicks originating from eggs laid 7 days after the hens were infected with serotype 7 AAV. Reece et al. (1985) isolated AAV in cell culture from the caecal tonsil of the birds which had been infected with serotype 8 AAV but not from composite samples of oviducts or tracheas. They failed to isolate virus from any of the eggs produced by these birds. Dawson et al. (1981) isolated adenovirus in cell culture from 20% of the fertile eggs laid during the week following infection of birds with serotype 1 AAV (CELO). The successful isolations followed concentration of the allantoic fluid with polyethylene glycol. Direct detection of AAV antigens by ELISA in egg materials strongly supports the hypothesis that transovarian transmission of AAV occurs if infection is present in breeder flocks.

In other studies, AAV antigens have been detected by ELISA in the duodenum, ileum, caecum, caecal tonsil and faeces for up to 20 weeks after a local serotype 8 strain of AAV

was administered by oral inoculation into two-day-old SPF chickens (see Chapter 6). These birds all had detectable VN antibodies to the infecting strain. It appears that persistent infection occurs frequently after initial exposure. Presumably at sexual maturity virus spreads to the oviduct and infects the developing eggs, thus ensuring transmission of virus to the next generation (Cowen et al., 1978b).

While transovarian transmission may be important for initial entry of virus into a flock it may not be the most important factor in maintaining and spreading infection. AAVs are very resistant in the environment and survive for long periods in litter, water or feed which has been contaminated with faeces from infected birds.

To aid in the maintenance of New Zealand's privileged position with respect to freedom from many infectious diseases, particularly IBD, a policy of only permitting the import of embryonating eggs has been enforced. On the basis of this study, such a policy would not exclude the entry of strains of AAV capable of causing IBH in susceptible birds.

Because the ELISA used in this present study detects a group-specific antigen, it cannot be stated with certainty that the viral antigens detected in the eggs were derived from serotype 8 strains or from one of the other 11 AAV serotypes. However, antibodies only to serotype 8 AAV were detected in this breeder flock so there is no evidence for infection with any other serotype of AAV. It is important to note that these eggs were derived from groups of birds which had high levels of neutralizing antibody against serotype 8 AAV. It is known from earlier studies, as already mentioned, that persistent infection can exist in the presence of neutralizing antibodies. Chickens which are hatched from eggs derived from infected flocks may have passively derived antibody and, from the results of this present study, be infected with AAV. As the antibody levels wane by around 3 weeks of age there would be the opportunity for suitable strains of AAV to spread from the intestinal tract and cause IBH. Fadly and Winterfield (1973) reported that chickens, derived from breeder flocks infected with AAV, were refractory to challenge with the same strains of AAV up to 3 weeks of age

but were susceptible after 4 weeks.

The use of an ELISA to screen fertile eggs from breeder flocks may be useful if it is desired to select breeder flocks which are free of AAV, particularly as parents of SPF flocks.

SUMMARY

The outbreaks of disease resulting in up to 30% mortality in broiler flocks in New Zealand were diagnosed as IBH on the basis of histopathology and isolation and identification of AAV. Increased mortality was noticed by 2 weeks; mortality peaked at the fourth week and declined to normal levels by fifth week of age. Significant changes observed at post mortem examination included a swollen haemorrhagic liver with focal necrosis, aplastic bone marrow and atrophy of the bursa and thymus with marked lymphocytic depletion. Eosinophilic INIBs were often observed in the hepatocytes.

Adenoviruses were isolated in the SPF CKC cultures from the liver tissues derived from a number of affected flocks. In the affected broiler flocks there was no evidence of any immunosuppressive agents or factors including IBD virus, CAA, MD virus and mycotoxin. All the sera tested were negative to IBD virus.

Most of the breeder flocks contained high levels of VN antibody titres against serotype 8 AAV whereas the clinically affected broiler flocks had very low levels of antibody against all the prototype strains of AAV.

AAV antigens were detected by ELISA directly in the egg yolk and egg albumen of about 25% and 10%, respectively, of the eggs derived from conventionally raised broiler-breeder flocks. Direct detection of AAV antigens in egg materials strongly supports the hypothesis that transovarian transmission of AAV occurs if infection is present in breeder flocks.

CHAPTER THREE

ISOLATION AND IDENTIFICATION OF AVIAN ADENOVIRUSES FROM FLOCKS WITH INCLUSION BODY HEPATITIS

INTRODUCTION

Several living cell systems, including cell cultures, embryonating eggs and animals, have been used for viral propagation. No single system will support the growth of all viruses; hence, the researcher is faced with the problem of selecting culture environment(s) suitable for isolation and identification of a particular viral agent. Cell cultures are the host system most frequently used for virus isolation or cultivation because of their broad spectrum of susceptibility and the relative ease with which they are maintained.

Adenoviruses are strictly host cell-specific. The most suitable and sensitive cell cultures for HAds are human laryngeal carcinoma and human cervical carcinoma cell lines and primary human embryonic kidney cells and, for AAVs, primary chicken embryo liver and kidney cells (Leland and French, 1988). There is no cell line available for the cultivation of AAVs.

Theoretically, viruses can be identified using various methods such as serology, immunocytochemistry, ELISA, and even by direct isolation of their genomes or structural proteins by electrophoresis. Alternatively, the genome may be cleaved enzymatically and certain fragments can be identified. Such direct methods require a relatively high concentration of virus and their use is therefore limited (Ranki et al., 1983). On the other hand, one of the most important methods of viral diagnosis remains propagation of virus in cell cultures in which identification can be based on susceptible cell types, morphological changes induced, growth rate, and other features.

The isolation of a virus is followed by confirmation of its identity. Both immunological and nonimmunological methods are used in the identification of an isolated virus and for detection of viral antigens within cell cultures or infected tissues or materials. Immunological methods include IF, immunoperoxidase (IP) staining, ELISA, VN, HAI, passive agglutination (PA), radioimmunoassay, and immune adherence HA. IF, IP staining, HAI, ELISA and PA provide rapid identification of viruses, whereas VN is more costly and time consuming. Nonimmunological methods include histological staining, EM, haemadsorption (HAD), HA, challenge interference (CI) and DNA probes. HAD, HA, and CI

are used primarily to detect viruses which do not produce a CPE within cell cultures (Leland and French, 1988).

The development of DNA probes has provided clinical virology with a powerful diagnostic tool for the detection and identification of viruses. This technique has been applied for detection of several viruses including HAds (Berninger et al., 1982; Enzo Biochem, 1985; Hyypia, 1985; Gissmann et al., 1986).

Although EM has long been recognized as a useful diagnostic tool (Nagler and Rake, 1948; Van Rooyen and Scott, 1948), virologists have been rather slow to use it for detecting viruses in clinical specimens. The sensitivity of direct EM examination may not be as high as other diagnostic methods but a relatively new technique, solid-phase immunoelectron microscopy, has been shown to increase the sensitivity of EM (Doane, 1986).

In this chapter the isolation and characterization of AAVs from chickens in flocks experiencing outbreaks of IBH are described. Isolates were identified by growth in CKC cells and their morphological appearance and typed serologically and by restriction endonuclease analysis.

Wild type isolates that display extensive cross-reactivity in VN tests or are difficult to type because of their fastidious properties can be efficiently assigned to the correct subgroup or genus by DNA restriction enzymes (Adrian et al., 1986). Serologically indistinguishable Group II AAVs have been separated by analysing their genomes using restriction enzymes (Zhang and Nagaraja, 1989). In this chapter, attempts were made to distinguish AAVs of the same serotype on the basis on their biological characteristics such as virulence and by restriction enzyme digestion patterns of their genome.

MATERIALS AND METHODS

SPF chickens

Birds used in this study were hatched from eggs derived from a White Leghorn (WLH) SPF flock maintained at MAF Qual Laboratories, Wallaceville, New Zealand. The parent flock was seronegative to 12 serotypes of AAV, IB virus, MD virus, leukosis virus, avian influenza virus, Newcastle disease (ND) virus, avian encephalomeylitis (AE) virus, infectious laryngotracheitis virus, IBD virus, EDS virus, avian reovirus, reticuloendotheliosis virus, *Mycoplasma gallisepticum* and *M. synoviae* and *Salmonella pullorum* and *S. gallinarum*. Originally the parent flock was received as fertile eggs from the CSIRO SPF Poultry Laboratory, Australia. The parent flock in the CSIRO Laboratory was a mixture of pure WLH and a synthetic SPF line of WLH (8th generation). The latter flock is best described as a 'parafield mini' bird containing a dwarf gene. The genetic status of the parent flock in New Zealand is not known.

Preparation of CKC culture

SPF chickens up to two weeks old were used for CKC culture. The chickens were tested and found to be free of VN antibodies to any of the 12 strains of AAV. One-day-old, conventionally raised chickens were also evaluated as a source for CKC culture. The CKC cultures were grown in MEM (pH 7.4) containing 10% FBS, 10% tryptose broth and antibiotics (penicillin 100 units/ml, streptomycin 100 ug/ml and kanamycin 100 units/ml; PSK) and maintained at confluency in MEM containing 2% FBS, 2% tryptose broth and similar amounts of PSK. Sometimes HEPES buffer (Sigma Chemical Co. USA) (pH 7.4) was used in the growth medium at a concentration of 0.5% (w/v).

Culture of liver cells from two-day-old SPF chickens was also evaluated following a similar procedure.

Chickens were stunned and then killed by cutting the throat. The skin, feathers, legs and wings were removed to reduce the risk of contamination. The kidneys were then collected aseptically into a sterile petri dish inside a positive-pressure, laminar flow cabinet. The cortical portions of the kidneys (free of blood clots and tubular structures) were separated and minced. The tissue fragments were washed several times in warm PBS (Ca^{++}, Mg^{++} free) containing antibiotics (PSK) to remove soluble substances and erythrocytes, and then

suspended and stirred for 5 min at 37^{o}C in 25 ml of an antibiotic-trypsin-versene (ATV) solution containing 0.05% trypsin (Sigma Chemical Co. USA). The supernatant was discarded, fresh ATV solution added, and stirring continued for about 25 min. The cell-suspension was passed through a wire mesh into a universal bottle containing 2 ml of FBS (approximately 10% of the total volume of cell suspension) and centrifuged at 200 xg for 10 min. The cell pellet was mixed with 10 ml of warm growth medium by gentle shaking. A 200 ul aliquot of cell suspension were mixed with 1.8 ml of Trypan blue and the transparent live cells were counted. The number of cells per ml was obtained by multiplying, by the factor x10^{5}, the average number of live cells counted per square. The cells were distributed at a concentration of approximately 2-5x10^{6}/ml of growth medium in a range (25, 80, 150 cm^{2}) of tissue culture flasks (Nunc, Delta, Denmark) depending on requirements, and incubated at 37^{o}C. At confluency, the growth medium was replaced by maintenance medium and incubation continued sometimes until the monolayer degenerated. The cells at confluency were occasionally passaged and used for virus isolation or propagation.

Passaging of the cells

At confluency of the monolayer, the maintenance medium was poured off and the cells washed with warm PBS. One to three ml of ATV solution (depending on the size of the flask) were spread on the monolayer surface and incubated at 37^{o}C for 2-5 min. The detached cells were resuspended in 5-10 ml of growth medium containing 10% FBS. The cells were counted and distributed in the original, and in new cell culture flasks, at a concentration of 1-2 x 10^{6} cells/ml of growth medium. By the second day of incubation, monolayers had usually reached confluency, and they were then repassaged or used for virus inoculation. CKC cultures were used up to the third passage level for viral propagation.

Preparation of inoculum

In most of the cases liver tissue, and occasionally bursa or intestinal tissue, derived from clinically sick or moribund birds was used for isolation of virus. During post mortem examinations, 13 infected livers (716W, 717B, 718, 18832, 716B, Dock39, RMCWB, 723, 19358, 18975, 820, 723 and 717C), 1 bursa (WV6642) and 1 intestinal (WV4513) tissue, each from a different flock, were collected aseptically into sterile containers. One liver sample (736NI) derived from a broiler flock which had no clinical signs of disease was similarly processed. Approximately 10% (w/v) suspensions of the tissues were prepared in

PBS (Ca^{++}, Mg^{++} free) containing antibiotics (penicillin 200 units/ml, streptomycin 200 ug/ml and kanamycin 200 units/ml) using a tissue homogenizer or stomacher machine (Stomacher 400, Colworth Ltd London). After 2-3 cycles of freezing and thawing, each tissue suspension was centrifuged at 800 xg for 20 min and the supernatant was stored at -75^{o}C as the inoculum.

Cultivation of virus in cell culture

a) Isolation of virus in CKC

For initial isolation of virus, 25 cm^2 tissue culture flasks containing established monolayer cultures of CKC were used. At confluency, the growth medium was poured off and 1.0 ml of each of the 16 tissue preparations was added onto a monolayer and allowed to adsorb for 1 hr at 37^{o}C. About 7 ml of maintenance medium containing 2% FBS was added to each flask and incubation at 37^{o}C was continued. In the control flask, the growth medium was also replaced by maintenance medium and similarly incubated. The flasks were examined daily with an inverted microscope for the appearance of CPE consistent with viral growth. On the 5th day pi, the inoculated cultures were frozen and thawed three times and reinoculated onto freshly prepared monolayer cultures of CKC. When CPE was observed it was allowed to progress to involve about 90% of the cells which were then disrupted by 2 cycles of freezing and thawing. The resulting suspension was stored at -75^{o}C as 'new isolate' in 500 ul aliquots until further examination. At least 3 blind passages of inoculated cultures were performed before any sample was considered negative.

b) Propagation of virus in CKC

The local isolates of AAV were cloned by three limit dilutions in CKC before they were titrated and propagated in large volumes. For the propagation of large amounts of virus, larger sizes of tissue culture flasks (80 or 150 cm^2) were used. At confluency, 1-2 ml of each viral isolate (total number 12) were inoculated onto the established monolayer culture of CKC and allowed to adsorb for 1 hr at 37^{o}C before the maintenance media was added. The flasks exhibiting >80% CPE were frozen and thawed 3 times before harvesting the suspension of virus and cellular debris. This suspension was clarified by low speed centrifugation and stored at -75^{o}C. Twelve prototype viruses (listed before) were also similarly propagated in CKC.

Titration of virus in CKC

Ninety-six well, flat-bottom, tissue culture plates (Nunc, Delta, Denmark) were used for the titration of virus. Ten-fold serial dilutions (10^{-1} to 10^{-8}) of each viral isolate in growth medium were added to quadruplicate rows of the microtitre plates (50 ul/well). One hundred and fifty microlitres of CKC suspension containing $3x10^5$ cells in growth medium were added to each well from the highest to lowest dilution of virus. The plates were incubated at 37°C in an atmosphere of air supplemented with 5% CO_2. Usually after 5 days of incubation, the end-point of the infectivity titration of each virus was determined under an inverted microscope or by staining with crystal violet solution. The titre of virus, expressed as $\log_{10}$ $TCID_{50}$ per unit volume, was calculated by the method of Reed and Muench (1938).

Cytopathology

CKC cultures grown on a sterile coverslip inside Leighton tubes were used to study the cytopathology produced by each of the viral isolates. A 200 ul inoculum of each virus was added onto a confluent monolayer of CKC in each tube and allowed to adsorb for 1 hr at 37°C before 1.5 ml of maintenance medium was added to each tube. The inoculated tubes, and control tubes containing non-infected CKC, were incubated at 37°C. At 12, 24, 36 and 48 hr pi inoculated and control tube for each isolate was fixed in Bouin's fluid. After 15 hr the cells were washed with 70% ethyl alcohol and stained with HE following standard methods before being examined under a light microscope.

Cultivation of virus in embryonating eggs

A 200 ul aliquots of each of the viral isolates 716W, 717B, 736NI and WV6642/88 were inoculated into the AC of each of 5 embryonating eggs (10-11 days old), which were derived from SPF birds, and incubated at 37°C. The eggs were examined daily for mortality or lesions consistent with viral growth. The embryos which died up to 24 hr pi were discarded. The remaining embryos were observed carefully until 6 days pi. The dead embryos, following the second day pi, and the live embryos after 6 days pi, were chilled for at least 2-4 hr. The allantoic fluid was collected aseptically and the embryos were examined for any lesions. The allantoic fluid was clarified by low speed centrifugation and reinoculated into a new batch of 5 embryonating eggs. For certain viruses up to 7 blind passages were performed.

EM examination

The infected cell cultures showing >80% CPE were disrupted by 3 cycles of freezing and thawing and clarified by centrifugation (2000 xg for 15 min). The supernatant was ultracentrifuged at 200000 xg for 3 hr and the resulting pellet was resuspended in 500 ul of distilled water. The viral suspension was negatively stained with 2% PTA (pH 7.2-7.4) and examined under a transmission EM (Philips 201C).

Sensitivity to chloroform

The sensitivity of the viral isolates 717B and 716W to chloroform was determined following the method described by Feldman and Wang (1961). A 1.9 ml aliquot of viral suspension was added to 100 ul of reagent-grade chloroform and the virus-chloroform mixture was shaken for 10 min at room temperature (20-25^{o}C). The mixture was centrifuged at 500 xg for 15 min. The virus in the upper aqueous layer was removed and assayed for infectivity in CKC culture. For comparison, the infectivity of the respective control virus (without chloroform treatment) was also assayed in CKC under similar conditions.

Temperature sensitivity

The stability of viral isolates 717B and 716W was tested at 50^{o}C and 4^{o}C for various periods. Aliquots of cell culture virus were placed in a water bath at 50^{o}C and at 1, 2, 3, 4 and 5 hr intervals one aliquot of each isolate was transferred to -75^{o}C until the residual infectivity was titrated in CKC. Aliquots of the same viral isolates were kept at 4^{o}C and tested for infectivity in CKC after 3 months, 6 months and 1 year. Each time the infectivity was compared by titrating the control virus which was directly taken from the -75^{o}C freezer.

Preparation of viral antigen

Antigens prepared from 8 prototype strains representing serotypes 1, 4, 5, 7, 8, 10-12 of AAV and the New Zealand isolates, 717B and 716W, both representing serotype 8 AAV, were used for the production of antisera in rabbits and chickens. Eighty millilitres of cell culture stock virus were mixed with 40 ml of reagent grade chloroform and homogenized for 30 sec in a high speed homogenizer (DuPont Instruments Co, USA). The virus-chloroform mixture was centrifuged for 20 min at 1000 xg. Above the chloroform layer, an opaque,

interphase layer was observed. The uppermost clear aqueous layer was collected and ultracentrifuged at 130000 xg for 2 hr at 4^{o}C. The resulting pellet at the bottom of the tube was resuspended in 8 ml of distilled water and stored at -75^{o}C in four 2 ml aliquots. The prototype strains, were inactivated by adding 2% (v/v) glutaraldehyde (25% aqueous solution, Serva, Feinbiochemica, USA) for 30 min before ultracentrifugation.

Production of antiserum

Antisera against the 8 prototype strains and the two New Zealand isolates were produced in 7-month-old rabbits. Antisera against 717B and 716W isolates were also produced in 3-month-old SPF chickens. Four ml of stock antigen were blended with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA) and 1 ml was injected into each thigh muscle of 2 rabbits and 2 chickens. After 3 weeks a second inoculum was prepared by blending 4 ml of antigen with 4 ml of incomplete Freund's adjuvant (Difco Laboratories) and administered to the rabbits and chickens. Two weeks later, the sera were tested in VN assays against the homologous adenovirus. Three weeks after the second inoculation, blood was collected from the rabbits and chickens and the serum harvested. Serum was inactivated at 56^{o}C for 30 min and stored at -75^{o}C. Negative control sera included preinoculation sera and sera collected from healthy rabbits and SPF chickens.

Viruses and antisera used in neutralization tests

Eleven prototype strains (CELO, SR48, SR49, KR5, 340, CR119, YR36, HVI, A2, C2B and 380) of AAVs representing serotypes 1 to 8 and 10 to 12, together with homologous rabbit antisera and 3 Australian isolates (VRI85, Qld and WA) representing serotype 8 AAV were supplied by Dr. D.A. Barr, Veterinary Research Institute, Melbourne, Australia. Strain 764 (serotype 9) and homologous rabbit antiserum were obtained from Dr. J.B. McFerran, Veterinary Research Laboratories, Belfast, Ireland. Eleven local isolates (716W, 717B, 718, 18832, 716B, Dock39, RMC, 736NI, 19358, WV6642 and WV4513) of AAVs together with rabbit antisera against AAV isolates 716W and 717B were also used. Because of the small volumes of antisera supplied, additional antisera against 8 prototype strains (CELO, KR5, 340, YR36, HVI, A2, C2B and 380) were produced in rabbits as described above.

Neutralization test procedure

VN tests were carried out in 96-well, flat-bottom tissue culture plates (Nunc, Delta, Denmark). Twenty-three viral strains (12 prototype strains and 11 local isolates) and 14 antisera (12 against the prototype strains and 2 against local isolates 716W and 717B) were compared in two-way (cross) neutralization tests in primary CKC cultures. Each serum was diluted in two-fold steps (1:10 to 1:20480) and 50 ul of each dilution was reacted with an equal volume containing approximately 100 $TCID_{50}$ of each of the viruses for a minimum of 1 hr at room temperature. One hundred and fifty microlitres of CKC suspension containing 3×10^5 cells in growth medium were added to each well. The plates were incubated at 37^oC inside a modular plastic chamber supplied with 5% CO_2. After 5 days, the plates were stained with 0.75% crystal violet solution. The VN antibody titre was taken as the reciprocal of the highest dilution of serum which completely inhibited the viral CPE in the cell culture. The tests were run in duplicate. During the VN test, each of the viruses used was titrated to determine the exact amount of virus ($TCID_{50}$/50 ul) being used.

Staining of the cell culture plates

After 5-6 days of incubation, the tissue culture fluid was removed by briskly shaking the plates into an autoclavable container inside the laminer flow hood. The plates were immersed in crystal violet working solution (stain/virus inactivant) for at least 10 min to inactivate the virus and to stain the plates. The plates were rinsed thoroughly with tap water and examined. The formula of the staining solution is given in Appendix I.F

Extraction of viral DNA

Six selected strains of serotype 8 AAVs (locally isolated strains 717B and 716W, the Australian isolates QLD, VRI and WA and the reference strain HVI) were extracted with chloroform, pelleted by ultracentrifugation (130000 xg for 3 hr), suspended in 3-4 ml of distilled water and dialyzed for 48 hr at 4^oC against TE buffer (0.1 M Tris, pH 8.5-0.01 M Na_2EDTA) with changes of buffer at 12 hr intervals. All reactions were performed in Eppendorf (Eppendorf Geratebau, Hamburg, Germany) centrifuge tubes and all reagents and materials were autoclaved or filter sterilized before use. Deionized water (Milli-Q, Reagent water system, USA) was used for all buffers and solutions. A 500 ul aliquot was digested with SDS (final concentration 0.5%) and proteinase K (1 mg/ml) for 2-3 hr at 37^oC. The tube was gently shaken once during the incubation period. An equal volume of

buffered phenol-chloroform (1:1) mixture was added, mixed gently by inverting the tube 20 times and centrifuged for 5 min (maximum speed) at room temperature.

The aqueous phase was gently collected and added to an equal volume of phenol-chloroform mixture (1:1), mixed gently as above and centrifuged. The aqueous phase was collected, mixed with an equal volume of chloroform and centrifuged. Finally, the volume of the aqueous phase was measured and 1/10 of a volume of 3 M sodium acetate (pH 4.8) was added. The total content was divided into 2 tubes. Three volumes of 95% ethanol which was stored at -20^{o}C were added to each tube and left overnight at -20^{o}C to precipitate the DNA. The tubes were centrifuged for 1 hr at 4^{o}C. The supernatant was decanted and the precipitate was washed with 1 ml of 70% ethanol (stored at -70^{o}C) for 15 min at room temperature. The ethanol was decanted and the DNA precipitate was vacuum dried for 15-30 min before it was suspended in 20 ul (each tube) of TE buffer (10 mM Tris, pH 7.5-1 mM Na_2EDTA) and stored at 4^{o}C.

The approximate amount of extracted DNA was estimated by electrophoresis of a mixture of 3 ul of DNA, 6 ul of TE buffer and 1 ul of sample loading buffer (bromophenol blue dye, 10x, Appendix IV)) on a horizontal 0.7% agarose (Bio-Rad Laboratories, California) mini gel (Horizon^{TM} 58, Bethesda Research Laboratories, USA) in TAE (Tris-acetate EDTA, pH 7.8) buffer at 100 mA voltage for one hour at room temperature. The gel was stained with ethidium bromide (1 ug/ml) for 15-30 min and rinsed with distilled water before being visualized under UV light. Photographs were taken using Polaroid film (type 667 ASA 3000 and type 665 ASA 80) and the amount of DNA required for the endonuclease digestion was estimated by the thickness of the band.

Digestion of DNA by restriction endonucleases

The restriction enzymes EcoRI and BamHI (Bohringer Mannheim, West Germany) which recognize six-base pair DNA sequences were used for the digestion of DNAs from the 6 different strains of serotype 8 AAV. An aliquot of 3-6 ul of DNA (depending on the concentration) was incubated for 1 hr at 37^{o}C in the presence of 2 ul of high salt incubation buffer (Appendix IV), 2 ul (20 units) of restriction enzymes and 10-13 ul of deionized water. The mixture was dipped into ice to stop the enzymic reaction and 2 ul (1:10) of bromophenol blue dye added before electrophoresis.

The fragments of digested DNAs were separated by electrophoresis on a 20x15x0.3 cm (well 1.5 mm width) horizontal 0.7% agarose slab gel (LKB 2012, Sweden) in TBE (tris-

borate EDTA, pH 8.3) buffer (Appendix IV) for 13 hr at 33 mA at room temperature. The gel running period was adjusted by evaluating the extent of migration of the DNA fragments in several gels for various times. The DNA of the bacteriophage lambda (Bohringer Mannheim) was digested by HindIII enzyme and run concurrently as a MW (size) marker. The gels were stained with ethidium bromide, rinsed with water and photographs were taken under UV light using polaroid film and a yellow filter.

Analysis of the restriction patterns of the genomes of AAVs

The number and size of DNA fragments generated by EcoRI and BamHI of each strain of virus were compared.

RESULTS

Isolation and identification of virus in CKC

In preliminary experiments it was found that CKCs derived from younger (<7 days) chickens were most suitable for primary isolation of viruses. Many conventionally raised chickens had VN antibody titres to serotype 8 AAV. Also AAVs were occasionally present in cell cultures established from them so only kidneys from SPF chickens were used for virus isolation and characterization.

Out of 16 tissue samples tested, 11 isolates of AAVs were obtained at various passage levels (1-4). One isolate of reovirus (717C) was also obtained at the 3rd passage. Four samples were considered negative after 4 passages. With most of the viral isolates, several blind passages were required before CPE appeared on CKC (Table 3.1). With one liver sample (RMCWB), CPE appeared on the first passage. All of the viral isolates grew very fast in CKC, within 2-3 days pi the CPE involved >80% of the cells (Fig. 3.1) and almost completely destroyed the monolayer within 3 days pi. The CPE appeared similar to that produced by the prototype viruses and the viruses grew to high titres (up to 10^8 $TCID_{50}$/50 ul) in CKC although there was considerable variation between isolates (Table 3.1).

Development of CPE by serotype 9 AAV (prototype strain 764) was very slow and the rounded cells appeared to be much smaller in size than with the other serotypes. The monolayer was not completely destroyed by this virus. Development of CPE following inoculation of serotype 6 AAV (strain CR119) was also comparatively slower. The prototype viruses varied significantly in their cell culture infectivity titres.

All prototype viruses and the New Zealand AAV isolates were found to produce basophilic INIBs in CKC but they often varied in morphology and frequency (see Chapter 7). Some strains produced a larger number of inclusions compared with other strains at similar times pi.

Table 3.1 **Passages required before detection of a cytopathic effect in chicken kidney cell cultures and titre of each virus isolate.**

Virus isolate (tissue used)	Passages required (No.)	Titre *
716W (liver)	2	$10^{7.0}$
717B (liver)	4	$10^{6.5}$
718 (liver)	2	$10^{5.5}$
18832 (liver)	3	$10^{6.0}$
716B (liver)	3	$10^{6.5}$
Dock39 (liver)	2	$10^{8.0}$
RMCWB (liver)	1	$10^{7.5}$
19358 (liver)	3	$10^{5.5}$
WV6642 (bursa)	3	$10^{5.5}$
WV4513 (intestine)	3	$10^{6.5}$
736NI (liver) **	2	$10^{6.0}$
717C (liver)	3	$10^{6.5}$ (reovirus)

* Log_{10} $TCID_{50}$ /50 ul, calculated by the method of Reed and Muench (1938) from titrations in quadruplicate.

** Obtained from birds in a flock with no evidence of inclusion body hepatitis.

Cultivation of virus in embryonating eggs

Only the isolate 736NI (serotype 1) consistently produced changes in embryonating eggs such as dwarfing, haemorrhages on the whole body surface, haemorrhage and necrosis of liver tissue and death of embryos. Other isolates did not cause embryonic death but did cause slight dwarfing and minor haemorrhages on the body surface even after 7 passages in eggs.

Table 2.5 **Virus neutralizing antibody levels to avian adenoviruses (AAVs) in broiler and broiler breeder chicken flocks in New Zealand.**

* Reciprocal of the highest dilution of serum which completely inhibited cytopathic effect of 100 mean tissue culture infective dose of the respective virus.

** Geometric mean titre of 8 sera from each flock.

*** White Leghorn breeder flock.

NT Not tested.

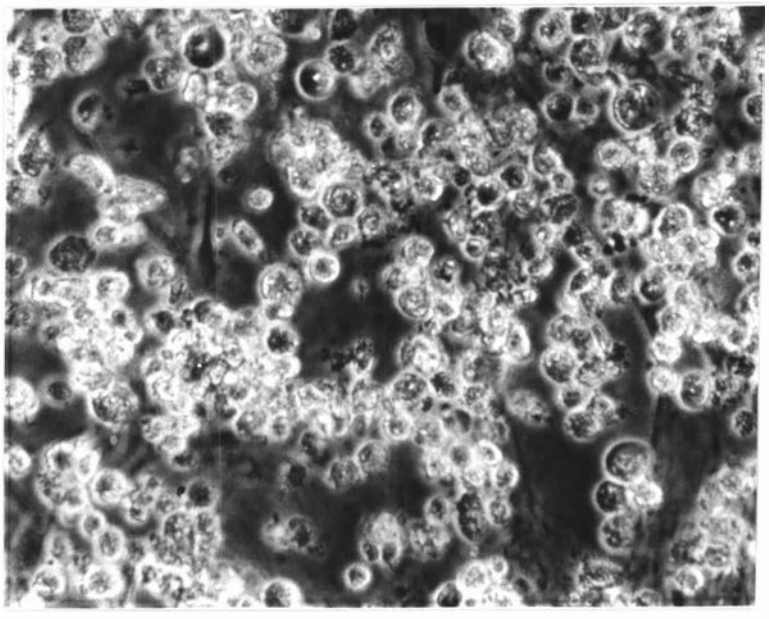

Fig. 3.1 **Advanced cytopathic effects on monolayer cultures of chicken kidney cells caused by locally isolated avian adenovirus (716W) at 2 days pi (unstained, x10).**

EM examination

All of the local viral isolates, when examined under the EM, contained icosahedral viral particles which were 70-80 nm in diameter (Fig. 3.2). No other viral particles were observed in any of the preparations except in one isolate (WV6642) where a large number of small (20-22 nm) viral particles were observed in association with typical adenoviruses. These are presumed to be adeno-associated parvoviruses (Fig. 3.3).

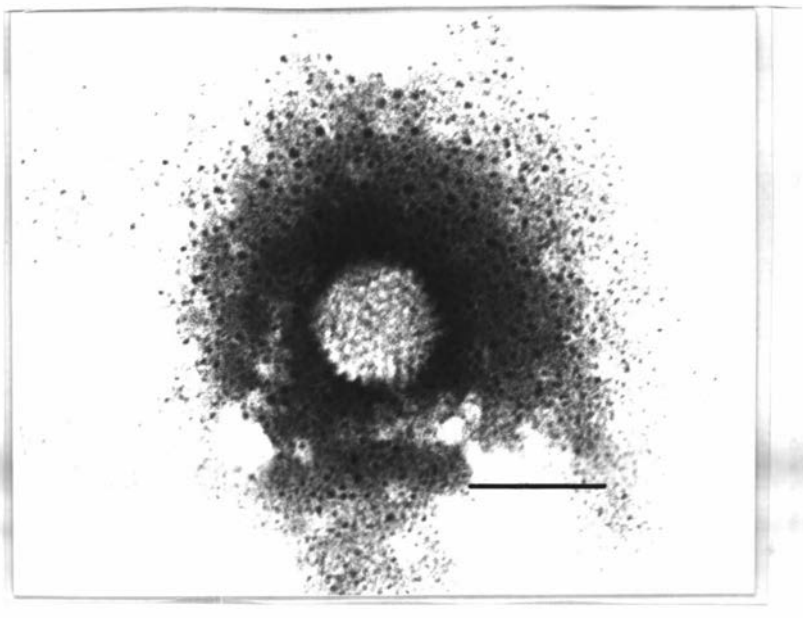

Fig. 3.2 **Icosahedral adenovirus particles observed with a locally isolated strain of AAV which had been propagated in cell culture (Bar = 100 nm).**

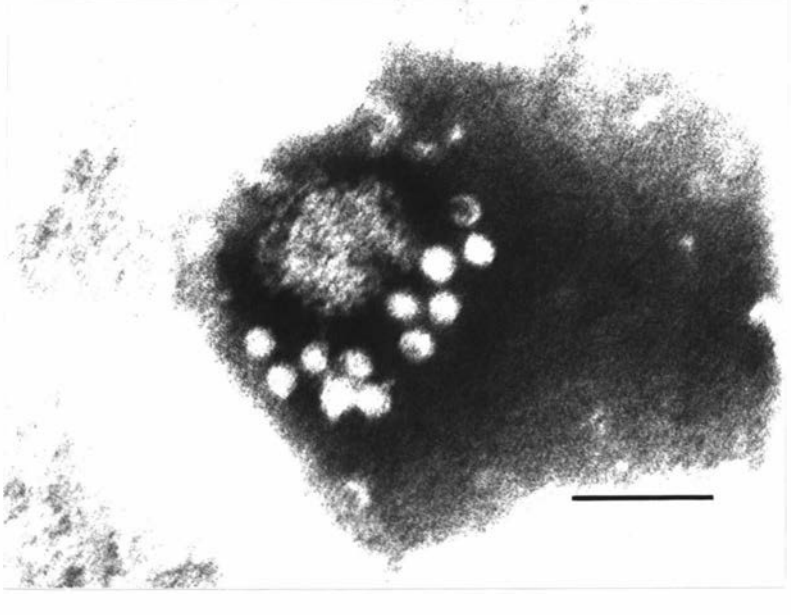

Fig. 3.3 **Adeno-associated parvovirus particles observed in association with a locally isolated avian adenovirus (WV6642) (Bar = 100 nm).**

Resistance

Both 716W and 717B viral isolates were found to be resistant to chloroform. No marked drop in infectivity titres was observed in any of the chloroform-treated viral isolates when compared with those of the control viruses (Table 3.2).

Table 3.2 **Sensitivity of avian adenoviruses to chloroform.**

Virus	Control virus	Chloroform treated virus
716W	$10^{6.50*}$	$10^{6.20}$
717B	$10^{7.00}$	$10^{6.75}$

* Log_{10} $TCID_{50}$ / 50 ul

The AAV isolates 716W and 717B were found to be fairly resistant to exposure to 4°C and 50°C. Although there was a drop of up to 80% in infectivity, high titres of infectious virus remained after 5 hr at 50°C and after 1 year at 4°C (Table 3.3).

Table 3.3 **Sensitivity of avian adenoviruses to various temperatures.**

Virus	at 50°C					at 4°C			Control virus
	1hr	2hr	3hr	4hr	5hr	3mo	6mo	1yr	(-75°C)
716W	$10^{6.5*}$	$10^{6.5}$	$10^{6.4}$	$10^{6.2}$	$10^{6.0}$	$10^{6.5}$	$10^{6.4}$	$10^{6.0}$	$10^{6.5}$
717B	$10^{7.0}$	$10^{7.0}$	$10^{6.8}$	$10^{6.7}$	$10^{6.4}$	$10^{7.0}$	$10^{6.6}$	$10^{6.3}$	$10^{7.0}$

* Log_{10} $TCID_{50}$ / 50 ul

Serotyping of AAVs

Homologous VN antibody titres recorded were 1280 to 10240 with all AAVs except prototype strain 764 (serotype 9) where the homologous titre was only 640 (Table 3.4). There were some cross-reactions noticed between prototype viruses and heterologous antisera. The CELO strain (serotype 1) of AAV was neutralized by antiserum to the YR36 strain (serotype 7) at a titre of 640 which was 16 times less than the homologous titre (10240). However, the YR36 virus was not neutralized by antiserum to CELO virus. Also the 340 strain (serotype 5) of AAV was neutralized by antiserum to CELO virus at a titre of 1280 which was 8 times less than the homologous titre (10240) but CELO virus was not neutralized by antiserum against strain 340. The KR5 strain (serotype 4) was neutralized by antiserum against the C2B strain (serotype 11) at a titre of 640 which was 8 times less than the homologous titre (5120). Also the C2B strain was neutralized by antiserum to the KR5 strain with a titre of 1280 which was 4 times less than the homologous titre (5120).

As shown in Table 3.4 nine New Zealand isolates were neutralized by antiserum to serotype 8 (strain HVI), 1 isolate to serotype 1 and 1 isolate to serotype 12.

Table 3.4 **Cross neutralization between 12 prototype avian adenoviruses (AAVs) and AAV isolates in New Zealand.**

* Reciprocal of highest dilution of serum which completely inhibited cytopathic effect of approximately 100 $TCID_{50}$ of the respective virus.

** Isolated from liver tissue of birds in flock with no evidence of inclusion body hepatitis.

*** Isolated from bursa of birds in flock affected with inclusion body hepatitis.

\- Antibody titre <10.

VIRUSES	ANTISERA 716W (8)	717B (8)	CELO (1)	SR48 (2)	SR49 (3)	KR5 (4)	340 (5)	CR119 (6)	YR36 (7)	HVI (8)	764 (9)	A2 (10)	C2 (11)	380 (12)
Prototype strains														
CELO	-	-	10240*	-	-	-	-	-	640	-	-	-	-	-
SR48	-	-	-	1280	-	-	-	-	-	-	-	-	-	-
SR49	-	-	-	-	1280	-	-	-	-	-	-	-	-	-
KR5	-	-	-	-	-	5120	-	-	-	-	-	-	640	-
340	-	-	1280	-	-	-	10240	-	-	-	-	-	-	-
CR119	-	-	-	-	-	-	-	1280	-	-	-	-	-	-
YR36	-	-	-	-	-	-	-	-	5120	-	-	-	-	-
HVI	2560	2560	-	-	-	-	-	-	-	10240	-	-	-	-
764	-	-	-	-	-	-	-	-	-	-	640	-	-	-
A2	-	-	-	-	-	-	-	-	-	-	-	10240	-	-
C2B	-	-	-	1280	-	-	-	-	-	-	-	-	5120	-
380	-	-	-	-	-	-	-	-	-	-	-	-	-	10240
New Zealand Isolates														
716W	10240	10240	-	-	-	-	-	-	-	5120	-	-	-	-
717B	10240	10240	-	-	-	-	-	-	-	5120	-	-	-	-
718	5120	10240	-	-	-	-	-	-	-	5120	-	-	-	-
18832	5120	5120	-	-	-	-	-	-	-	2560	-	-	-	-
716B	5120	5120	-	-	-	-	-	-	-	5120	-	-	-	-
DOCK39	10240	5120	-	-	-	-	-	-	-	2560	-	-	-	-
RMC	5120	5120	-	-	-	-	-	-	-	2560	-	-	-	-
736NI**	-	-	5120	-	-	-	-	-	-	-	-	-	-	-
19358	5120	2560	-	-	-	-	-	-	-	2560	-	-	-	-
WV6642***	-	-	-	-	-	-	-	-	-	-	-	40	-	2560
WV4513	5120	5120	-	-	-	-	-	-	-	5120	-	-	-	-

Restriction endonuclease analysis

Satisfactory preparations of DNA were obtained by extraction of each of the viral strains and a single dense band was evident after electrophoresis. Some variation in density of the viral DNA band was noted between strains.

For the DNA of each strain, the number of fragments produced by each enzyme (EcoRI and BamHI) and their migration distances were different. Also, for either of the enzymes, the pattern of bands produced with the DNA of any one strain was different from that of any of the other strains (Fig. 3.4). In spite of the differences, two major types of patterns were observed. One type included the New Zealand isolates and the reference strain, the other included the three Australian isolates.

The DNA of New Zealand isolate 717B was cleaved into 10 and 9 segments by EcoRI and BamHI respectively. One less band was apparent in the pattern of DNA fragments of strain 716W with each enzyme. The migration patterns of the DNA fragments generated by EcoRI significantly varied between the two New Zealand isolates, particularly with the low MW fragments, whereas the sizes of the fragments cleaved by BamHI were more similar.

The DNA patterns of the three Australian isolates appeared to be similar in the number and size of the high MW fragments cleaved by each enzyme but varied between the fragments which were below 2.3 kilobase pair. The DNA patterns of the New Zealand isolates generated by both enzymes revealed obvious differences from the Australian isolates. The DNA of the reference strain HVI, after cleavage by either EcoRI or BamHI, produced a pattern which was similar to those of the New Zealand isolates but markedly different from those of Australian isolates.

A schematic drawing of the restriction patterns of the 6 different strains of AAV generated by EcoRI and BamHI are presented in Figure 3.5.

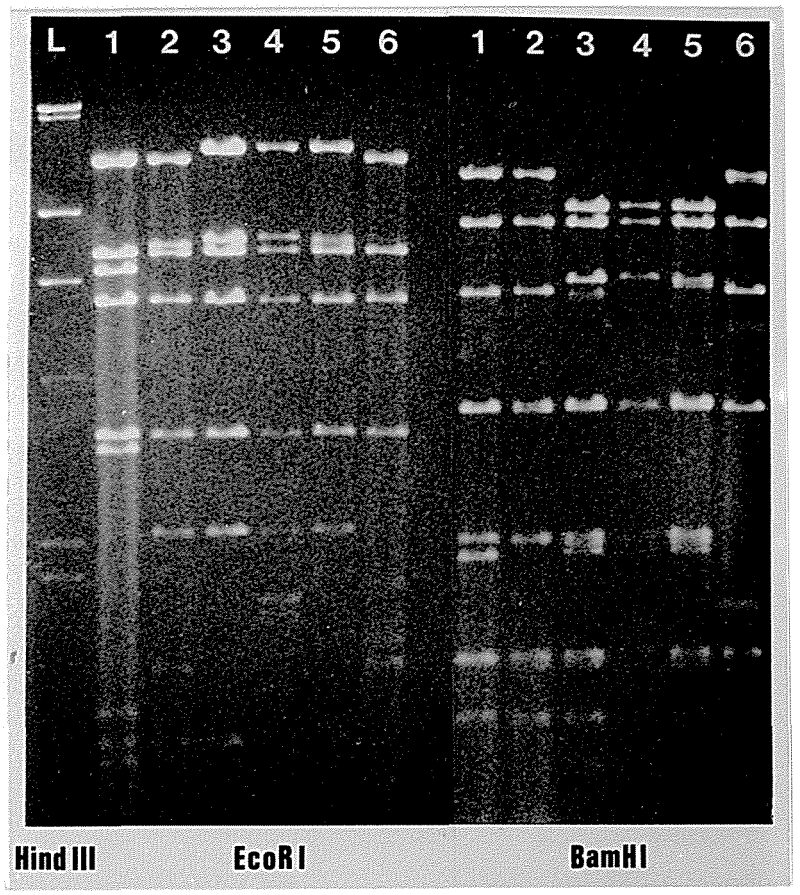

Fig. 3.4 **Electrophoretic patterns of DNAs of six different strains of serotype 8 avian adenoviruses generated by digestion with the restriction endonucleases EcoRI and BamHI. Lanes 1-6 respectively represent viruses 717B, 716W, QLD, VRI, WA and HVI. Bacteriophage lambda (L) DNA, digested with HindIII, used as a molecular weight marker.**

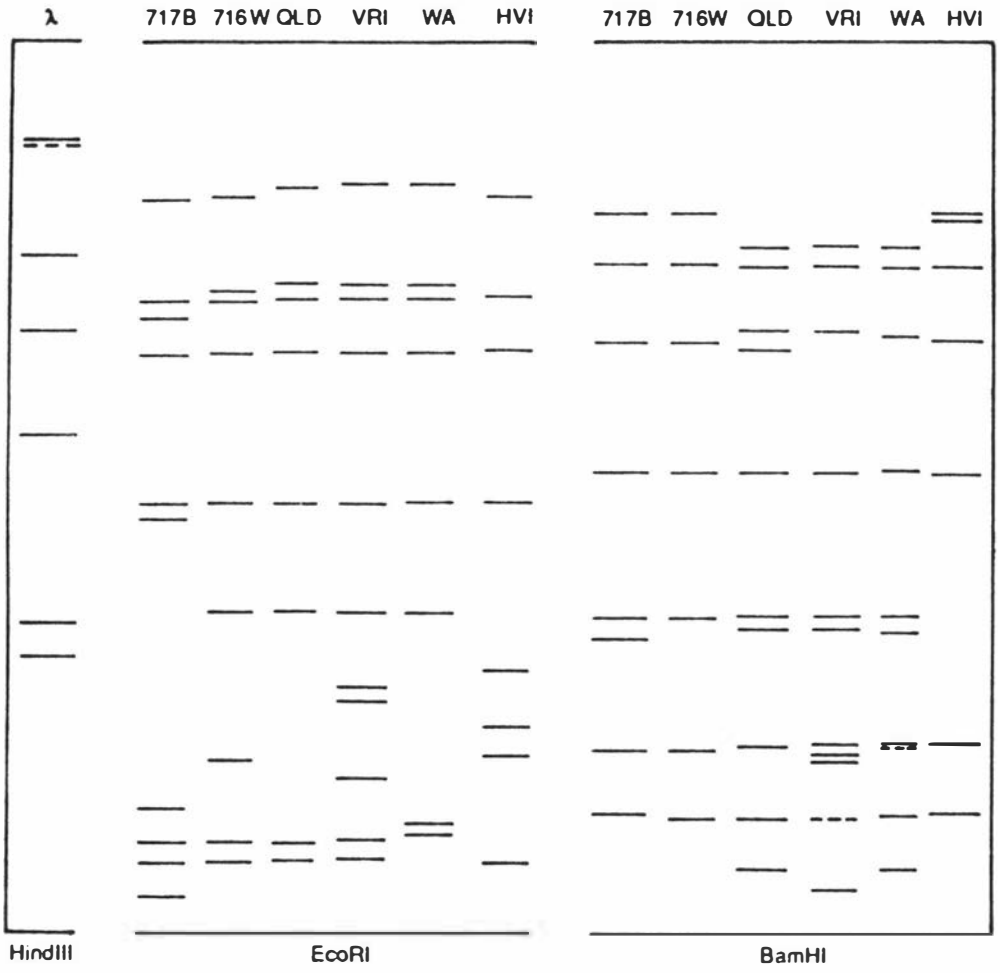

Fig. 3.5 **Schematic drawing of the restriction patterns of the six strains of serotype 8 AAVs generated by EcoRI and BamHI.**

DISCUSSION

Although some workers have found that chick embryo liver cells are superior to kidney cells for primary isolation of virus (Sharpless et al., 1958), CKC cultures have been more extensively used for isolation of AAVs (Kawamura et al., 1964; Khanna, 1964; McFerran et al., 1972). It was not considered worthwhile to take the risk of using the cells from conventional chickens as AAVs are commonly distributed in apparently healthy birds in New Zealand (Green et al., 1976).

Most of the viral isolates required multiple passages in cell culture before they produced a CPE, as also described by McFerran et al. (1976b) and Cook (1983). While it is probable that there is a direct relationship between the amount of virus in the liver and the severity of hepatitis this did not always relate to the number of passages in cell culture that were required before a CPE was observed. The major reason for this was that many of the passages were not able to be maintained for the appropriate time (5-6 days) because the cell sheet detached and occasionally samples were contaminated with bacteria and required filtration before repassaging.

Most of the viral isolates were made from liver tissues but one was from bursa and one from intestine. Although IBH viruses have been occasionally isolated from various tissues and faeces (McDougall and Peters, 1974; McFerran et al., 1976b), liver tissue has been preferred by several authors (MacPherson et al., 1974; McFerran et al., 1976b; Cubillos et al., 1986; Reece et al., 1986a).

Only basophilic inclusion bodies with a clear halo were observed in CKC infected with each of the AAVs isolated in New Zealand. Inclusions were more frequently observed with strains 716W and 717B. Different strains of other serotypes have been shown to produce either basophilic or eosinophilic inclusion bodies in cell culture (Adair, 1978).

Most of the isolates were identified by VN test as serotype 8 AAV and mostly they were involved with IBH outbreaks of variable severity. In particular, strains 716W and 717B were associated with high mortality in broiler flocks but some strains were associated with milder forms of the disease. Not surprisingly, serotype 8 has been frequently found to be associated with IBH outbreaks in other parts of the world (McFerran et al., 1976a; Grimes et al., 1977b; Reece et al., 1986a). The severity of the disease may be either related to the virulence of a particular strain of virus, intercurrent disease, the immune status of the flock or other complicating management factors. The serotype 1 (strain 736NI) isolate was

derived from a normal broiler flock in the North Island.

Although the VN test is considered the most sensitive and specific method for the identification of adenovirus (Adair, 1976) significant cross-reactions are observed between different prototype strains (Kawamura et al., 1964; McFerran et al., 1975; Grimes and King, 1977). Out of 11 isolates in New Zealand, 9 were serotype 8, 1 was serotype 1 and 1 serotype 12. There was no cross-reaction between serotype 8 AAVs (both New Zealand isolates and the prototype strain of serotype 8) and any other strain. One isolate (WV6642), identified as serotype 12, cross-reacted with antiserum to prototype strain A2 (serotype 10). The isolate 736NI identified as serotype 1 did not react with any antiserum other than antiserum to CELO virus.

The homologous antibody titres with the serum samples obtained from overseas were markedly lower than with the antisera that were produced here in rabbits. No details are available for the production methods used overseas but presumably differences in antigenic mass and purity, use of adjuvants and timing of inoculations would be significant.

Although it is probable that all AAVs multiply in embryonating eggs, not all serotypes caused recognizable lesions in the embryos. Following inoculation into the AC, serotype 1 AAV (736NI) always caused death of the embryo whereas two isolates of serotype 8 (716W, 717B) did not cause embryonic death. Many isolates of serotype 1 AAV, including CELO virus, even though they have been derived from clinically normal birds, produce lethal infections of chicken embryos. Kawamura et al. (1964) found that only serotypes 1 (Ote strain), 5 (TR22 strain) and 6 (CR119) killed embryos when high concentrations of virus were inoculated into AC but in lower concentration only serotype 1 killed embryos. Field experience supports the value of cell culture for isolation of AAVs. Thus Burke et al. (1959) made only 3 isolates in embryonating eggs compared with 45 in cell cultures.

A-AVs were only observed in association with the WV6642 isolate which was involved with a relatively mild form of IBH. A-AVs were not found in association with any of the isolates of AAVs in New Zealand which caused severe disease with high mortality rates. A-AVs may have some inhibitory effects on the virulence of the AAV involved as has been suggested previously by Pronovost et al. (1978).

Reovirus was isolated from the liver tissue from birds in one flock affected with IBH. Its identity was established by failure to react in VN tests using 12 prototype AAV antisera, typical morphological appearance by EM examination and the typical electrophoretic pattern of its RNA segments (Saifuddin et al., 1989). Reovirus has also been isolated from cases of

IBH by others (McFerran et al., 1976b). From one outbreak only reovirus was isolated while in another two outbreaks both reovirus and adenovirus were recovered. Like AAVs, avian reoviruses are frequently isolated from apparently healthy birds and their role in specific disease syndromes is often unclear (Robertson and Wilcox, 1986).

Six serologically similar strains of serotype 8 AAVs, two New Zealand isolates, three Australian isolates and one reference strain, were studied by restriction endonuclease fingerprinting technique. The lambda DNA, previously digested with HindIII, was used as a MW marker.

Ideally, viruses should be classified according to the sequence homologies of their genomes since the evolution of a virus is the result of gradual changes in the nucleotide sequence of the genome (Alestrom et al., 1982).

Analysis of the DNA fragments of 717B and 716W, generated by EcoRI and BamHI, revealed that the New Zealand isolates resembled each other but were quite distinct from the Australian isolates. The three Australian isolates also resembled each other. This evidence does not support the hypothesis that the virulent New Zealand strains of IBH virus have been recently introduced from Australia.

Analysis of restriction patterns has been extensively used for tracing the origin of herpes simplex virus in outbreaks in humans (Buchman et al., 1978; 1979). Restriction enzyme analysis of the viral DNA seems to be an excellent tool to detect subgroups of bovine herpesviruses and even to recognize variants in different strains (Ludwig, 1982). In some cases, recombinant genomes may be detected by restriction analysis, and this is well documented for typing of recombinants between herpes simplex virus 1 and 2 (Mores et al., 1979).

The migration pattern of the DNA fragments of HVI virus was somewhat similar to that of the New Zealand strains and very similar to the pattern reported for this strain by Zsak and Kisary (1984).

Bovine adenoviruses have also been analysed by restriction endonuclease fingerprinting for strain identification and for subgroup classification (Benko et al., 1988). It is noteworthy that there is no common restriction site for EcoRI, HindIII and BamHI between the genomes of BAV7 and BAV3 though they share common group-specific CF antigens (Hu et al., 1984).

SUMMARY

Out of 16 tissue samples tested, 11 isolates of AAV were obtained at various passage levels in SPF CKC cultures. All of the isolates were found to produce basophilic INIBs surrounded by clear halos in cultures of CKC.

Nine of the AAV isolates were identified by VN tests as serotype 8 and mostly they were involved with IBH outbreaks of variable mortality. There was no cross-reactivity between the locally isolated serotype 8 AAVs and prototype strains of other serotypes.

Restriction endonuclease analysis of six serologically indistinguishable strains of serotype 8 AAV revealed that the two New Zealand isolates resembled each other but were markedly different from three Australian isolates. The reference strain (HVI) was somewhat similar to the New Zealand isolates and the three Australian isolates resembled each other.

One isolate of serotype 1 AAV was obtained from liver tissue from an unaffected flock and one isolate of serotype 12 was obtained from bursal tissue from a flock with IBH.

Two of the serotype 8 isolates tested were found to be resistant to exposure to chloroform and temperatures of $4^{o}C$ and $50^{o}C$.

Only the isolate 736NI (serotype 1) was found to consistently produce changes in embryonating eggs such as dwarfing, haemorrhages on the body surface, haemorrhage and necrosis of liver and death of embryos.

A-AVs were only observed in association with the isolate WV6642 which was involved with a relatively mild form of IBH.

CHAPTER FOUR

REPRODUCTION OF INCLUSION BODY HEPATITIS IN CONVENTIONALLY RAISED CHICKENS AND AN ASSESSMENT OF THE IMMUNOSUPPRESSIVE EFFECTS OF INCLUSION BODY HEPATITIS VIRUS

INTRODUCTION

Twelve serotypes of AAV have been recognized in chickens. Some of these can be readily isolated from both apparently healthy as well as diseased flocks and this has led to conflicting views on their role as pathogens (McFerran and Connor, 1977; Winterfield, 1984). Marked differences in virulence among isolates of the same and different serotypes of AAV have also been demonstrated (Winterfield, 1984). Although many investigators (McDougall and Peters, 1974; MacPherson et al., 1974; Rosenberger et al., 1974; McCracken et al., 1976; Grimes et al., 1977b). have been able to reproduce the IBH syndrome following inoculation of virus by a parenteral route, clinical IBH following a natural route of exposure with any serotype or isolate has been infrequently reported (Gallina et al., 1973; Winterfield et al., 1973; Cook, 1983; Reece et al., 1987). A number of field and experimental studies have emphasized the predisposing role probably played by IBD infections (Rosenberger et al., 1975; Fadly et al., 1976; Winterfield, 1984). However, New Zealand's freedom from IBD is officially recognized (Government of New Zealand, 1984).

In recent years, IBH has been a significant problem in the Australian poultry industry (Reece and Beddome, 1983; Reece et al., 1986a) and serotype 8 AAVs have commonly been isolated from the affected flocks (Kefford and Borland, 1979; Kefford et al., 1980; Reece et al., 1986a). In earlier reports, most of the IBH outbreaks in Australia were associated with IBD infection whereas, in recent years, no evidence of IBD has been reported in some of the outbreaks (Reece and Beddome, 1983; Reece et al., 1986b).

Although hepatitis with INIBs in the hepatocytes is characteristic of IBH (Howell et al., 1970; Pettit and Carlson, 1972) the involvement of bone marrow and lymphatic organs particularly the bursa, thymus and spleen has also been reported by several workers (MacPherson et al., 1974; Fadly et al., 1980). Similar lesions in lymphoid organs, following experimental infection, have also been observed (Hoffmann et al., 1975; Grimes et al., 1977b; Fadly et al., 1980).

It has been suggested that prior infection with IBD virus enhances the pathogenicity of AAV and that the bursal lesions described in natural outbreaks of IBH may have been due to IBD virus infection (Rosenberger et al., 1975; Fadly et al., 1976). However, results from both natural outbreaks (MacPherson et al., 1974; Christensen and Saifuddin, 1989) and experimental infections (Grimes et al., 1977b; Fadly et al., 1980) suggest that the IBD virus, or any other factor, is not always necessary for IBH to occur following infection with AAV. In fact, infection with some strains of AAV directly causes lymphoid depletion of the bursa, thymus and spleen and thus has an effect on both humoral and cell mediated immunity (Hoffmann et al., 1975). Some of the previous reports of lymphocytic depletion in lymphoid organs in association with IBH are shown in Table 4.1.

It is of considerable interest to note that the bursa of Fabricius has been constantly involved in IBH infection. Whether the AAV isolates which induce severe lymphoid depletion in bursa, thymus, spleen, caecal tonsil and bone marrow are immunosuppressive for chickens remains to be determined. New Zealand is free from IBD (Howell et al., 1982; With, 1985; Jones, 1986; With and Christensen, 1987) and the field outbreaks of IBH which occurred in 1987 (Christensen and Saifuddin, 1989) were unrelated to other identifiable immunosuppressive agents. Lymphoid depletion of the bursa and thymus was apparent on histological examination of tissues derived from affected chickens in these outbreaks.

This Chapter describes several aspects of both natural and experimental infections with an AAV isolate, obtained from a field outbreak of IBH in New Zealand, and particularly examines its possible association with immunosuppression in chickens.

Table 4.1 **Previous reports of lymphocytic depletion in lymphoid organs of chickens which were affected with inclusion body hepatitis.**

Lymphocytic depletion in					Reported evidence of IBD or other immunosuppressive factors (F or L)	References
Bone marrow	Bursa	Thymus	Spleen	Caecal tonsil		
+	+	NE	+	NE	No (F)	Pettit and Carlson, 1972
+	+	NE	+	NE	No (F)	Bickford, 1973
NE	+	+	+	NE	No (L)	Grimes et al., 1977b
+	+	+	+	NE	No (F)	Hoffmann et al., 1973
NE	+	NE	+	NE	No (L)	Fadly et al., 1980
+	+	+	+	+	Sero +ve to IBD virus (F)	Hoffmann et al., 1975
+	+	+	+	+	Sero +ve to IBD virus (L)	Hoffmann et al., 1975
NE	+	NE	+	NE	No (F)	Reece et al., 1986b
NE	+	NE	+	NE	No (F)	MacPherson et al., 1974
+	+	NE	+	NE	No (F)	Wells et al., 1977
+	+	NE	+	NE	No (F)	Itakura et al., 1974
+	+	+	NE	NE	No (F)	Christensen and Saifuddin, 1989

IBD = Infectious bursal disease
+ = Histological evidence of lymphocytic depletion
F = Field ; L = Laboratory
NE = Not examined

MATERIALS AND METHODS

Virus

Strain 717B virus (serotype 8) was used in both parts of the experiment. It was recovered from an outbreak of IBH in a New Zealand broiler flock which experienced about 30% cumulative mortality by the age of 6 weeks. The virus was isolated from a 10% suspension of pooled liver tissue which was inoculated onto monolayer cultures of SPF CKC. After cloning by three limit dilutions in CKC, the isolate was serotyped using rabbit antisera to the 12 standard prototypes of AAV. A stock of virus was prepared at the seventh passage level.

Birds

In the first part of the experiment (reproduction of IBH), conventionally raised broiler chickens (2 days old) were used. The chicks were derived from a breeder flock which, it was later discovered, had reasonably high levels of VN antibodies (320-1280) to serotype 8 AAV. In the second part (evaluation of immunosuppressive effects), 2-3 days old chicks, derived from a WLH SPF flock (see Chapter 3) were used for challenge with the same strain of virus. The parent flock was tested and found to be free of VN antibodies to any of the 12 serotypes of AAV.

REPRODUCTION OF IBH

Challenge of birds

Blood samples were collected, into 2-4 plain capillary tubes (Terumo Corporation, Tokyo, Japan) from each of 80 conventionally raised, 2-day-old broiler chickens which had been marked with individual wing tags. The birds were then randomly divided into four groups of 20 birds. An inoculum of 500 ul of virus suspension, containing 10^6 $TCID_{50}$, was administered orally to each bird in one group and intraperitonially to each bird in a second group. These two groups were housed together in a positive-pressure bubble isolator along with a third group of uninoculated "contact" birds. They were supplied with autoclaved feed and water *ad libitum.* The fourth group served as controls and was kept in strict isolation but with similar housing and feed.

Monitoring of the effects of IBH infection

Birds were closely examined clinically each day and any dead birds were subjected to post mortem examination including histological examination of selected tissues. Blood was collected from birds considered moribund and they were then killed and similarly examined. At 2 weeks pi all survivors in the IP and orally inoculated groups, 10 contact and 10 control birds were weighed, bled and killed. Selected tissues were examined histologically. At 5 weeks pi the remainder of the contact and control birds were bled, killed and similarly examined.

Reisolation of virus from the infected birds

Attempts were made to isolate virus from the liver tissues of birds which had been exposed to IBH virus. Liver samples were collected aseptically from the moribund birds and from birds which were killed at 2 and 5 weeks pi. Ten percent (w/v) suspensions were prepared by pulverising (Stomacher 400, Colworth Ltd. London) the liver tissue for 30 min in PBS containing antibiotics (PSK) and inoculated onto established monolayer cultures of CKC. After 5 days of incubation, the cells were frozen and thawed 3 times and reinoculated onto freshly prepared monolayers of CKC. Up to 3 passages of inoculated cell cultures were performed before any of the liver samples were considered negative. Later, 10% percent suspensions of liver samples were also assayed for AAV antigens by ELISA (see Chapter 5 for the development of this assay).

Measurement of VN antibodies

Serum was collected by spinning (Micro-capillary centrifuge, International Equipment Company, Needham, USA) the capillary tubes for 3 min. The sera were inactivated at $56^{o}C$ for 30 min and tested for VN antibodies against serotype 8 AAV using approximately 100 $TCID_{50}$ of virus in 50 ul. Assays were conducted in monolayer cultures of SPF CKC using 96-well tissue culture plates (Nunclon, Delta, Denmark). The tests were prepared in duplicate and the VN antibody titres were expressed as the reciprocal of the highest dilution of serum which completely inhibited the CPE produced by the virus.

IBH VIRUS AS AN IMMUNOSUPPRESSIVE AGENT

Examination of birds naturally infected with IBH virus

Twenty dead or moribund chickens, from a 3-week-old broiler flock of 15000 birds with sudden high mortality, were received for laboratory examination. At necropsy, portions of the liver, spleen, bursa, thymus, caecal tonsil, gizzard and femur were fixed in 10% buffered formalin for histopathological examination by HE staining and 10% suspensions of individual tissues were prepared in PBS for the identification of AAV antigens by indirect ELISA. A suspension (10% w/v) of pooled liver tissue was prepared and inoculated onto monolayer cultures of SPF CKC for the isolation of AAV and into the MDCC-MSB1 cell line for the isolation of CAA. Blood samples were collected from the moribund birds to measure PCV and VN antibody titres against AAV serotype(s). The virus isolated from liver tissue was negatively stained with PTA and examined by EM. Virus was serotyped in VN tests using antisera to the 12 serotypes of AAV. The sensitivity of the virus to chloroform was tested according to the method of Feldman and Wang (1961).

Examination of birds experimentally infected with IBH virus

Eighteen 2-day-old SPF birds received oral inoculations of 500 ul (10^6 $TCID_{50}$) of AAV isolated from naturally infected liver tissue. At 2, 3, 4, 5, 7 and 9 days pi, 7 tissues (liver, spleen, bursa, thymus, caecal tonsil, gizzard and femur) were collected from each of 3 birds including those which had died or were moribund. Portions of the selected tissues (except liver and gizzard) were fixed in 10% buffered formalin for histopathology and immunocytochemistry (see Chapter 5 for details) and 10% suspensions of the tissue in PBS were tested for viral antigens by indirect ELISA. Liver and gizzard tissues were fixed in 10% buffered formalin only for histopathology. ELISA results were expressed as ELISA specific absorbance (ESA) obtained by subtracting the absorbance obtained with SPF tissue from that obtained with test tissues. The results of immunocytochemistry were expressed as intense (+++), less intense (++), trace (+) and negative (-) development of a brown to dark brown colour which corresponded to the amount of antigen present in the tissues (see Chapter 5).

Humoral immune response in birds challenged with IBH virus

Forty-six SPF birds (3-day-old) were individually marked with wing tags. Blood samples

were collected from 10 randomly selected birds. Each of the birds received an IM inoculation of 1.0 ml of 10% sheep red blood cells (SRBC) in PBS homogenized with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA). They were then divided into two groups consisting of 20 and 26 birds. The 26 birds in one group each received 500 ul (10^6 $TCID_{50}$) of AAV suspension (seventh tissue culture passage of 717B strain) orally and were housed separately in a positive-pressure bubble isolator and supplied with autoclaved feed and water. The control group consisting of 20 birds was kept in isolation but with similar housing and feed. All of the birds in the control group and the survivors in the virus inoculated group were bled at 2 weeks pi and reinoculated IM with 1.0 ml of 10% SRBC homogenized with incomplete Freund's adjuvant (Difco Laboratories). After a further 2 weeks, blood samples were collected from all of the birds. Serum samples were inactivated at 56^{o}C for 30 min and tested in a HA assay using 0.5% SRBC in PBS.

Post mortem examinations were conducted on birds which died and samples of liver, spleen, bursa, thymus, caecal tonsils and bone marrow were fixed in 10% buffered formalin for histopathology.

HA test

The HA tests were carried out in 96-well, V-bottom microtitre plates (Linbro, Flow Laboratories, USA). Fifty microlitres of each serum sample were diluted (2 fold steps) in PBS and an equal volume of 0.5% SRBC in PBS was added to each well. After a minimum of 1 hr of incubation at room temperature (20-25^{o}C) the results were recorded. The tests were run in duplicate and the HA titre was expressed as the reciprocal of the highest dilution of serum which caused complete agglutination of the added SRBC suspension. Blood samples collected from the noninoculated birds were similarly tested as controls.

The isolation and identification of viruses in cell culture, serotyping of virus isolates by neutralization test and the procedures for direct detection and quantification of AAV antigens in different tissues by ELISA have been described elsewhere (Chapters 3 and 5).

RESULTS

FIELD OUTBREAKS OF DISEASE

History of the disease

The course of the disease appeared to be quite rapid; the mortality peaked on the third day after losses were first noticed, remained high for two days and was largely over by day seven. The total mortality recorded was about 30%. The predominant signs of the disease included reluctance to eat, ruffled feathers, occasionally white pasty droppings, marked weakness, depression and eventual prostration and death.

Post mortem lesions

In naturally infected birds the liver was pale, swollen and often had multiple petechial haemorrhages. Multiple haemorrhages were seen in the thymus, breast muscle and intestinal tract. Severe erosions were observed in the gizzard of a few birds. The bursa of Fabricius and thymus were smaller than in the healthy birds. The spleen was swollen in some birds. The bone marrow was markedly pale and the PCV was between 10-18%.

Histopathology

In naturally infected birds, extensive liver lesions included multiple coalescing foci of vacuolated hepatocytes, focal necrosis, degeneration, and haemorrhages with eosinophilic INIB in hepatocytes. Lymphocytic and heterocytic infiltrations were noticed in the liver parenchyma. Pancytopaenia in the bone marrows was observed in a few cases. Lymphocytic depletion occurred in the bursa, thymus and spleen. The bursa had severely damaged follicles surrounded by increased fibrous tissue in a few birds. Occasionally, scattered cysts in the medullary areas of the lymphoid follicles and INIB in the lining epithelium of the bursa were observed. In the spleen, the perivascular aggregations of lymphoid cells were lacking and a prominent proliferation of reticular cells including macrophages was frequent around the arteries. Lymphoid cells were decreased, especially in the red pulp. The thymus had very small or missing cortices and haemorrhages were obvious throughout the medulla. Scattered petechial haemorrhages were present in the lamina propria of the intestine in a few birds. Extensive necrosis, severe haemorrhages and

lymphocytic infiltrations were observed in the submucosa of the gizzard.

Isolation and identification of virus from naturally infected birds

A CPE, characterized by rounding of cells, was observed by the second passage of liver suspensions inoculated onto CKC monolayers. Typical icosahedral, adenovirus particles (size 70-80 nm) were demonstrated in the infected cell culture suspension. The virus was resistant to chloroform and it was grouped as serotype 8 by VN tests and designated as strain 717B. No evidence of the growth of CAA in the MDCC-MSB1 cell line which was inoculated with the same infected liver suspension was detected after 5 passages. Variable amounts of AAV antigens were detected by ELISA in all of the tissues collected from naturally infected birds (data not shown). The VN antibody titres to serotype 8 AAV in the sera collected from birds in the affected flock were <5-40. The antibody titres against other serotypes of AAV were <5 .

REPRODUCTION OF IBH

Clinical features

Nine of 20 and 6 of 20 birds in the IP and orally inoculated groups, respectively, died within 3-8 days pi (Table 4.2). None of the contact or control birds died. While some birds died suddenly without showing any signs of illness others showed drowsiness, ruffled-feathers, decreased food intake and occasionally white pasty faeces for a few hours to 1 day before they died. All of the survivors from the IP inoculated group, half of those from the orally inoculated group and 7 of the contact birds showed signs of illness and their body weights at 2 weeks of age were depressed by up to 41% in comparison with those of the control birds. Birds inoculated via the IP route usually died earlier than those inoculated orally (Table 4.2).

Post mortem and histopathology

Birds which died suddenly or were killed when considered moribund had enlarged, pale livers with small (1-2 mm) haemorrhages on the serosal surface (Fig.4.1). The bursae of Fabricius and thymuses were greatly reduced in size. Haemorrhages were present in the thymuses and spleens and the kidneys were swollen. Changes apparent on histological examination included severe depletion of lymphocytes in the bursae (Fig. 4.2), thymuses

and spleens (Fig. 4.3), widespread haemorrhages in thymuses, spleens and livers and hepatic necrosis associated with many basophilic and a few eosinophilic inclusion bodies in the nuclei of many hepatocytes (Fig. 4.4).

Table 4.2 **Mortality and growth response of conventionally raised chickens following exposure to avian adenovirus strain 717B at 2 days of age.**

	Route of exposure			
	Intraperitoneal (n = 20)	Oral (n = 20)	Contact (n = 20)	Control (n = 20)
Mortality (%)	9 (45)	6 (30)	0	0
Mean death time in days (range)	3.89 (3-6)	5.67 (4-8)	–	–
Mean body weight in gms of survivors at 2 weeks (range)	115* (100-130)	156* (110-190)	165* (130-210)	195 (160-225)

* $p < 0.0005$

Recovery of virus

Adenovirus was isolated in SPF CKC cultures at 1-3 passage levels from the liver of each of the birds which died following IP or oral inoculation of AAV. Also high levels of viral antigens (ESA 0.56 - 1.75) were detected by ELISA in the same liver suspensions. Virus was not isolated in CKC cultures from any of the liver suspensions derived from the inoculated birds which were killed at 2 and 5 weeks pi. Viral antigens were also not detected by ELISA in 10% liver suspensions derived from these birds. Virus was not isolated, nor were viral antigens detected in the liver suspensions derived from control birds.

The viruses isolated from the liver suspensions of experimentally infected birds were confirmed as serotype 8 AAV by VN tests.

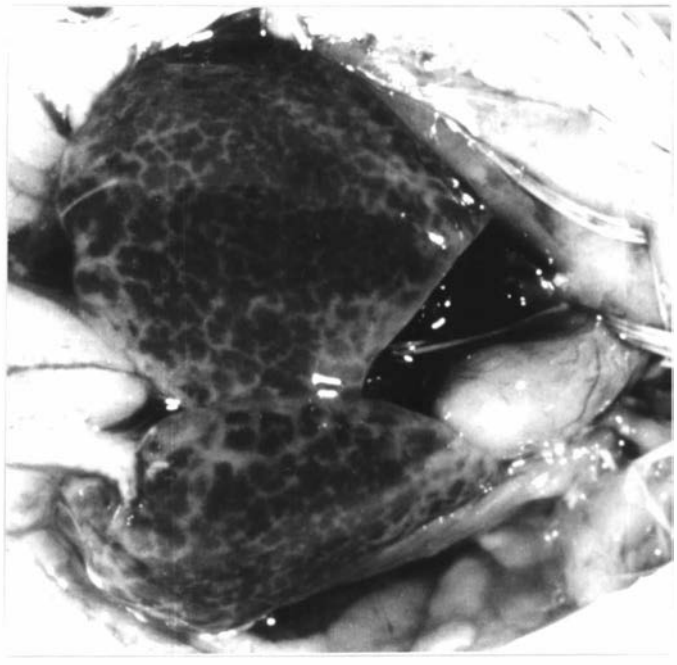

Fig. 4.1 **Enlarged and mottled liver of chicken which died 5 days after receiving an oral inoculation of serotype 8 avian adenovirus.**

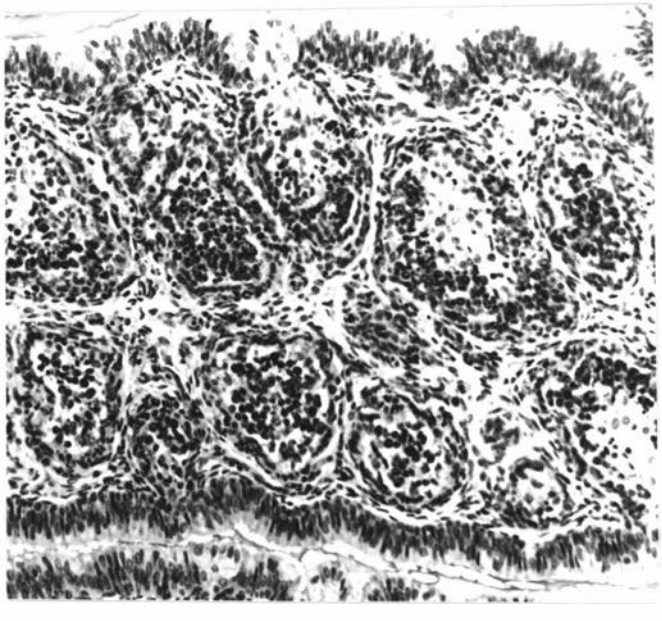

Fig. 4.2 **Lymphoid depletion in bursa of Fabricius of a chicken which died 5 days after receiving an oral inoculation of serotype 8 avian adenovirus (haematoxylin and eosin, x10).**

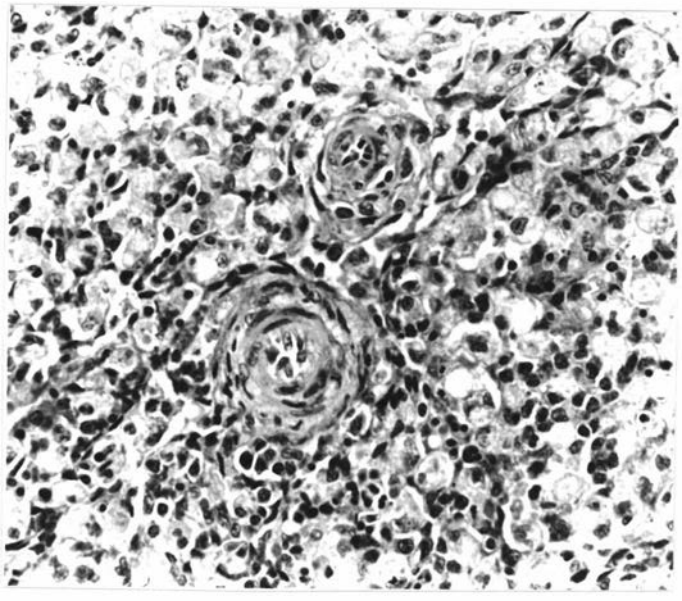

Fig. 4.3 **Severe lymphoid depletion in peri-arteriolar area of spleen of a chicken which died 5 days after receiving an oral inoculation of serotype 8 avian adenovirus (haematoxylin and eosin, x20).**

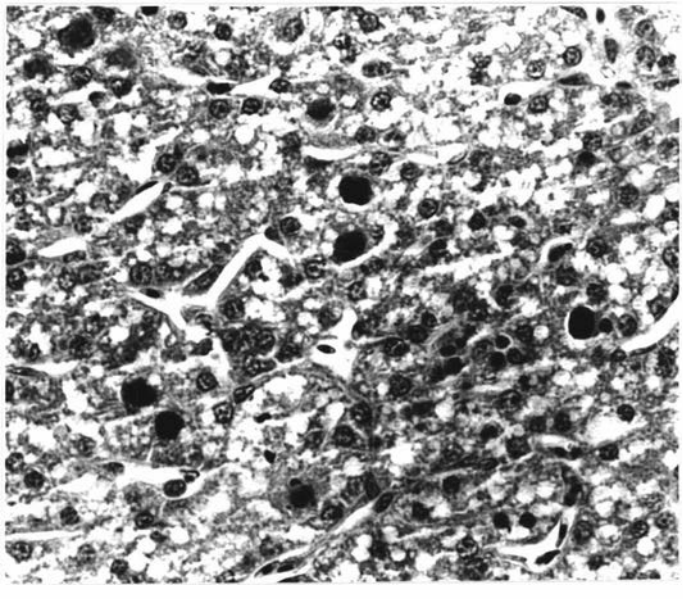

Fig. 4.4 **Liver of chicken which died on day 5 following oral inoculation of strain 717B of serotype 8 avian adenovirus. Extensive necrosis and intranuclear inclusion bodies are apparent (haematoxylin and eosin, x40).**

Antibody response

At 2 days of age, prior to exposure to AAV, most birds in each of the four groups had VN antibodies to serotype 8 AAV (Table 4.3). At the age of 2 weeks 7 of 10 and 10 of 10 of the survivors from the IP and the orally inoculated groups, respectively, 8 of 10 of the contact birds but none of the control birds which were tested, had VN antibodies detectable in their sera (Table 4.3). During this time, 36% and 71% of the survivors from the IP and orally inoculated groups and 60% of the contact birds had increased (2-4-fold) antibody titres (data not shown). None of the inoculated or contact birds which had VN antibody titres of 64 or greater at 2 days of age had increased VN antibody titres or had died by the age of 2 weeks. But, at the age of 5 weeks, all (10/10) of the remaining contact birds, irrespective of whether they had maternally derived antibody titres >64 or not, had high VN titres (640-2560). Rises in antibody titres were not detected in any of the control birds.

Table 4.3 **Detection of virus neutralizing antibody in sera of conventionally raised chickens before and after exposure to avian adenovirus (strain 717B).**

	Route of exposure			
Age	Intraperitoneal	Oral	Contact	Control
2 days	17/20*	16/20	13/20	7/10
(Preinoculation)	8-128 (45)**	8-128 (41)	8-64 (24)	8-32 (18)
2 weeks	7/10	10/10	8/10	0/10
	8-64 (33)	16-512 (130)	8-128 (45)	(<2)
5 weeks	NT	NT	10/10	0/10
			640-2560 (1664)	(<2)

*Numerator = number of birds with antibody
Denominator = number of birds tested
** = range and mean () antibody titres
NT = not tested

IBH VIRUS AS AN IMMUNOSUPPRESSIVE AGENT

Post mortem lesions

In experimentally infected birds, the post mortem changes in the livers, spleens, bursae, thymuses, intestinal tracts and bone marrows were similar to those in naturally infected birds except that, in some of the severely affected birds, white pin-head necrotic foci were observed on the swollen livers' surfaces and atrophy of the bursae and thymuses was more obvious. Neither haemorrhages in the muscles nor erosions in the gizzard were observed in experimentally infected birds.

Histopathology

In experimentally infected birds similar liver lesions to those observed in natural infections were seen, including many basophilic INIBs which were large and filled the whole nucleus, and a few eosinophilic INIBs which were smaller and surrounded by a clear halo. Changes in other tissues were similar to those in the naturally infected birds although no gizzard lesions were observed. The lymphocytic depletion in the bursa and spleen was similar to that shown in Figures 4.2 and 4.3 and lymphocytic depletion in the thymus is shown in Figure 4.5.

Detection of viral antigens in lymphoid tissues

The level of viral antigens detected by ELISA in different lymphoid tissues is shown in Table 4.4. In caecal tonsils, high levels of viral antigens were detected by ELISA from 2-9 days pi with a peak (ESA 2.03) at 5 days. Viral antigens were demonstrated in bursae at 2-9 days and in spleens at 3-7 days pi. The level of viral antigens in bursae and spleens peaked at 5 days pi. In thymuses and bone marrows, the viral antigens were detected from 3-5 and 3-7 days pi with peak levels (ESA 0.28 and 0.34, respectively) at 4 days pi.

Viral antigens were located by immunocytochemical staining in the lining epithelium of caecal tonsils (Fig. 4.6) at 2-9 days pi, in the epithelial and follicular cells of the bursae and in the cellular components of the white and red pulps of the spleens from 4-5 days pi (Fig. 4.7). Low levels of viral antigens were demonstrated in lymphoid aggregates of the caecal tonsil at 4-7 days (Fig. 4.6) and of the thymuses at 4 days pi (Fig. 4.8). A comparison of the level of viral antigens detected by ELISA and immunocytochemistry in various lymphoid

tissues is illustrated in Table 4.5.

High levels of viral antigens were detected in the livers of experimentally infected birds by ELISA and immunocytochemistry during 3-7 days pi (data not shown). Viral antigens were not detected in gizzard tissue.

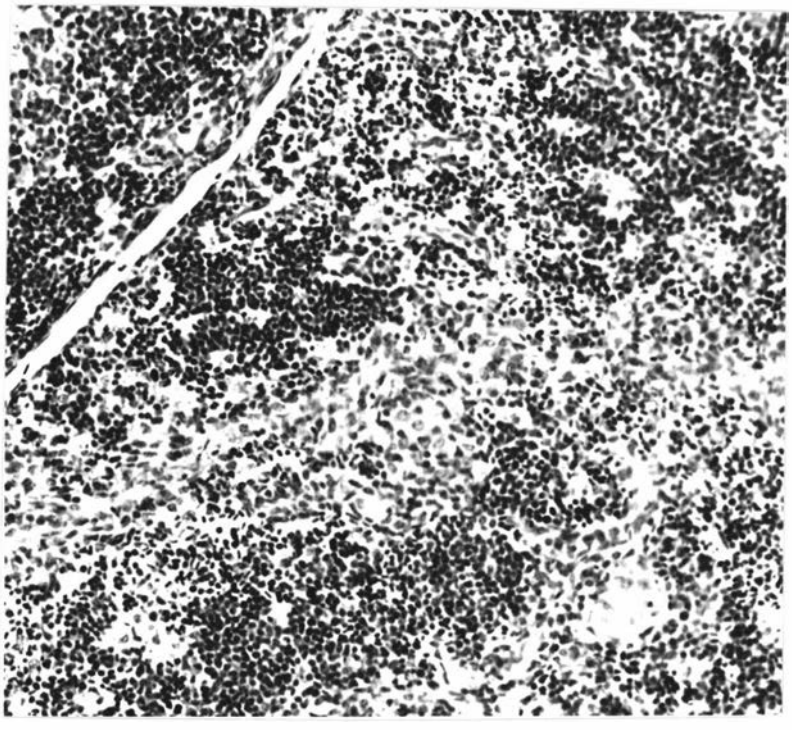

Fig. 4.5 **Lymphocytic depletion in thymus from experimentally infected chicken at 4 days post infection (haematoxylin and eosin, x10).**

Table 4.4 **Detection of viral antigens in lymphoid organs by ELISA after oral inoculation of strain 717B (serotype 8) avian adenovirus in 2-day-old SPF chickens.**

Tissues examined	Days post inoculation					
	2	3	4	5	7	9
Caecal tonsil	1.77 *	1.80	1.92	**2.03**	1.79	1.21
Bursa	0.25	0.34	0.42	**0.55**	0.30	0.15
Thymus	0.04	0.16	**0.28**	0.19	0.04	0.03
Spleen	0.03	0.18	0.47	**0.69**	0.35	0.04
Bone marrow	0.03	0.17	**0.34**	0.29	0.13	0.03

* Mean ESA value of three birds. ESA greater than 0.06 is considered positive. This represents more than three standard deviations above the mean ELISA absorbance value for 22 SPF chicken tissues.

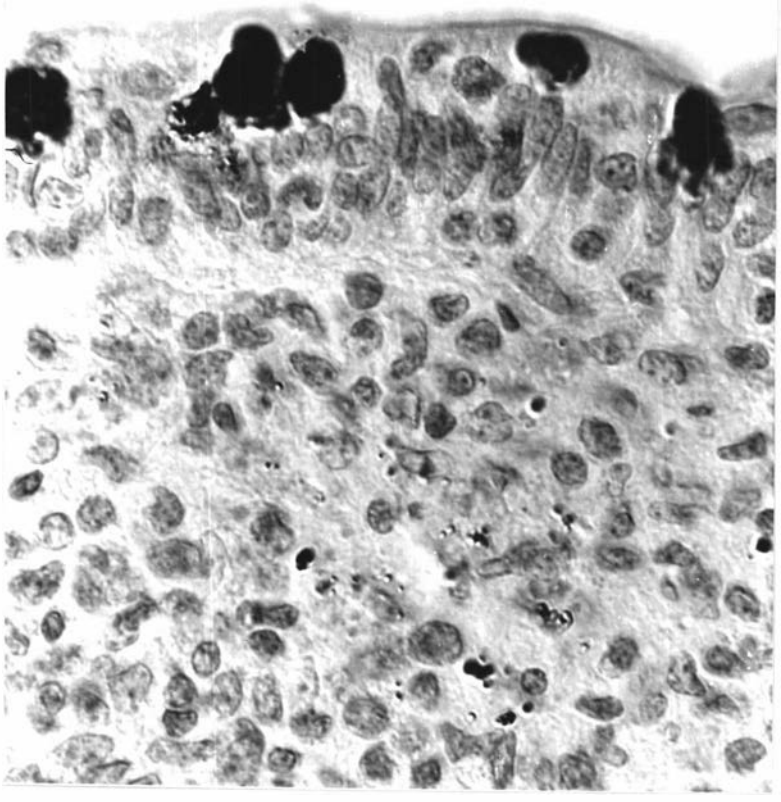

Fig. 4.6 **Viral antigens in epithelial cells and lymphoid tissue of the caecal tonsil detected by avidin-biotin peroxidase complex technique at 5 days post infection (haematoxylin counterstain, x20).**

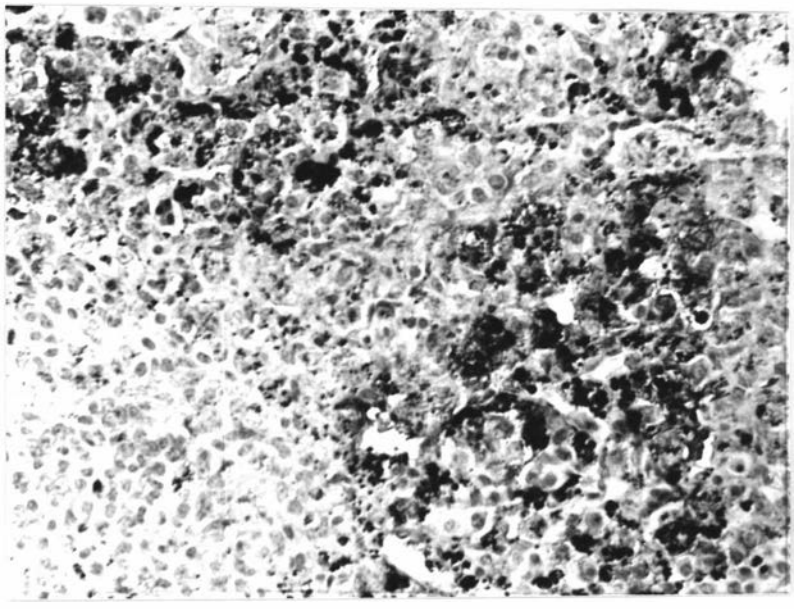

Fig. 4.7 **Viral antigens in the lymphoid tissues of spleen detected by avidin-biotin peroxidase complex technique at 5 days post infection (haematoxylin counterstain, x20).**

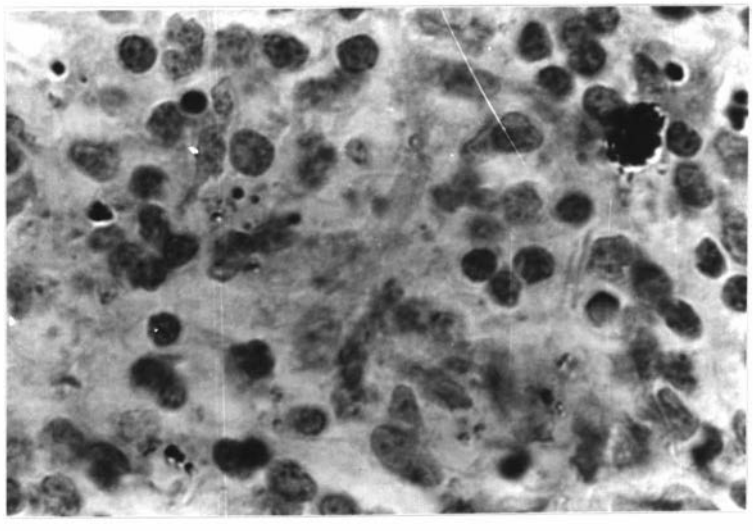

Fig. 4.8 **Viral antigens in lymphoid aggregates of thymus detected by avidin-biotin peroxidase complex technique at 4 days post infection (haematoxylin counterstain, x40).**

Table 4.5 **Comparison of the level of viral antigens detected by ELISA and by Immunocytochemistry in various tissues following oral administration of strain 717B (serotype 8) avian adenovirus to 2-day-old SPF chickens.**

Tissues examined	Mean peak ESA (day of peak)	Immunocytochemistry (intensity of colour)
Caecal tonsil	2.03 (5)	++
Bursa	0.55 (5)	++
Thymus	0.28 (4)	+
Spleen	0.69 (5)	+++
Bone marrow	0.34 (4)	NT

ESA = ELISA specific absorbance (value >0.06 considered positive)
+++ = intense dark brown stain in many cells
++ = moderately intense staining
+ = faint staining in few cells
NT = Not tested

Antibody response to SRBC

Five out of 26 of the birds, which had received an inoculation of virus, died between 3 and 7 days pi with hepatic lesions and INIBs in the hepatocytes. After 2 weeks pi the geometric mean HA titre (GMT) of anti-SRBC antibodies was 6 (range <2-32) in the control group and 3 (range <2-16) in the virus inoculated group (Table 4.6, p <0.025). In the control group 17/20 (85%) of the birds had detectable HA antibodies compared to only 13/21 (62%) of the virus inoculated group (p <0.1). At 4 weeks following the first inoculation (2 weeks after the booster dose) the GMTs obtained were 113 and 69, respectively, in the control and the virus-infected groups of chickens (p <0.09).

Table 4.6 **Humoral antibody response to sheep RBC (SRBC) in 3-day-old SPF chickens which were inoculated intramuscularly with SRBC at the same time as oral administration of 717B strain (serotype 8) of avian adenovirus.**

Noninfected birds (weeks post inoculation)		Birds infected with 717B virus (weeks post inoculation)	
2	4	2	4
4*	80	4	80
16	160	8	80
4	160	4	40
32	320	<2	160
8	160	8	80
16	80	4	20
<2	40	16	160
8	160	<2	80
16	320	<2	40
8	160	8	160
4	80	4	80
16	80	4	160
<2	40	<2	<10
4	80	4	160
16	320	<2	80
4	80	<2	160
8	80	8	80
<2	40	<2	20
8	160	8	160
8	160	<2	160
		4	80
GMT = 5.38	GMT = 165	GMT = 2.65	GMT = 101

* Reciprocal of the highest dilution of serum which completely agglutinated 50 ul of 0.5% sheep RBC.

DISCUSSION

In this study IBH was reproduced in conventionally raised broiler chickens by both oral and IP administration of a locally isolated strain of serotype 8 AAV. Also the effects of IBH virus, demonstrated by ELISA, immunocytochemistry and histology, on various lymphoid organs in chickens following natural and experimental infections have been described.

Isolates of the same or different serotypes of AAV may vary in their pathogenicity for conventional chickens (Winterfield, 1984). A variable mortality rate (31% and 9%) and duration of viral persistence in tissues, following oro-nasal administration of two strains (HV7 and GAL) of serotype 2 AAV to day-old SPF chickens under similar conditions, has suggested that the pathogenicity of AAVs is not necessarily serotype-dependent (Cook, 1983). The high mortality, significant depression in body weight gain, and the antibody response in a large percentage of birds detected after oral exposure to 717B virus (serotype 8) indicate that this isolate, which was recovered from a natural outbreak of IBH in New Zealand, is a highly pathogenic strain of AAV. Cook (1983) observed a mortality rate of less than 3% after oronasal administration to day-old SPF chicks of serotype 8 (strain TR59 and H6) AAV, whereas, Reece et al. (1987) demonstrated 30% mortality following oral infection of day-old SPF chicks with the VRI-33 strain of serotype 8 AAV. Our results are similar to the findings of Reece et al. (1987). This New Zealand isolate of serotype 8 AAV may be similar to recent Australian isolates of serotype 8 since, in both cases, field outbreaks have been associated with high mortality rates in the absence of evidence of concurrent IBD. In this report, some IBH infected birds died suddenly without showing any illness. Appel (1987) suggested that this type of sudden death occurs in cases of ICH due to anaphylactic shock at the time of an early immune response. However, birds which died suddenly had extensive hepatic necrosis and death may have been due to organ failure.

From this study, it is clear that there is a relationship between the titre of maternally-derived VN antibody and the severity of the disease. No birds having maternally-derived antibody titres of 64 or greater died even after parenteral (IP) inoculation. Some birds with maternal antibody titres greater than 64 became sick but recovered. Antibody responses were also not detected in these birds by two weeks but by 5 weeks all the contact birds, no matter what their maternally-derived antibody titres were, had significant rises in antibody titre (640-2560). Other studies have also shown with serotype 5 or 8 of AAV, that birds with maternal antibody titres of <20 suffer significant mortality rates following challenge at 2-3 days of age with the respective viruses whereas birds with higher antibody titres were protected (Fadly and Winterfield, 1973; Grimes and King, 1977b). It has previously been demonstrated that

chickens can be experimentally infected with GAL virus (serotype 2) in the presence of circulating antibodies against this virus (Kohn, 1962). Infection of the respiratory or intestinal tract is known to occur in other viral diseases such as poliomyelitis, influenza, and with adeno, coxsackie or enteroviruses in spite of antibodies being present in the blood of infected animals. However, an active antibody response is not elicited until the maternal antibody titre has dropped below a certain level (Sabin and Cincinnati, 1956; Kohn, 1962).

In this study, birds which received IP or oral inoculations of AAV, or were in contact with the infected birds, had body weight gains which were significantly lower than those of the controls. This has also been recorded by others who used a variety of serotypes of AAV including serotype 8 (Grimes and King, 1977b; Cook, 1983).

This New Zealand isolate is capable of producing high mortality rates following exposure by a natural route and of causing severe growth retardation in those birds which survive infection.

Natural infection of IBH was associated with high mortality, characteristic hepatic changes with INIBs, and depletion of lymphocytes in the bursa, thymus and spleen. The bursa and thymus were grossly atrophied, the bone marrows were markedly pale and the PCV was very low. Variable amounts of AAV antigens were also detected by the indirect ELISA in all of the lymphoid tissues that were examined and in the liver. Serotype 8 AAV was isolated from the liver tissue but there was no evidence of CAA in the same tissue. This naturally occurring disease was not associated with infection with IBD virus. Recently, severe outbreaks of IBH causing up to 30% mortality in several broiler flocks in New Zealand and associated with lymphocytic depletion in bursa and thymus have been reported (Christensen and Saifuddin, 1989). These outbreaks also were not associated with IBD virus infection or any other identifiable immunosuppressive factors (Christensen and Saifuddin, 1989). The serotype 8 AAV isolated from the infected liver tissue from birds in these outbreaks was found to be highly pathogenic for conventionally raised and SPF chickens.

The severity of the clinical signs observed in any particular outbreak of an infectious disease, such as IBH, will depend on the pathogenicity of the infecting strain of virus as well as the presence of other agents or factors which may lower the individual's ability to control the infection. It has been demonstrated that the mortality rate was dramatically increased (up to 40%) in some broiler chicken flocks in Australia when IBH was associated with diseases such as coccidiosis, colibacillosis, paratyphoid or respiratory disease complex

(Reece et al., 1986a). The importance of strain variation has been emphasized by Rivas and Fabricant (1988) who suggested that there is a direct relationship between MD virus strains of higher pathogenicity and more profound immunodepression.

Although the presence of bursal changes in many outbreaks of IBH has been claimed to be due to IBD virus infection (Rosenberger et al., 1975; Fadly et al., 1976), there are several reports of the involvement of lymphoid organs including bursa, thymus, spleen and caecal tonsil in naturally occurring IBH infections without any evidence of IBD or any other immunosuppressive factors (Pettit and Carlson, 1972; Hoffmann et al., 1973; Itakura et al., 1974; MacPherson et al., 1974). .

SPF birds infected with the AAV isolated from the naturally infected birds in this study, showed severe lymphocytic depletion in their bursae, thymuses and spleens in addition to the typical hepatic lesions with a large number of INIBs. Significant amounts of viral antigens were detected by ELISA in all of the lymphoid organs. Viral antigens were localized by immunocytochemistry in the lymphoid aggregates both in the white and red pulps of the spleen and in the lymphoid follicles of the bursa. Similarly lymphoid tissue of the caecal tonsil and thymus were also specifically stained. Some workers have also reported lymphoid depletion of the bursae, thymuses and spleens of birds experimentally infected with IBH virus (Grimes et al., 1977b; Fadly et al., 1980).

In addition to this morphological and antigenic evidence of viral involvement and damage to lymphoid tissue, there is evidence that birds infected with a pathogenic strain of AAV have a decreased ability to mount an antibody response to unrelated antigens. In the IBH infected group, both the number of birds which had detectable HA antibody to SRBC in their serum and the mean HA titre were lower than in the noninfected control group at 2 weeks pi. At 4 weeks following the first inoculation (i.e., 2 weeks after the booster dose), the mean HA antibody titre in the serum of infected birds was also lower than that of the noninfected control group. Hoffmann et al. (1975) reported that infection with some strains of AAV causes lymphoid depletion of bursa, thymus and spleen so that both humoral and cell mediated immunity is compromised.

While it has been previously thought that IBH occurs in birds which are infected with a strain of AAV and suffering concurrent immunodepression due to some other agent or factor, it now appears that some strains of AAV are highly pathogenic in their own right. Further, some strains can cause significant immunosuppression due to their direct effects on lymphoid tissues.

SUMMARY

A strain of serotype 8 AAV was isolated from a natural outbreak of IBH in broiler flocks. Post mortem changes included characteristic liver lesions with INIBs in the hepatocytes and severe lymphocytic depletion in the bursa, thymus and spleen.

Fatal hepatitis, resembling IBH, occurred in 30% of conventionally raised chickens following their inoculation orally with the locally isolated serotype 8 virus while a 45% mortality was recorded in those inoculated IP. Severe growth depression was recorded in the survivors and in birds in-contact. Control birds were healthy and their body weights were expectedly normal.

Birds which had maternally-derived VN antibody titres of 64 or greater, at the time of viral exposure, did not succumb to fatal infection, but their growth rates were significantly depressed.

There was obvious depletion of lymphocytes in the bursae, thymuses and spleens of the experimentally infected birds, and AAV antigens were detected by ELISA and immunocytochemical staining in various lymphoid tissues.

Humoral antibody responses against SRBC, detected by a HA test, were markedly decreased in the chickens infected with IBH virus.

It is concluded that this isolate of AAV causes depression of the immune system of infected chickens by its direct effects on various lymphoid organs following either natural or experimental infection.

CHAPTER FIVE

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY AND AN IMMUNOCYTOCHEMICAL PROCEDURE TO DETECT, QUANTIFY AND LOCATE ADENOVIRAL ANTIGENS IN CHICKEN TISSUES

INTRODUCTION

Diagnosis of an avian adenoviral infection currently depends on demonstrating virus-specific antibody or on isolating the virus, either in cell culture or in embryonating eggs, and its subsequent typing by cross-neutralization tests with specific antisera. Frequently, in attempts to isolate AAV from tissue samples, several blind passages are required before a CPE is observed (Green et al., 1976; McFerran et al., 1976b; Cook, 1983), so it is often not possible to titrate the amount of infectious virus in tissues of infected birds.

To study the pathogenesis of IBH caused by infection with a local isolate of serotype 8 AAV, there was a need to develop an assay which permitted ready detection and quantitation of virus in a range of tissues at various times after infection. An ELISA to detect viral antigens seemed most likely to satisfy this need.

The ELISA is an inexpensive, highly sensitive and easily conducted serological test which is widely used to detect and quantify a range of antigens and antibodies directly in serum, tissues, excretions and secretions. The ELISA has been extensively used for detecting antibodies to influenza A virus (Hammond et al., 1980), IBD virus (Marquardt et al., 1980), IB virus (Marquardt et al., 1981; Nandapalan et al., 1982; Perrotta et al., 1988), AE virus (Garrett et al., 1984; Smart and Grix, 1985), avian reo virus (Slaght et al., 1978) and ND virus (Snyder et al., 1983; Wilson et al., 1984). ELISAs to detect viral antigens have been developed for leukosis virus (Smith et al., 1979; Clark and Dougherty, 1980; Clark et al., 1981; Ignjatovic and Bagust, 1982; Crittenden et al., 1983; De Boer et al., 1983; Crittenden et al., 1984), AE virus (Shafren and Tannock, 1988) and ND virus (Snyder, 1986).

Other ELISAs have been developed to detect antibody in chicken serum against AAV, and extensive cross-reactions between 10 different serotypes have been reported (Calnek et al., 1982). If this cross-reactivity was also found in an ELISA to detect adenoviral antigens, then such an assay may also be useful for screening SPF flocks for the presence of any AAV.

An ELISA to detect and quantify AAV in tissues does not, however, reveal the precise site of viral replication within a particular tissue so it has limitations for studies of viral pathogenesis. An immunocytochemical technique utilizing an avidin-biotin peroxidase complex (ABC) was considered suitable for monitoring the effects of host-virus interaction at the cellular level in various tissues.

The high sensitivity of the ABC technique is based on the strong affinity between the large glycoprotein molecule avidin (MW 68000 daltons) and the small molecule of the vitamin biotin (Hsu et al., 1981). The affinity between avidin and biotin is over one million times higher than that of antibodies for most antigens and is essentially irreversible (Bourne, 1983; Faran et al., 1986; Coghill et al., 1987; Vector Laboratories, 1987). The ABC method was originally designed for use in frozen or paraffin-embedded tissue sections. However, the ABC technique has also been used in ELISAs, nucleic acid hybridization (Southern blot), protein detection (Western blot) and in immunoaffinity techniques (Coghill et al., 1987).

The ABC technique was selected and developed to localize AAV antigens in paraffin-embedded tissue sections to determine the location of viral replication following oral exposure of chickens to a locally isolated, serotype 8, AAV.

MATERIALS AND METHODS

Viruses

Twelve prototype strains of AAVs (CELO, SR48, SR49, KR5, 340, CR119, YR36, HVI, 764, A2, C2B and 380) representing serotypes 1-12, 2 New Zealand isolates (736NI and 716W) representing serotype 1 and 8 and 1 Australian isolate (Qld) representing serotype 8 were used in this study. The 716W strain of AAV was isolated from liver suspension derived from a natural outbreak of IBH with high mortality in broiler flocks. A locally-isolated avian reovirus (Saifuddin et al., 1989) was also used as a negative control in assessing the specificity of reactions by immunocytochemistry.

Preparation of gamma globulin

Gamma globulins were prepared from rabbit and chicken immune sera to 716W virus (serotype 8) by saturated ammonium sulphate (SAS) precipitation. Gamma globulins, from non-immune rabbit and chicken sera, used for the standardization of the reagents in ELISA, were also prepared by SAS precipitation. Serum was clarified by centrifugation at 10000 xg for 30 min at 10^{o}C. SAS was slowly added to precooled serum to a final concentration of 45% (v/v) with gentle stirring at 4^{o}C. After stirring overnight, the serum-SAS mixture was centrifuged as above. This precipitate was resuspended in 45% SAS in distilled water and recentrifuged. The precipitate was dissolved in PBS and clarified by centrifugation under similar conditions. After 2 further precipitations with 40% SAS the final precipitate was dissolved in PBS to half the volume of the original serum and dialyzed at 4^{o}C against 500 ml of PBS with 4 changes at 15 hr intervals. After dialysis, a 10% solution of barium chloride was used to check whether the gammaglobulin preparation contained any ammonium sulphate residues or not (barium chloride forms a white precipitate in presence of sulphate ions). A drop of barium chloride solution was mixed with a drop of gammaglobulin solution on a black surface.

The globulin fraction of immune rabbit serum was incubated with an equal volume of a 10% SPF chicken liver-kidney cell suspension for 1 hr at room temperature and was centrifuged at 10000 xg for 30 min at 10^{o}C. The supernatant was stored at -75^{o}C as stock gamma globulin preparation.

Monitoring of the recovery of gamma globulin

The gamma globulin contents of rabbit and of chicken antiserum to 716W virus were compared with that of the non purified antiserum using a GD test, cellulose acetate electrophoresis and VN activity against homologous and heterologous viruses.

a) GD test

The test procedure followed the technique described in the bovine leukemia glycoprotein immunodiffusion kit (Pitman Moore Inc. Washington Crossing, N.J. USA). One hundred millimetre petri dishes, each containing 15 ml of 0.9% Sea Plaque Agarose (FMC Corp. USA) prepared in 0.15 M NaCl were used. Three patterns of wells consisting of 1 central and 6 peripheral wells (5 mm in diameter) were cut in each petri dish. The gamma globulin preparation of immune rabbit serum was added to two peripheral wells; whole pre-immune and immune rabbit sera, and PBS were added to the remaining peripheral wells and pig anti-rabbit IgG whole molecule (Eivai bios laboratories, UK) was added to the central well. The dishes were incubated at room temperature in a humid box and after 24-48 hrs the result was read using a strong narrow beam of light against a black background.

b) Cellulose acetate electrophoresis

The gamma globulin preparations were electrophoresed on cellulose acetate strips (60 x 76 mm) according to the technique described by Helena Laboratories, Beaumont Texas, USA. The whole immune and pre-immune chicken and rabbit sera were also run in parallel with the globulin preparations. The gamma globulin level was scanned at a wave length of 525 nm and quantitated by a computerized integrator.

c) Neutralization test

The VN activity of SAS-precipitated immune chicken and rabbit globulins was tested in neutralization tests against the homologous 716W virus and compared with the titres obtained with whole immune sera. The detailed procedure for the neutralization test is described in Chapter 3.

ELISA

Preparation of antigen

Antigen prepared from the strain 716W (serotype 8 AAV) was used for the standardization of chicken and rabbit antibodies to 716W virus used in ELISA. Virus was propagated in CKC and clarified by homogenization with reagent grade chloroform (2:1) followed by centrifugation at 1000 xg for 20 min. The clear supernatant was then centrifuged at 130000 xg for 3 hr at 4^{o}C. Further details of the method were described in Chapter 3.

Preparation of tissues

Twenty tissues (liver, gall bladder, spleen, kidney, pancreas, thymus, brain, trachea, lung, pharynx, oesophagus, proventriculus, gizzard, duodenum, ileum, caecal tonsil, colon, cloaca, bursa and muscle) from SPF chickens and liver tissue from AAV infected chickens were pulverised individually (Stomacher 400, Colworth Ltd. London) for 30 min in PBS to prepare 10% (w/v) suspensions of each sample. Additionally, samples of blood, faeces, egg yolk and egg albumin were collected from SPF chickens and similarly processed. After 2 cycles of freezing and thawing, aliquots of each suspension were stored at -75^{o}C until they were assayed for viral antigens by ELISA.

ELISA procedure

The indirect ELISA was based on the procedure of Engvall and Perlmann (1971) with minor modifications. The test was carried out in 96-well, flat-bottom, polystyrene microtitre plates (Nunc, Inter Med, Denmark) and all components of the test were added in 100 ul volumes. The plates were always incubated at room temperature (20-25^{o}C). Carbonate-bicarbonate buffer, 0.05 M, pH 9.6, and 0.02 M PBS, containing 0.05% Tween-20 (PBS-Tween, pH 7.5), were used for diluting the coating antibody (rabbit anti-AAV) and for washing the plates, respectively. PBS-Tween, containing 2% bovine serum albumin (PBS-Tween-BSA, pH 7.5), was used for diluting test samples, chicken anti-AAV and horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Heavy + Light) (Miles Laboratories, Elkhart, Indiana). The substrate was made by diluting ortho-phenylenediamine (OPD) (Merck, Darmstadt, West Germany) in 0.1M phosphate citrate buffer (0.04% w/v), and adding 0.012% H_2O_2 (30%). After washing 4 times the plates were washed once for 1 min with phosphate citrate buffer before the substrate was added. The ELISA absorbance (EA) values were measured

at a wavelength of 486 nm using a computerized spectrophotometer (SLT 210; Labinstruments Ges. m.b.H, Salzburg, Austria). The outline of the ELISA is shown in Figure 5.1.

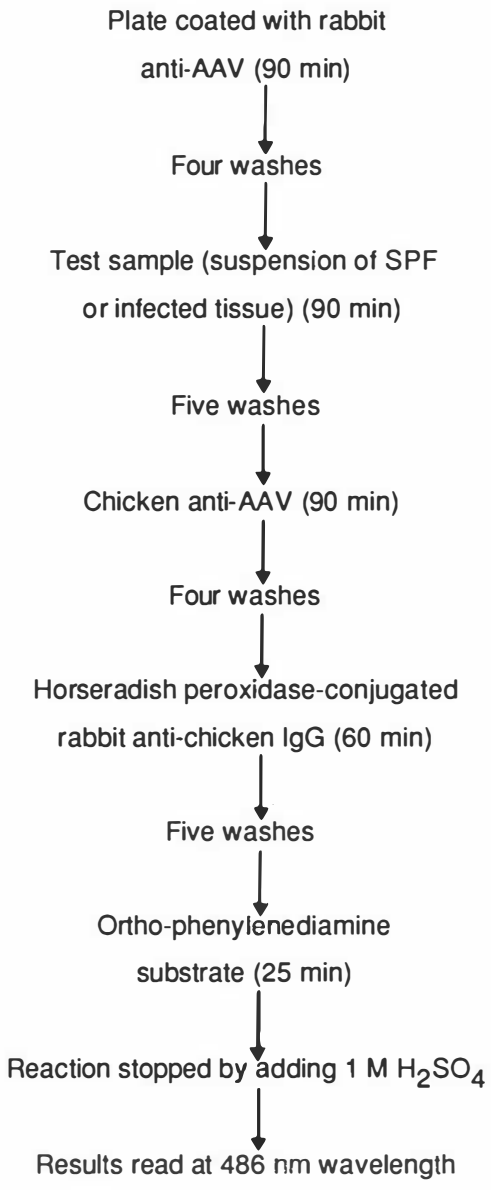

Fig. 5.1 **Flow diagram for the ELISA.**

Standardization of the reagents

Before the ELISA was used for the routine detection of viral antigens, the working strengths of the immune sera and conjugate were determined by checkerboard titration. Initially, the chicken antibodies to AAV strain 716W (second antibody) were titrated against the conjugate. The inner 40 wells of a microtitre plate were coated with purified 716W viral antigen (1:100). Two-fold dilutions of immune (anti-716W) and non-immune chicken globulin preparations ranging from 1:100 to 1: 1600, respectively, were distributed to each vertical half of the coated plate. After incubation and washing, serial two-fold dilutions (1:250 to 1:2000) of the conjugate were added to the horizontal rows of wells. The plate was incubated for 60 min, washed and substrate was added to all wells. After 25 min, EA values were recorded. The highest dilution of the immune chicken globulin and conjugate giving an EA value of approximately 1.0 was taken as the working dilution.

The next step was the titration of immune rabbit globulin (coating antibody). Two-fold dilutions (1:125 to 1:2000) of immune (anti-716W) and non-immune rabbit globulin were added to wells for 90 min. Routine washing was followed by the addition of purified 716W antigen (1:100) and the working dilution of 716W chicken antibody preparation and conjugate. The highest dilution of immune rabbit globulin giving an EA value of about 1.0 was considered the working dilution.

Interpretation of the ELISA result

The EA values were determined for 10% suspensions of the different tissue samples from SPF chicken and of normal CKC culture. The mean EA value and the standard deviation (SD) were calculated.

Comparison of EA value with titre of infectious virus

A suspension containing equal parts of a 10% (w/v) suspension of SPF chicken liver tissue and cell-culture virus (716W) of known titre was serially diluted in PBS-Tween-BSA buffer (twofold steps, 1:10, 1:50 to 1:409600). Each dilution was tested four times in the ELISA to determine the reproducibility and sensitivity of the assay in the presence of normal liver suspension. Also, a 10% (w/v) suspension of infected liver tissue of known virus titre was similarly diluted and each dilution was tested by ELISA. The arithmetic mean EA values of the infected liver suspension and the suspension of 10% SPF liver tissue with the cell-

culture virus were plotted separately against the $\log_{10}$ of the calculated titre of the infectious virus for each dilution. Titres were expressed as $\log_{10}$ of the mean tissue-culture infective dose ($TCID_{50}$) per gram of tissue.

Cross-reactivity between adenoviruses in VN test and ELISA

Each of the 12 prototype strains and 2 New Zealand isolates, 716W and 736NI, representing serotype 8 and 1, respectively, was tested by VN test against rabbit anti-716W (serotype 8) serum in 96-well, flat-bottom tissue-culture plates. Serum was diluted in two-fold steps from 1:10 to 1:20480 and each dilution was reacted with approximately 100 $TCID_{50}$ of each of the viruses. The tests were prepared in duplicate and the neutralization titre was taken as the reciprocal of the highest dilution of serum completely inhibiting the CPE of the virus in monolayers of CKC.

For comparison with the ELISA, the plates were coated with a 1:1000 dilution of rabbit antibodies to strain 716W, and a 1:10 dilution of each virus that had been propagated in SPF CKC was added. The tests were run in duplicate with the previously determined working concentration of immune chicken antibody preparation and conjugate. A 1:10 dilution of uninfected CKC was used as the negative control.

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Fixation of tissues

Five different tissues (liver, spleen, ileum, pancreas and breast muscle) were collected at 3, 4, 5 and 9 days pi from each of 3 SPF birds which had each received 500 ul of the locally-isolated serotype 8 AAV (10^6 $TCID_{50}$) orally. Portions, not larger than 2 cm^2 x 4mm, of the tissues were fixed in Bouin's fluid and in 10% buffered formalin for 12, 18, 24 and 48 hr at room temperature. Tissues fixed in Bouin's fluid were washed with 70% ethyl alcohol and embedded in paraffin (heated at not higher than 60^oC) following the standard procedure. Similar tissues fixed in Bouin's fluid were stored in 70% ethyl alcohol and tissues fixed in formalin were stored in formalin for about 6 months before being embedded. Ten percent (w/v) suspensions of another portion of the tissues were prepared in PBS and examined for viral antigens by the indirect ELISA. Tissues were collected from non-infected SPF birds and fixed similarly for negative controls.

Preparation and handling of the sections

Five micron thick sections were cut from paraffin-embedded tissues, fixed on microscope slides coated with polyvinyl acetate and dried thoroughly at 37^{o}C for at least 48 hr. The sections were deparaffinized and hydrated through a series of xylene and graded alcohol to water.

Development of optimum staining procedure

The immunological staining was carried out in a humid box at room temperature. The slides were freed of excess fluid before adding each reagent to the sections. Two percent BSA in 0.01 M PBS containing 0.1% sodium azide was used for diluting the blocking agent, primary antibody and secondary antibody. The washing buffer and diluent for H_2O_2, trypsin, BSA, conjugate and substrate was 0.01 M PBS.

Before the technique was applied for routine use, the optimum dilution and the period of incubation for each of the reagents needed in the procedure was determined as follows, using liver sections derived from birds which had been infected with the locally-isolated serotype 8 virus. The 10% (w/v) suspension of this liver tissue contained a large amount of viral antigens as determined by indirect ELISA.

a) Endogenous peroxidase

Liver sections were treated with 3% H_2O_2 in PBS and methanol for 10, 15, 20, 25 and 30 min for the evaluation of satisfactory inactivation of endogenous peroxidase present in the liver tissue. The sections were briefly washed in distilled water and then in PBS for 2 min.

b) Unmasking of intracellular antigen

The same liver sections were treated with warm 0.1% trypsin (pH 7.8) solution for 10, 15, 20, 25 and 30 min to expose the intracellular viral antigen. The sections were washed in PBS for 5 min.

c) Blocking of non-specific binding

Two percent BSA and 2% normal goat serum (NGS) (Vector Laboratories, Burlingame, California, USA) in 2% BSA were separately used for 10, 20 and 30 min for blocking any

nonspecific reactions. The sections were not rinsed but excess fluid was tapped off before adding primary antibody .

d) Primary antibody

Dilutions of 1:100, 1:250, 1:500, 1:1000, 1:2000 and 1:5000 of immune rabbit gamma globulin were applied to the liver sections for 30, 60, 70 and 90 min. The sections were then washed three times in PBS for 15 min each.

e) Indicator system

A 0.5% solution of biotinylated goat anti-rabbit IgG (Vector Laboratories) was applied for 15, 30, 45 and 60 min followed by washing with PBS for 15 min (3 changes). Subsequently, 1% HRP-conjugated biotin-avidin (Vectacin ABC, Vector Laboratories) was applied for 15, 30, 45 and 60 min. The washing procedure was repeated before the substrate solution, diaminobenzidine (DAB) tetrahydrochloride (Sigma Chemical Co. USA) (prepared by adding 5 mg DAB and 12 ul H_2O_2 to 10 ml of PBS) was added for 4 to 7 min. The reaction was stopped by rinsing the slides in tap water.

f) Counter staining

Finally the sections were counter stained with haematoxylin following the standard procedure and examined by light microscopy to assess the specificity of the immunostaining reaction.

Controls used with each preparation were known positive and negative sections, each run with and without the primary antibody, secondary antibody, conjugate (ABC) or substrate (DAB). The optimum dilution and the period of incubation for each reagent was represented by the slide with the most intense specific stain and the least amount of background staining after a minimum period of incubation.

The ABC assay was compared with a Biotin-streptavidin system using biotinylated HRP-conjugated streptavidin complex (Amersham, UK) on similar liver sections under similar conditions.

Expression of the results

The amount of viral antigen present in the tissues was expressed as the intensity of the

brown to dark brown colour which developed at the sites of the antigen-antibody reactions. The intensity of the colour was subjectively graded as highly intense (++++), intense (+++), less intense (++), trace (+) and negative (-) depending on the amount and distribution of colour in different tissues. The intensity of the colour developed in each infected tissue was also compared with the ESA value obtained with a 10% suspension of the same tissue.

Cross-reactivity between adenoviruses in ABC technique

Purified virus suspensions were made in distilled water of each of the 15 previously listed AAV strains. Two hundred and fifty microlitres of each suspension were mixed with 250 ul of a 1:500 dilution of the primary antibody to the locally-isolated 716W strain of AAV for 12 hr at room temperature. Each of the virus-antibody mixtures was centrifuged (Eppendorf Geratebau, Hamburg, West Germany) for 10 min at highest speed. Each absorbed antiserum was then used as primary antibody on the infected liver sections following the standardized ABC staining protocol. As controls, liver sections were also tested with a 1:1000 dilution of unabsorbed primary antibody and with primary antibody which had been absorbed with an unrelated avian virus, namely, a local isolate of avian reovirus.

RESULTS

ELISA

Cellulose acetate electrophoresis of immune rabbit serum and the fraction precipitated with SAS indicated that more than 90% of the gamma globulins were recovered, with little contamination by other serum proteins. Reactions in GD tests against antiviral rabbit globulin revealed a line of identity with immune serum, SAS-precipitated gamma globulin fraction and the SAS-precipitated fraction after it had been absorbed with SPF chicken liver-kidney cell suspension. The titre of VN antibodies in the absorbed rabbit gamma globulin preparation was 1:5120 while that of the original immune rabbit serum was 1:10240.

Dilutions of 1:800 and 1:1000 of immune chicken serum and conjugate, respectively, gave an EA value of 1.08 with a 1:100 dilution of purified antigen coated onto the plate. An EA value of 1.1 was obtained when a 1:1000 dilution of immune rabbit globulins was used to coat the plate. In the wells with higher dilutions, the EA values were less than 1.0. In the case of negative rabbit or chicken globulins, the values determined in all dilutions were less than 0.1. No false-negative or false-positive results were obtained with known positive or negative globulins, respectively.

Incubation of the plate at $37^{o}C$ produced nonspecific EA values with negative samples, so all incubations were carried out at room temperature.

The EA values of 21 different SPF tissues, including blood, faeces, egg yolk and egg albumin from SPF birds, ranged from 0.12 to 0.18 with an arithmetic mean of 0.15 and an SD of 0.02. The cut-off point between ELISA-positive and ELISA-negative absorbance values was taken as the mean value of the negative SPF samples plus 3 SD, that is, 0.21. The undiluted suspension containing cell-culture virus and 10% (w/v) SPF liver suspension gave an absorbance value of 2.45. This corresponded to $10^{7.6}$ $TCID_{50}$ /gm. The lowest positive absorbance value with this suspension was 0.21, which was obtained with a dilution of 1:25600 and corresponded to a titre of 1.56×10^{4} $TCID_{50}$ /gm. The highest mean absorbance value (2.58) was obtained with the undiluted 10% suspension of infected liver tissue. This corresponded to a titre of $10^{5.3}$ $TCID_{50}$ /gm. The lowest positive absorbance value with this suspension was 0.22, which was obtained with a dilution of 1:25600, and this corresponded to 78 $TCID_{50}$/gm. The relationship between EA value and the titre of infectious virus is illustrated in Figure 5.2. The regression equations calculated on the

ELISA-positive values are given by EA = 0.77 $\log_{10}$ $TCID_{50}$ - 3.24 and EA = 0.82 $\log_{10}$ $TCID_{50}$ - 1.57, respectively, for the SPF liver suspension with cell-culture virus and for the infected liver suspension. In both cases, the correlation coefficient between absorbance value and the titre of infectious virus per gram of tissue was 0.98.

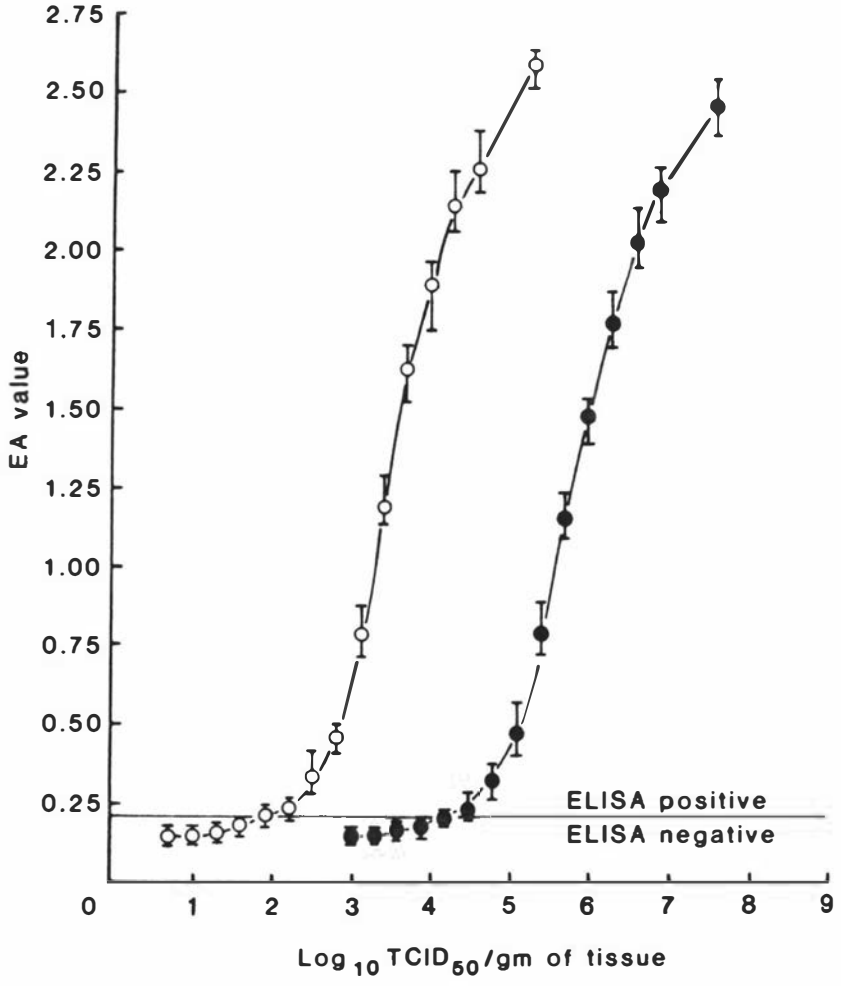

Fig. 5.2 **Correlation between ELISA absorbance (EA) value and concentration of infectious virus in infected liver tissue (O---O) and in suspension of SPF liver and cell-culture virus (●---●).**

Rabbit antiserum to strain 716W (serotype 8) inhibited the CPE caused by homologous virus up to a dilution of 1:10240 and up to 1:5120 with strain HVI (serotype 8), but not with any of the other strains. However, in the ELISA, the gammaglobulin preparation against 716W gave positive reactions with each of the 12 serotypes of virus (EA values 0.7 to 2.2), although the homologous value was the highest (Table 5.1).

Table 5.1 **Reactions between antiserum to avian adenovirus (AAV) 716W (serotype 8) and representatives of 12 serotypes of AAV in neutralization tests and by enzyme-linked immunosorbent assay (ELISA).**

Virus (serotype)	Neutralization test [A]	ELISA [B]
716W (8)	10,240	2.2
736NI (1)	<10	0.8
CELO (1)	<10	0.8
SR48 (2)	<10	1.4
SR49 (3)	<10	1.3
KR5 (4)	<10	1.0
340 (5)	<10	0.9
CR119 (6)	<10	1.7
YR36 (7)	<10	1.1
HV1 (8)	5120	1.8
764 (9)	<10	1.2
A2 (10)	<10	1.4
C28 (11)	<10	0.7
380 (12)	<10	1.4

[A] Reciprocal of the highest dilution of serum (antiserum to 716W) which completely inhibited cytopathic effect of approximately 100 mean tissue-culture infective dose of the respective virus.

[B] ELISA absorbance value at 486 nm.

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Tissues fixed in Bouin's fluid for 12-18 hr and in formalin for 24 hr presented satisfactory morphology after staining with HE. There was no significant difference between the tissues fixed in formalin for 24 or 48 hr. Tissues were not well-fixed in formalin within 18 hr whereas tissues became over-fixed and tended to get harder in Bouin's fluid after 24 hr. In both cases it was difficult to cut suitable thin sections. Blood-rich tissues, such as liver and spleen, seemed to have fixed better in formalin than in Bouin's fluid. Regardless of fixative and dilution of antibodies used in the ABC technique, no antigen was detected in any of the infected tissues stored in 70% ethyl alcohol or formalin for about 6 months.

Sections dried at 37^{o}C for less than 48 hr tended to detach from the slide during the staining procedure. Regardless of the timing of incubation, dilutions of 1:100 and 1:250 of the primary antibody (rabbit anti-viral gamma globulin) showed very intense staining but with an unacceptably large amount of background staining. A dilution of 1:1000 revealed intense staining with minimum background staining after about 60-70 min of incubation. Dilutions of 1:2000 and 1:5000 showed pale staining with a clear background after 90 min of incubation.

Biotinylated goat anti-rabbit IgG (second antibody) and HRP-conjugated biotin-avidin (ABC reagent) both after 30-45 min of incubation presented intense staining with a primary antibody dilution of 1:1000. Six to seven min of incubation was found suitable for adequate colour development after adding the substrate.

Omission of the primary antibody, secondary antibody, ABC reagent or DAB from the standard procedure resulted in the loss of specific staining. Sections or parts of sections, that were inadvertently allowed to dry, stained nonspecifically.

The endogenous peroxidase in liver sections was satisfactorily inactivated by 3% H_2O_2 in PBS and 3% H_2O_2 in methanol after incubating for 30 and 20 min, respectively. Adequate exposure of intracellular antigen was achieved after 15 min of incubation with 0.1% trypsin solution. Two percent NGS in 2% BSA was found suitable for blocking any nonspecific reactions by 20 min of incubation. The outline of the standardized ABC staining protocol is shown in Figure 5.3.

The specific colour reaction was stronger with the ABC procedure than with a Biotin-streptavidin system so this system was not evaluated any further.

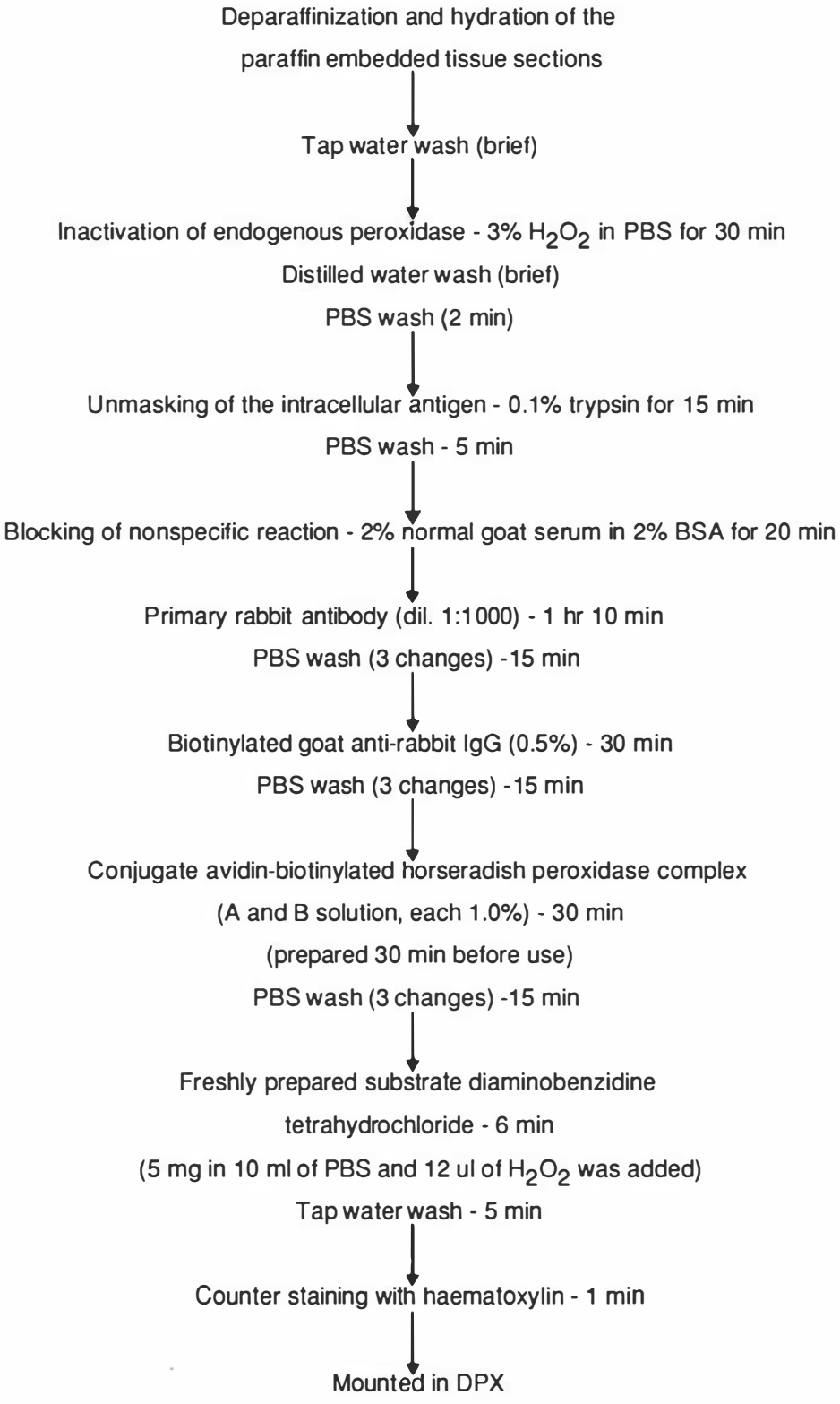

Fig. 5.3 **Flow diagram for the standardized staining procedure of avidin-biotin peroxidase complex technique.**

Viral antigens were detected by the ABC technique in liver, spleen, pancreas and ileum from 3-5, 4-5, 4-5 and 3-9 days pi, respectively (Table 5.2). At 3 and 4 days pi, viral antigens were mostly located in the nuclei of hepatocytes (Fig. 5.4a) and, at 5 days pi, antigens were detected both in nuclei and cytoplasm (Fig. 5.4b). In spleens, viral antigens were detected mainly in the peri-arteriolar areas (Fig. 5.4c), in ileum in the lining epithelium and in pancreas mostly in acinar cells. Viral antigen was not detected in muscle tissues. Likewise, liver sections from noninfected SPF birds did not show staining (Fig. 5.4d). In 10% suspensions of the same liver, spleen, pancreas and ileum samples, viral antigens were detected by ELISA from 3-9, 3-5, 3-5 and 3-9 days pi, respectively (Table 5.2). Viral antigen was not detected by ELISA in muscle tissue.

Table 5.2 **Detection of viral antigens by ABC and ELISA assays in tissues of birds infected orally with a locally isolated strain of serotype 8 avian adenovirus.**

Tissue	Days post inoculation			
	3	4	5	9
Liver	++ *	+++	++++	-
	(0.69) **	(0.92)	(1.15)	(0.18)
Spleen	-	++	+++	-
	(0.15)	(0.42)	(0.63)	(0.05)
Pancreas	-	+	++	-
	(0.20)	(0.50)	(0.72)	(0.03)
Ileum	++	++	+++	++
	(1.75)	(1.83)	(1.95)	(1.58)
Muscle	-	-	-	-
	(0.00)	(0.00)	(0.00)	(0.00)

* Intensity and extent of immunocytochemical staining graded from ++++ to - where ++++ means intense specific staining in many cells.

** Mean ESA (n = 3) Elisa specific absorbance obtained by subtracting the absorbance with SPF tissue from that obtained with test tissue. ESA greater than 0.06 is considered positive.

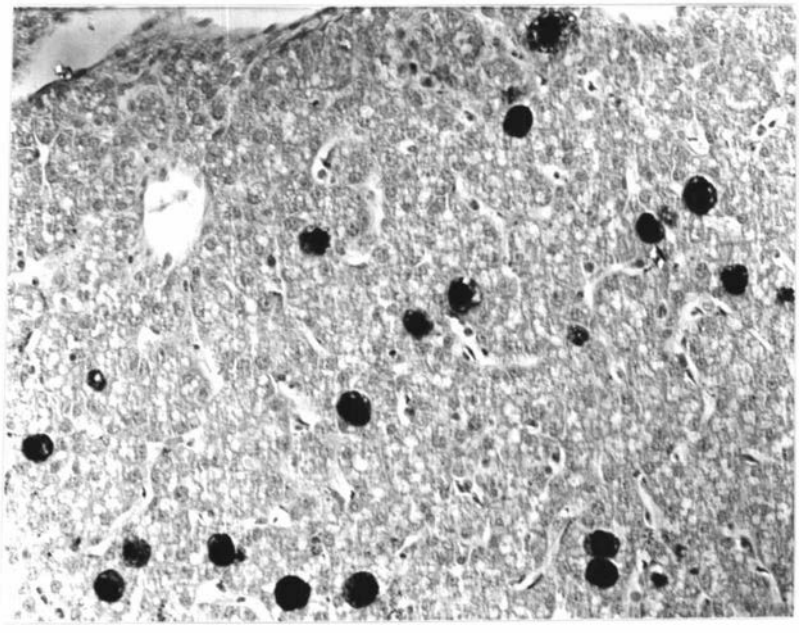

Fig. 5.4a **Immunoperoxidase staining of viral antigens in liver tissue following oral administration of serotype 8 avian adenovirus. Dense staining mainly in the nuclei of the hepatocytes without nonspecific background staining (4 days pi, haematoxylin counterstain, x10).**

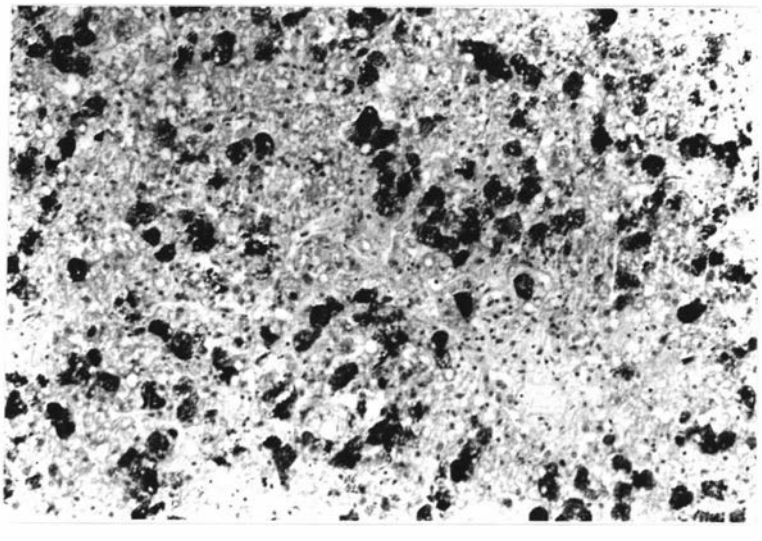

Fig. 5.4b **Dense staining of a large number of hepatocytes (both nucleus and cytoplasm) of a chicken which died at 5 days post inoculation (X20).**

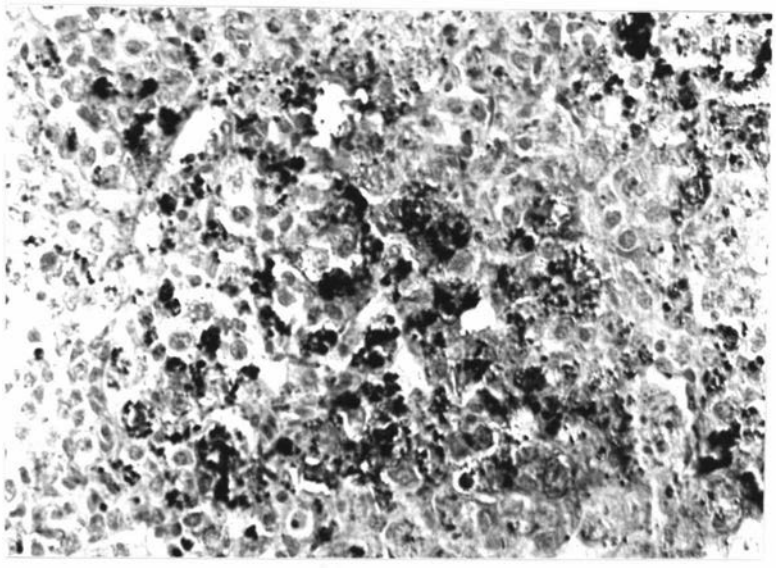

Fig. 5.4c **Section of spleen showing strong staining in groups of cells (5 days pi, x20).**

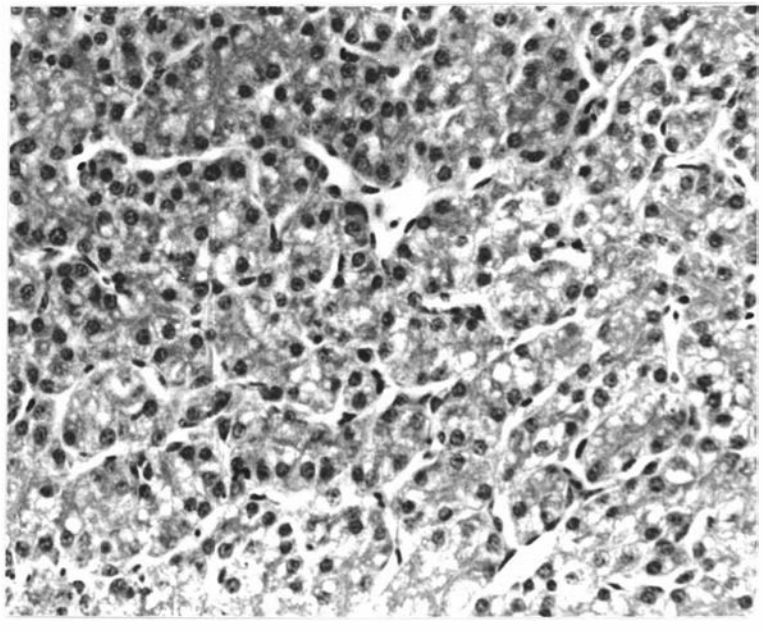

Fig. 5.4d **Section of liver from a non-infected SPF chicken showing no nonspecific staining (x20).**

Following absorption of the primary antibody with 6 of the prototype strains, one New Zealand isolate (716W) and the Australian isolate Qld, representing serotypes 1, 2, 6, 7, 8 and 9, no colour reaction was detected when the absorbed antibody was used in the ABC technique on sections of infected liver. Absorption with the 4 prototype strains, representing serotypes 4, 5, 11 and 12, and the New Zealand isolate, 736NI (serotype 1), resulted in a colour reaction which was about 25% of the intensity of the unabsorbed antibody. Absorption with prototype strains representing serotypes 3 and 10 resulted in a colour reaction which was about 10% of the intensity of the unabsorbed antibody. Absorption with the avian reovirus did not result in any decrease in staining intensity.

DISCUSSION

This chapter has described the optimum conditions for a simple, rapid, and sensitive ELISA to detect and quantify AAV antigens directly in infected tissues. The optimum conditions for an immunocytochemical procedure to study the site of viral replication within a particular tissue are also described.

The use of the gamma globulin fractions of the binding and detecting antisera and absorption with SPF liver-kidney cell suspensions probably both contributed to the specificity of these assays (Dawson et al., 1980).

The results of the ELISA were interpreted by comparison of positive and negative reference samples. A test sample was regarded as positive if the absorbance value was greater than the mean value of respective SPF control tissues plus 3 SD. This is consistent with the findings of Shafren and Tannock (1988), but De Boer et al. (1983) considered that a difference of only 2 SD was sufficent. Voller et al. (1976) suggested that the results of microplate ELISAs can be interpreted only in the presence of positive and negative reference samples and that test samples should be considered positive if they yield absorbance values above that of the normal (uninfected) individuals that provide the base-line level. From Figure 5.2, it is apparent that when absorbance values are plotted for samples containing less than 1.56×10^4 $TCID_{50}$/gm for the cell-culture virus or less than 78 $TCID_{50}$/gm of infected liver tissue, the slope very closely approaches the horizontal. This supports the use of the mean absorbance plus 3 SD as a cut-off point for the detection of viral antigen. Evaluation of the test using a mixture of virus propagated in cell culture with SPF liver tissue, was chosen as it was believed this would more closely approximate the situation with tissues from an infected bird than would straight cell-culture virus. This approach was useful for initially developing the test, but, as later discussed, the sensitivity of the test appeared to be much higher with tissue derived from infected birds.

The EA values of the 21 SPF control tissues ranged from 0.12-0.18 (mean 0.15, SD 0.02). The absorbance values of each two-fold serial dilution (1:10, 1:50 to 1:409600) of infected liver suspension were determined, and each of the values was plotted against the $\log_{10}$ of the calculated titre of each respective dilution of the tissue. The titre was expressed as $\log_{10}$ $TCID_{50}$ per gram of tissue. Thus, using a standard curve produced from the EA values of known infected tissues, the titre of virus per gram of any infective tissue could be predicted by comparison with its absorbance reading. This is in line with the suggestion of Voller et al. (1976) that the titre of unknown samples can be expressed as a dilution titre of

a known positive sample by ELISA. A 1:25600 dilution of the infected liver suspension was the highest dilution to still give a positive absorbance value (0.22). This corresponded to a titre of 78 $TCID_{50}$/gm of tissue and indicated that the test was highly sensitive. In an ELISA to detect leukosis viral antigens, a similar level of sensitivity was obtained (Ignjatovic and Bagust, 1982; De Boer et al., 1983), and a positive correlation between absorbance values and titre of infectious virus was demonstrated (Clark et al., 1981; De Boer et al., 1983). Also, when the ELISA has been used to detect antibody in avian serum, a positive correlation between VN titre and EA value of serum samples has been found with IB virus (Nandapalan et al., 1982), reovirus (Islam and Jones, 1988), AE virus (Smart and Grix, 1985), and ND virus (Snyder et al., 1983).

The ELISA was much more sensitive in detecting virus in infected liver tissue than in the suspension made up of a mixture of virus propagated in cell culture and SPF liver. In fact, positive absorbance readings were obtained with the dilutions of infected liver that were calculated to contain less than 1 $TCID_{50}$ in the 100 ul of suspension used in the test. This is probably because the ELISA detects viral antigens whether or not infectious virus is present. It appears that AAV replication in vivo leads to more viral antigen per infectious unit of virus than does replication in tissue culture. The test therefore was about 100 times more sensitive at detecting virus in liver tissue from infected birds than in detecting virus in cell culture.

Because more than one blind passage of tissue suspensions often is necessary before AAV produces CPE on monolayer cell culture (Green et al., 1976; Cook, 1983), it is most unlikely that the amount of virus in clinical or experimental samples could be quantified using cell cultures. By contrast, detection as well as quantitative estimation of AAV in the same type of materials is possible within 5-6 hr by the ELISA developed in the present study.

In the present assay, using antibody to 716W (serotype 8), positive reactions were obtained with all 12 serotypes of AAV, with EA values ranging from 0.70-2.20. The test therefore helps to identify group-specific AAV antigens. This is consistent with the cross-reactivity reported by Calnek et al. (1982) in an ELISA developed to detect antibody to AAV. Because of this group-specific reactivity, the present assay may be useful for the rapid diagnosis of outbreaks of IBH caused by any of the several serotypes of AAV that have been associated with field outbreaks of this condition (Kefford et al., 1980; McFerran, 1981a; Cubillos et al., 1986; Reece et al., 1986a).

The high sensitivity and broad-spectrum reactivity could make this ELISA the preferred test for the study of AAV pathogenesis, for laboratory diagnosis of IBH irrespective of the

serotype of AAV involved, and for surveying commercial and SPF flocks for the presence of AAV.

The ABC technique has proven to be very useful for detecting AAV antigens in various paraffin-embedded chicken tissues. Further, there are no health hazards associated with the reagents used. Tubbs and Sheibani (1982) have suggested that the substrate DAB is less dangerous than was previously thought (Weisberger et al., 1978).

Several factors may account for the negligible background and the intense specific staining of this ABC technique. The preliminary absorption of the primary antibody to serotype 8 AAV with SPF liver-kidney cell suspension probably contributed significantly to the minimum nonspecific staining of the uninfected cells. This is consistent with the findings of Katz et al. (1987). Also, treatment of the sections with NGS, the high dilution (1:1000) of primary antibody and the appropriately diluted ABC complex reduced the nonspecific background reactions. High concentrations of ABC may cause troublesome nonspecific background staining (Hsu et al., 1981). Presumably, inhibition of the endogenous peroxidase activity by using H_2O_2 also enhanced the specific staining.

The high sensitivity of the technique is based on the strong affinity ($10^{15}M^{-1}$) between the large glycoprotein molecule avidin (MW 68000 daltons) and the small vitamin biotin. Since avidin has four binding sites for biotin, one molecule of avidin can bind with three molecules of marker (biotinylated enzyme or flourescent dye) and still have one vacant biotin-binding site. This vacant site is available for binding biotinylated antibodies that have been bound previously to the antiviral primary antibody. The resulting complex can then be visualized by ordinary light microscopy following reaction with an appropriate chromogen such as DAB. The affinity between avidin and biotin is over one million times higher than that of antibodies for most antigens and is essentially irreversible even in the presence of high pH, protein denaturing agents, proteolytic enzymes and organic solvents (Bourne, 1983; Faran et al., 1986; Coghill et al., 1987; Vector Laboratories, 1987).

The ABC technique was compared with an indirect ELISA in a controlled set of experiments. Duplicates of tissues derived from chickens at various times after inoculation were prepared either as 10% suspensions for the ELISA or as paraffin-embedded sections for the ABC technique. In liver, spleen and ileum, high levels of AAV antigens were detected by ELISA at 4-5, 5 and 5 days pi respectively when an intense specific ABC staining was also obtained in a large number of cells. In ileum, high levels of viral antigens were detected by ELISA at 3, 4 and 9 days pi but only a limited number of cells was intensely stained by the ABC technique. In liver, spleen and pancreas, low levels of viral antigens were detected by ELISA at 9, 3 and 3 days pi, respectively, but no specific staining by ABC was detected at

these same periods. Thus there were some situations where positive ELISA reactions were obtained yet viral antigens were not detected by immunocytochemical staining. There can be considerable variation in intensity of immunocytochemical staining at different points within a section or between samples or tissues. Nevertheless it is not considered practicable to quantitate, with any degree of precision, the amount of the corresponding antigen at a particular location because of the kinetic variations in the reactions at the various stages of immunological and enzyme histochemical staining (Coghill et al., 1987).

Although the ABC technique was slightly less sensitive than the ELISA it did provide precise and permanent information on the site of AAV replication at the cellular level in particular tissues. This information was not provided by the ELISA and although other methods, such as fluorescent antibody, may yield the same information they do not give a permanent record. By allowing clear visualization of the antigens in various infected tissues, the spread of virus throughout the body can be revealed and thus the ABC technique provides a valuable tool for studying the pathogenesis of IBH in chickens.

Fixation of tissues in formalin or Bouin's fluid is essential to preserve the morphological structure of cells and to facilitate precise pathological studies but formalin fixation and paraffin embedding inhibits the penetration of labelled antibodies (Ohman et al., 1981). The treatment of tissue sections with 0.1% trypsin at 37^{0}C for 10 min prior to staining with the primary antibody gave an excellent exposure of AAV antigens with little or no detectable cell damage. Prolonged digestion, however, resulted in tissue disintegration and caused detachment of the section from the slide. Use of proteolytic enzymes to unmask viral antigens in formalin fixed paraffin embedded tissues has been reported to overcome similar difficulties with other viruses (Jonsson and Engstrom, 1986; Bryson et al., 1988; Allan et al., 1989). Most probably, the enzyme helps to break down the intermolecular linkages induced by formalin fixation (Brozman, 1978).

No viral antigens were detected in those tissues stored in 70% alcohol or in formalin for 6 months before being embedded in paraffin. Presumably, the antigens were somehow leached out from the tissue or were modified or degraded so that they were no longer recognized by the primary antibody. Others have also found that the fixation time, irrespective of the fixative, is a crucial variable in the preservation of viral antigenicity (Jonsson and Engstrom, 1986). Tissues fixed with either formalin or Bouin's fluid should preferably be embedded within 24 and 48 hr respectively. The antigenicity, as well as the cellular morphology, may be preserved for several years once the tissue has been embedded.

Absorption of the primary antibody (anti serotype 8) with the large range of AAVs illustrated

two features of the specificity of the reactions observed with this ABC technique. Firstly, absorption with purified homologous virus completely removed all reactivity, thus demonstraing that the reactions observed between primary antibody and infected liver sections were reactions with viral antigens present in the tissue. Secondly, the fact that absorption with any of the AAVs, representing all of the recognized serotypes of group I AAV, removed at least 75% of the reactivity of the primary antibody indicates that a common group-specific antigen(s) is being recognized. This latter finding is consistent with earlier studies where group reactivity was demonstrated using antiserum to serotype 1 AAV in immunofluorescent tests (Adair et al., 1980). Some variation in the degree of cross-reactivity between serotypes was apparent in the immunofluorescent study (Adair et al., 1980) and is also apparent in our present work. Also, using the same primary antibody preparation, we have shown that a common group-specific antigen (s) was revealed in the antigen-detecting ELISA, and some variation in the degree of cross-reactivity was found.

This study describes for the first time, the use of the ABC system for the detection of AAV antigens in various paraffin-embedded tissues from infected chickens. Because of the lack of reactivity with uninfected tissues, the high sensitivity and the broad-spectrum reactivity, this immunocytochemical technique could be preferred for studies of the pathogenesis of IBH and, as a complement to routine histopathological studies, the ABC technique can also be used as an important diagnostic tool for the detection of AAV antigens, irrespective of the serotype of infecting virus, in formalin-fixed and routinely embedded tissues.

SUMMARY

An indirect ELISA was developed to detect and quantify AAV in various chicken tissues including blood. A positive EA value was obtained with suspensions of infected liver tissue which contained less than 100 $TCID_{50}$ per gram. A positive correlation was observed between the EA values and titres of infectious virus in infected liver tissue. A group-specific antigen common to the 12 serotypes of AAV tested was demonstrated by this ELISA. Because its high sensitivity and broad spectrum reactivity this ELISA could be useful for the study of AAV pathogenesis, for laboratory diagnosis of IBH irrespective of the serotype of AAV involved and for screening commercial and SPF flocks for the presence of AAV.

An immunocytochemical technique utilizing ABC was developed to detect viral antigens in various tissues. A strong colour reaction was obtained with tissues from infected birds which contained at least the minimum level of viral antigens detectable by ELISA. No reaction was detected in sections of tissues obtained from SPF chickens and the reactivity with infected tissues could be removed by prior absorption of the primary antibody with purified AAV. A group-specific antigen common to the 12 serotypes of AAV was demonstrated by this technique. Because of the high sensitivity and broad-spectrum reactivity, this technique could be useful for studying the pathogenesis and laboratory diagnosis of IBH caused by several serotypes of AAVs. This technique has the advantage of producing a permanent record of the precise location of viral antigens within any particular tissue.

CHAPTER SIX

PATHOGENESIS OF INCLUSION BODY HEPATITIS IN CHICKENS

INTRODUCTION

IBH is an acute infectious disease of young chickens of 2-7 weeks of age caused by several serotypes of Group I AAV (McFerran, 1981a). Natural outbreaks are characterized by a sudden onset of sharply increased flock mortality (10-30%), a short clinical course, anaemia and necrotic hepatitis. Basophilic or eosinophilic INIBs are often observed in hepatocytes.

IBH virus is shed in faeces and usually spreads laterally through contaminated food, water, litter and sheds (McFerran, 1981a). Evidence for vertical transmission has also been presented (Reece et al., 1985). Very little is known about the pathogenesis of infection following exposure to IBH virus by a natural route. The pathogenesis of a number of other viral infections which result in hepatitis has been studied. Following ingestion, hepatitis A virus initially multiplies in the intestinal epithelium, spreads via lymphatic channels to the blood stream and infects the liver following a cell-free viraemia (White and Fenner, 1986). By contrast, ICH virus multiplies in lymphoid cells in tonsillar crypts and Peyer's patches and subsequently spreads to the local lymph nodes. Viral spread is in infected lymphoid cells and infection of the liver and other organs is via a cell-associated viraemia (Appel, 1987). Other modes of pathogenesis are also recognized (Mims and White, 1984).

Detection of IBH viral infection currently depends on either the demonstration of serum antibody or on isolation of the virus either in cell cultures or in embryonating eggs. More than one blind passage of infected tissue suspension is often necessary before the IBH virus produces a visible CPE on monolayer cell culture (Green et al., 1976; McFerran et al., 1976a; Cook, 1983). It is therefore not possible to determine directly by titration in cell cultures the amount of virus in chicken tissues for the study of pathogenesis. In an earlier study, the presence of virus was demonstrated in a variety of tissues at different intervals following oronasal inoculation of AAV, by isolation of virus in cell and organ cultures (Cook, 1983). Besides demonstrating the duration of virus, this study did not determine the first appearance or the amount of virus in a particular tissue, so the information obtained was limited. The development of a highly sensitive, indirect ELISA to detect and quantify AAV directly in chicken tissues including blood (Chapter 5) permits a more detailed study of the pathogenesis of IBH to be undertaken.

In this chapter, the results of a study of the pathogenesis of IBH and the pattern of viral shedding for a prolonged period following oral administration of a local isolate of serotype 8 AAV to 2-day-old SPF chickens are presented. The ELISA was used to detect and quantify virus in a range of tissues and faeces and an immunocytochemical procedure was used to locate the site of viral replication in specific tissues.

MATERIALS AND METHODS

Virus

The New Zealand isolate 717B, representing serotype 8 AAV, was used in this study at the seventh passage level (see Chapter 4).

Birds

Birds used in this experiment were hatched from eggs derived from a WLH SPF flock (details in Chapter 3). Once birds had been inoculated with virus they were kept in a positive-pressure bubble isolator and supplied with autoclaved feed and water *ad libitum*. Noninoculated, control birds were kept in strict isolation but with similar housing and feed.

Detection of viral antigen by ELISA

Ten percent (w/v) tissue suspensions in PBS were tested for viral antigens using the previously described indirect ELISA (Chapter 5). Results were expressed as ESA obtained by subtracting the absorbance with SPF tissue from that obtained with test tissue.

Detection of viral antigen by immunocytochemistry

Five micron-thick sections of paraffin-embedded tissues were used to locate the site of viral replication by using the ABC technique (Chapter 5).

Pathogenesis of AAV infection

a) Viraemia

Blood samples were collected in heparinized capillary tubes (Terumo Corporation, Tokyo, Japan) from five 2-day-old SPF chickens (each marked with individual wing tags) by wing vein puncture prior to and at intervals (12 hr, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13 and 15 days) following oral administration of 500 ul (10^6 $TCID_{50}$) of AAV strain 717B to each bird. Blood samples were separated into plasma and cellular fractions by centrifuging (Micro-capillary

centrifuge, International Equipment Company, Needham, USA) the capillary tubes for 3 min. Plasma and cellular fractions were diluted 1:10 with PBS and stored at -75^{o}C before they were assayed separately for viral antigens by ELISA.

b) Viral antigen in tissues

Sixty, 2-day-old SPF chickens were given 500 ul (equivalent to 10^6 $TCID_{50}$) of a suspension of strain 717B by the oral route and, at various times pi (12 hr, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13 and 15 days), 5 birds (including those which appeared moribund) were killed and 21 different tissues (brain, pharynx, trachea, lung, oesophagus, proventriculus, duodenum, ileum, caecum, caecal tonsil, colon, cloaca, bursa, thymus, bone marrow, liver, gall bladder, pancreas, spleen, kidney and muscle) were collected from each bird. Portions of selected tissues were fixed either in Bouin's fluid or in 10% buffered formalin for immunocytochemical examination (Chapter 5) and 10% suspensions of all tissues were prepared in PBS and tested for viral antigens by ELISA (Chapter 5).

Faeces were also collected from each bird and 10% suspensions prepared in PBS were tested for viral antigen by ELISA.

c) Antibody response

Serum samples were randomly collected from 10, 2-day-old, SPF birds before virus inoculation. Sera were also collected from each of 5 birds before they were killed at 12 hr, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13 and 15 days pi. All the sera were inactivated at 56^{o}C for 30 min and tested for VN antibodies to 717B virus in monolayer cultures of CKC (Chapter 3).

Persistent infection

Following the oral administration of 500 ul of 717B virus suspension (10^6 $TCID_{50}$), duodenum, ileum, caecum, caecal tonsils and faeces were collected from each of 2 infected birds at 3, 4, 6, 8, 10, 13, 16 and 20 weeks pi. A 10% suspension of each of the tissues and faecal samples was prepared in PBS and assayed for AAV antigen by ELISA. At each time, blood was collected from each bird and the serum tested for VN antibodies against 717B virus using monolayer cultures of CKC.

RESULTS

Viraemia

Viral antigens were detected only in the plasma fraction of the blood. Viraemia was first detected in 4 out of 5 birds at 24 hr pi (Fig. 6.1). The mean level of viral antigens in blood plasma reached a peak at 2 days pi, declined to a low level by 4 days pi, rose to a second peak at 7 days pi and then declined to an undetectable level by 11 days pi. While this biphasic pattern occurred with 4 of the 5 birds in the group, viral antigens were not detected in the fifth bird until 3 days pi (Fig. 6.1). In the fifth bird the level of viral antigens peaked at 7 days pi. The mean level of viral antigens in blood plasma was higher at the second peak (ESA 0.50) than at the first (ESA 0.40).

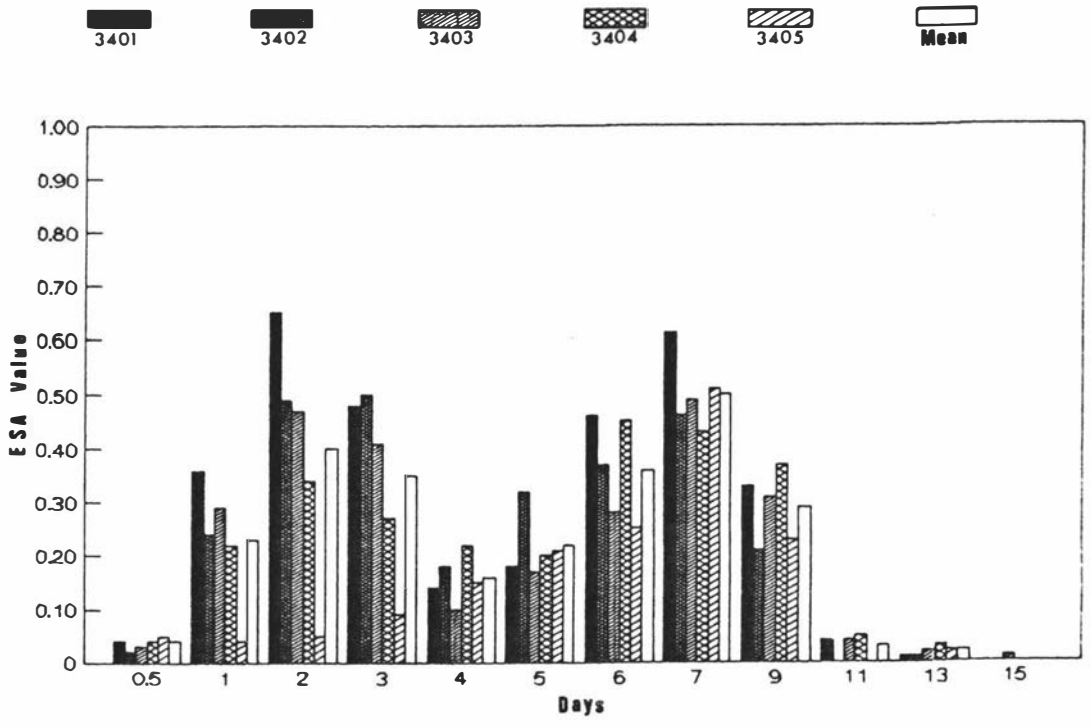

Fig. 6.1 **Detection of viral antigens in blood plasma collected from SPF birds following oral inoculation of 717B strain (serotype 8) of AAV at the age of 2 days.**

Viral antigen in tissues

The results of the detection of antigens by ELISA are summarised in Tables 6.1 and Figure 6.2. Data on individual birds are contained in Appendix VI. Viral antigens were demonstrated in duodenum, ileum, caecum and caecal tonsil in 2, 3, 4 and 4 birds, respectively, of the 5 which were sacrificed at 12 hr pi. Viral antigen was not detected in the colon until 24 hrs pi. The mean ESA values for ileum, caecum and caecal tonsil increased rapidly to 1.3, 1.4 and 1.6 respectively by 2 days and remained at high levels until day 9. They then declined to undetectable levels by 15 days except for 2 birds where low levels of antigen were still detected. High ESA values were recorded in the duodenum and colon from 2 days up to 7 days, but then declined sharply to undetectable levels by day 15. Viral antigen was detected in the cloaca between days 2 and 11 pi. Although the levels (mean ESA values 1.2 and 1.1) on the days 5 and 6 were quite high the overall concentration of viral antigen in the cloaca was less than that in other intestinal tissues. Viral antigens were detected in faeces between 2 and 15 days pi with a peak level (ESA 1.0) at day 6 (Fig. 6.2).

Viral antigens were first detected at very low levels in liver tissue at 2 days pi. The level rose to a peak by 6 days (mean ESA 0.70) and then decreased to become undetectable by 11 days (Table 6.1). Viral antigens were detected in the gall bladder between days 3 and 11 with a peak (mean ESA 0.41) at day 6.

Viral antigens were detected in the pancreas, bursa, oesophagus, trachea and spleen from 3-7, 2-9, 2-4, 2-5 and 4-7 days, respectively, (Table 6.1). Relatively low levels of viral antigens were detected in the proventriculus, bone marrow and thymus from 2-5, 4-6 and 3-6 days, respectively, after viral administration.

In the oesophagus, proventriculus, bone marrow and trachea, viral antigens were detected only in a few birds. Viral antigens were detected in the kidney of one bird at day 5 and one bird at day 6. Viral antigens were not detected in the brain, lung, muscle or pharynx.

Table 6.1 **Detection of viral antigens in different tissues by ELISA after oral administration of 717B strain (serotype 8) of AAV in chickens.**

Tissues examined	Duration of detection of viral antigens (day of peak level)	Peak ESA values (mean of 5 birds)
Duodenum	0.5-13 (6)	1.63
Ileum	0.5-15 (4)	1.79
Caecum	0.5-15 (4)	1.93
Caecal tonsil	0.5-15 (4)	1.97
Colon	1-13 (6)	1.90
Blood plasma	1-9 (2, 7)	0.40, 0.50
Cloaca	2-11 (5)	1.22
Bursa	2-9 (5)	0.33
Oesophagus	2-4 (2)	0.23
Proventriculus	2-5 (4)	0.15
Trachea	2-5 (4)	0.22
Liver	2-9 (6)	0.70
Pancreas	3-7 (5)	0.52
Thymus	3-6 (4)	0.10
Gall bladder	3-11 (6)	0.41
Spleen	4-7 (6)	0.26
Bone marrow	4-6 (4)	0.15
Kidney	5-6 (6)	0.09
Lung	-	-
Brain	-	-
Muscle	-	-
Pharynx	-	-

- = viral antigens not detected

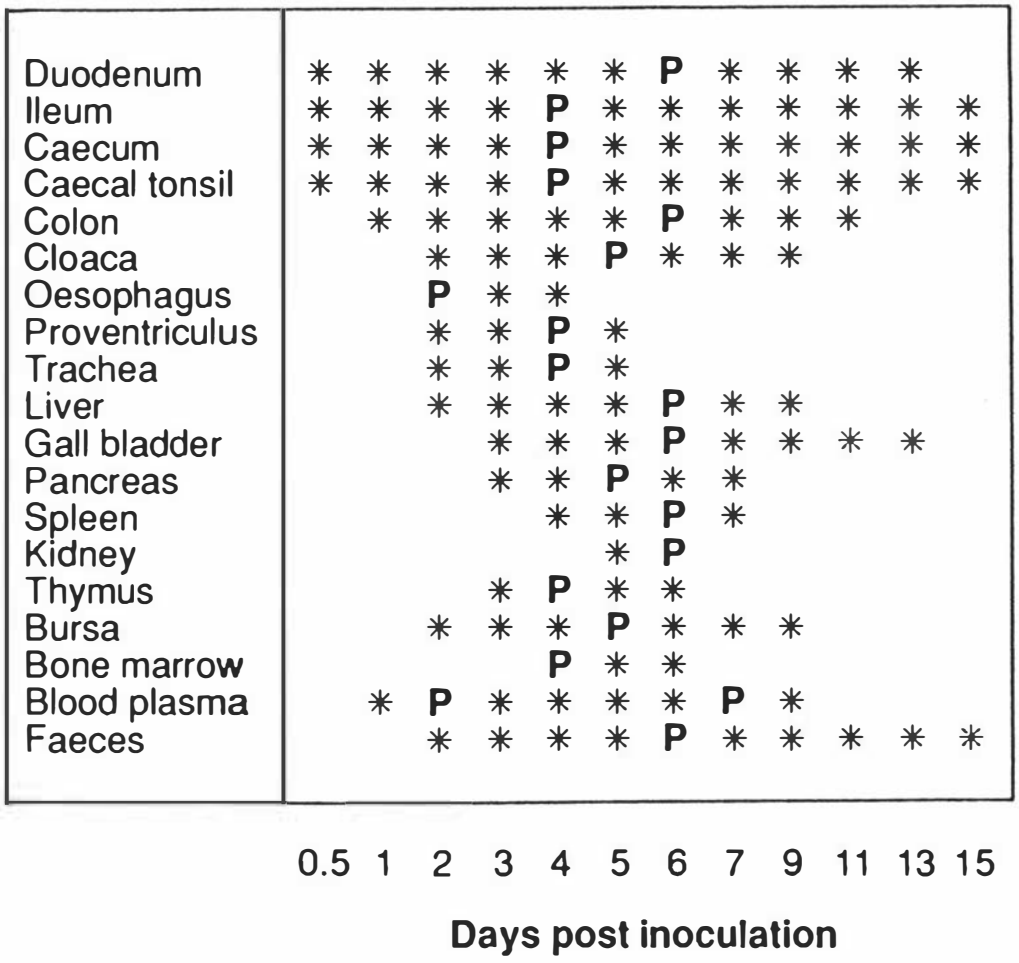

Fig. 6.2 **Adenoviral antigens in various tissues including blood and faeces following oral administration of 717B strain of AAV. ✳ represents the positive values (mean of 5 birds; ESA >0.06) and P the peak values.**

Immunocytochemistry

Viral antigens were detected in the epithelial cells of the intestinal villi from 12 hr to 13 days pi. Initial staining was confined to the surface of the epithelial cells (Fig. 6.3a) and was evenly distributed over the length of the villi but, by 24 hr pi, both nuclei and cytoplasm of the epithelial cells stained strongly (Fig. 6.3b). Many of the epithelial cells over the caecal tonsils stained strongly and small amounts of granular staining were observed in the subepithelial lymphoid tissue (Fig. 6.3c).

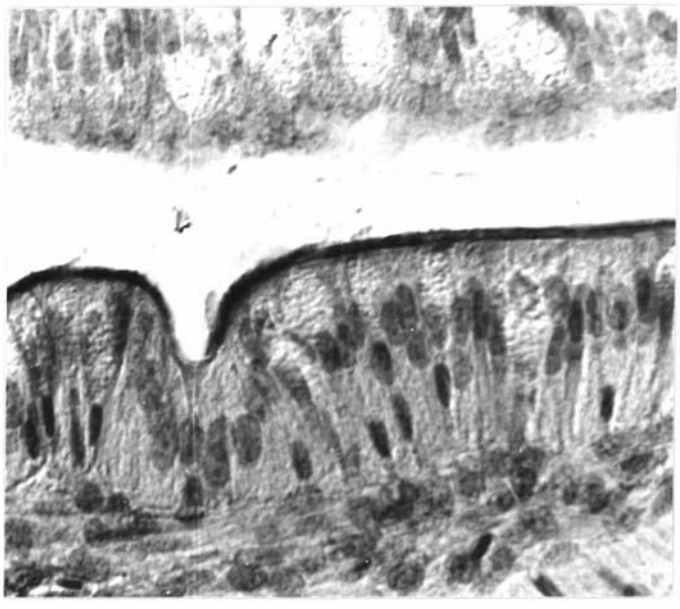

Fig. 6.3a **Immunoperoxidase staining of viral antigens in intestinal tissues of chickens following oral administration of serotype 8 avian adenovirus. Diffuse staining along the lumenal surface of the epithelial cells of an ileal villus (12 hours pi, haematoxylin counterstain, x20).**

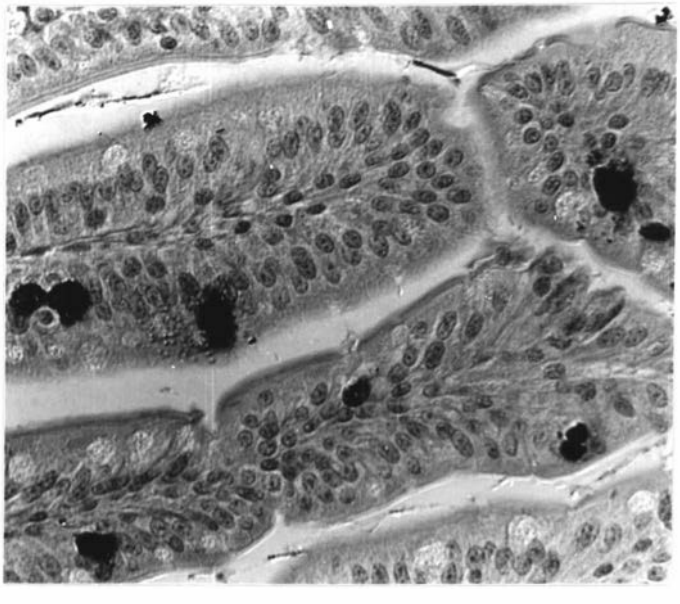

Fig. 6.3b **Dense staining in individual epithelial cells of intestinal villi (1 day pi, x20).**

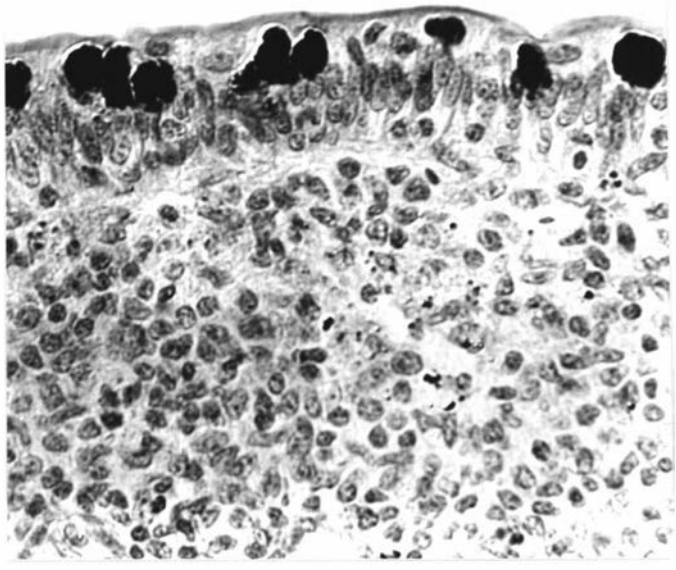

Fig. 6.3c **Dense staining in epithelial cells overlaying the caecal tonsil and small granules of stain within the lymphoid tissue (arrows) (3 days pi, x 40).**

At 2 days pi viral antigens were detected in cells, presumably Kupffer cells, lining the liver sinusoids (Fig. 6.3d). Viral antigens were not detected in hepatocytes until 3 days pi and it was apparent that hepatocytes adjacent to the sinusoids were the first to be involved (Figs. 6.3e and 6.3f). The number of cells in which antigens were detected increased up to 6 to 7 days pi when nuclei and cytoplasm of the hepatocytes were equally involved (Fig. 6.3g). At 9 days pi, antigens were not detected in the hepatocytes of those birds which had survived infection. Areas of lymphocytic and mononuclear cell infiltration were observed in the livers of these birds (Fig. 6.3h). Significant amounts of viral antigens were detected in the spleen both in the white and red pulps, and pancreas (mainly acinar cells) from 4 to 7 days pi. In the spleen, the staining was prominent in cells in the peri-arteriolar areas (Fig. 6.3i).

Only small amounts of antigens were detected in other tissues such as kidney, bursa and thymus and only from days 4 to 7 pi.

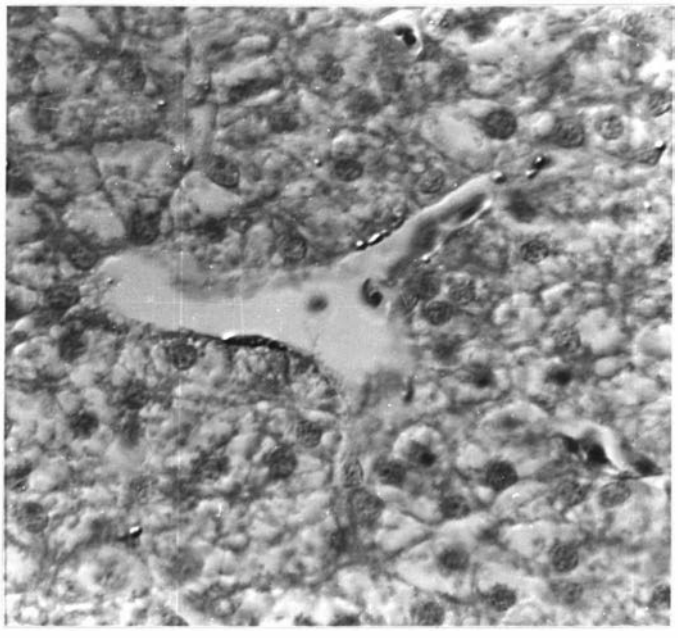

Fig. 6.3d **Discrete staining within cells lining liver sinusoids (presumably Kupffer cells) (2 days pi, x40).**

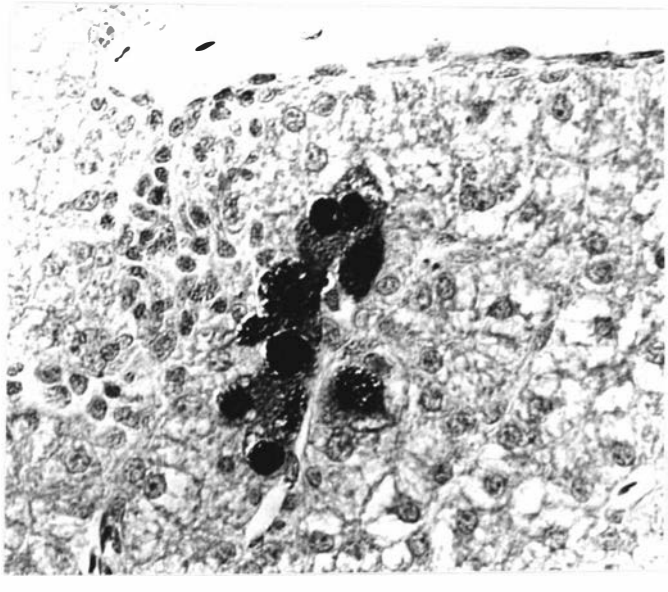

Fig. 6.3e **Dense staining of both nucleus and cytoplasm of hepatocytes. Stained hepatocytes are arranged linearly along a hepatic sinusoid (3 days pi, x20).**

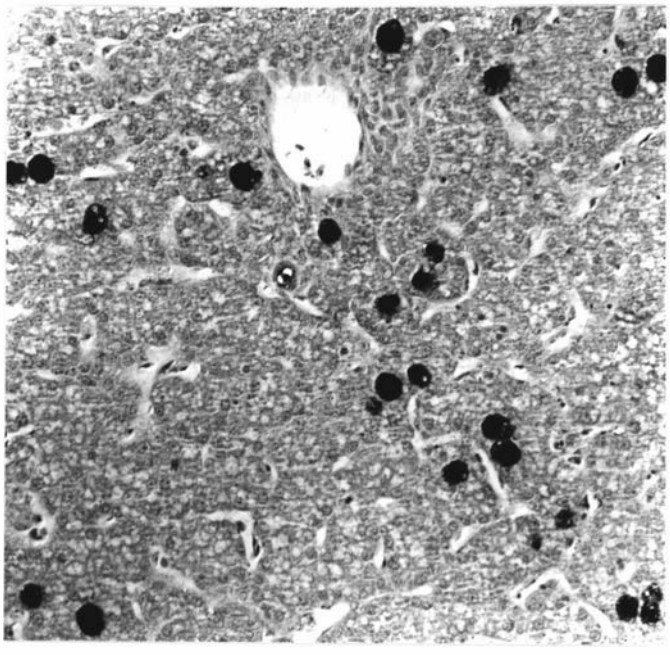

Fig. 6.3f **Extensive involvement of hepatocytes (4 days pi, x10).**

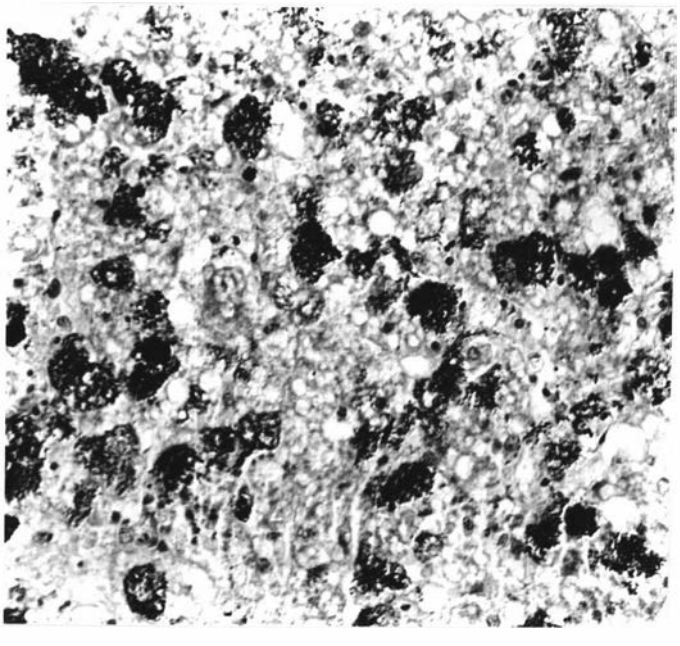

Fig. 6.3g **Staining of many hepatocytes (both nucleus and cytoplasm) in the liver of a chicken which died subsequent to inoculation (6 days pi, x20).**

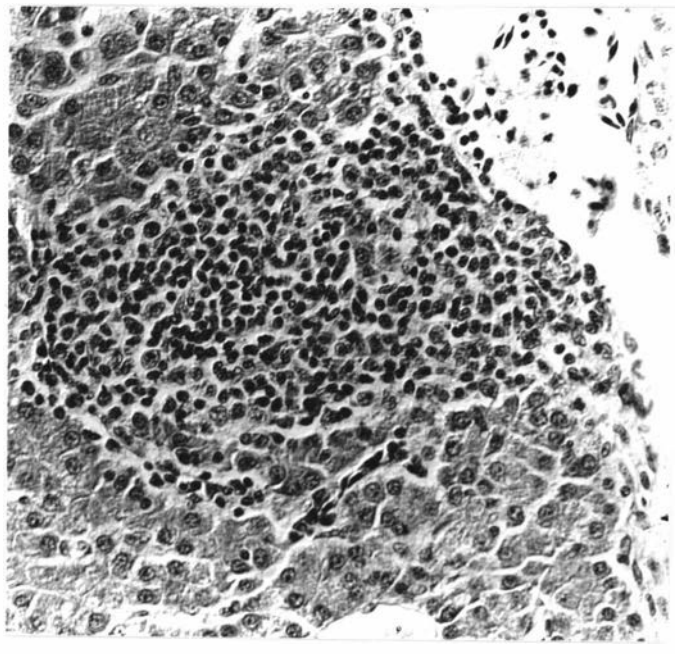

Fig. 6.3h **Infiltration of mononuclear cells in the liver of a chicken which survived infection (9 days pi, x20).**

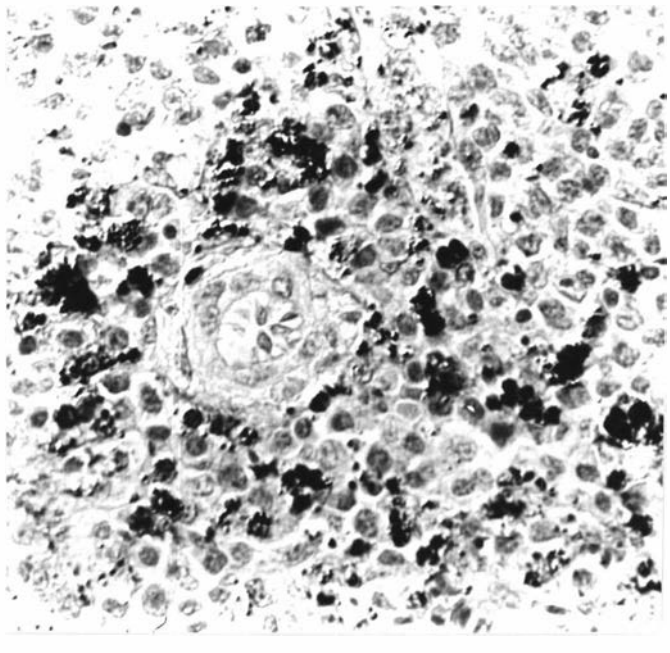

Fig. 6.3i **Dense staining of cells in the peri-arteriolar area of the spleen (5 days pi, x20)**

Antibody response

Prior to oral administration of virus, serum from all birds tested was negative (titre <10) for VN antibodies to AAV strain 717B (Appendix VI, Fig. 6.4). By 7 days pi, low levels of VN antibodies were detected in 4 of 5 birds where the GMT was 13. The mean titre rose steadily up to 1280 when the experiment was terminated at 15 days pi.

The relationship between the viral antigens in ileum, blood plasma and liver detected by ELISA and the appearance of VN antibodies is illustrated in Figure 6.4. The flow diagram in Figure 6.5 represents an hypothesis for the pathogenesis of IBH in the chicken.

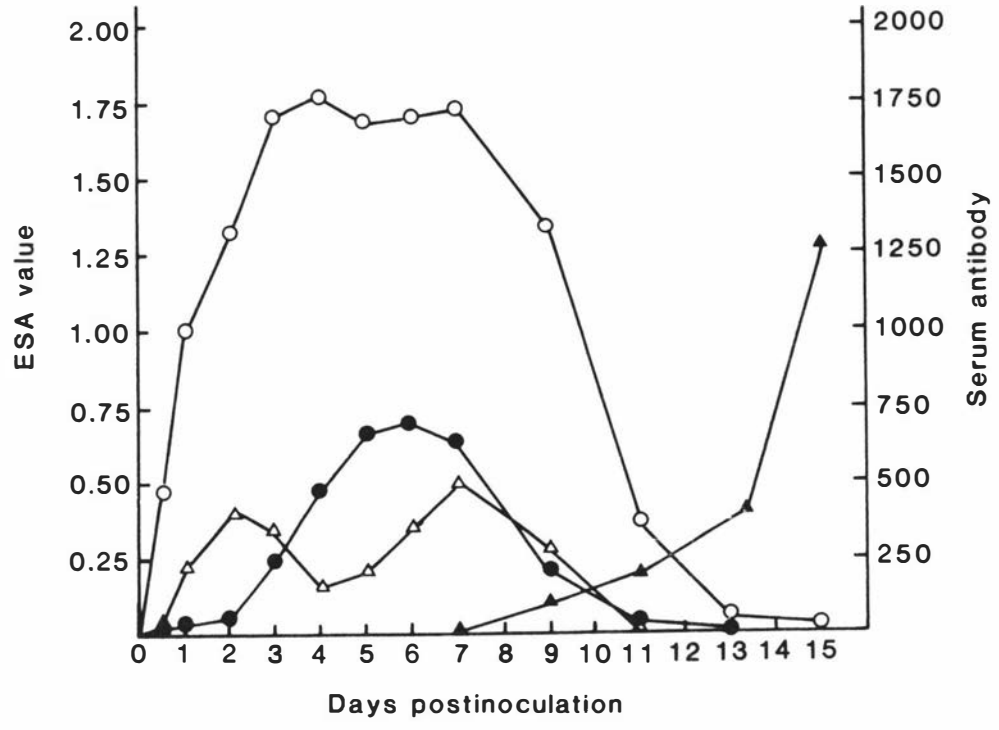

Fig. 6.4 **Detection of viral antigens in ileum (O----O), blood (△----△) and liver (●----●) by ELISA, and appearance of virus neutralizing antibodies (▲----▲) in serum of chickens following oral inoculation of serotype 8 avian adenovirus.**

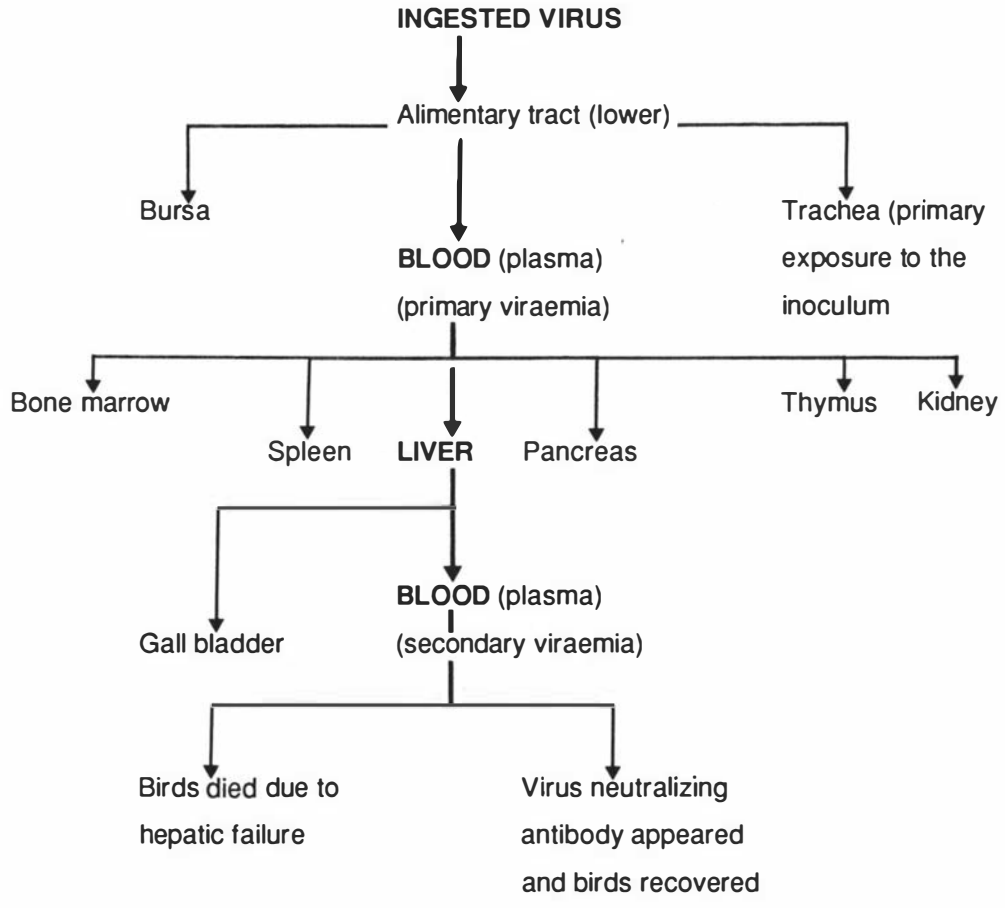

Fig. 6.5 **Schema of the spread of inclusion body hepatitis virus through the body based on qualitative and quantitative measurements of viral antigens by ELISA and avidin-biotin peroxidase complex techniques in various tissues following oral administration of locally isolated serotype 8 (strain 717B) avian adenovirus.**

Persistent infection

Viral antigens were intermittently detected in each organ (duodenum, ileum, caecum and caecal tonsil) up to 20 weeks pi when the experiment was terminated (Table 6.2). Significant amounts of viral antigens were also demonstrated in faeces up to 20 weeks pi. Overall, the amount of viral antigens detected was quite low except for a few birds. At 16 weeks pi, ESA values of 0.13, 0.16, 0.28, 0.26 and 0.29 were obtained with suspensions of duodenum, ileum, caecum, caecal tonsil and faeces, respectively, in 1 out of 2 birds. The antigen levels at 20 weeks pi were slightly lower than at 16 weeks pi.

Table 6.2 **Detection of viral antigens in various tissues and faeces of chickens by ELISA following oral administration of 717B strain (serotype 8) of AAV and recovery from clinical disease.**

Tissue	ESA values at weeks post inoculation							
	3	4	6	8	10	13	16	20
Duodenum	**0.09** *	0.05	0.04	0.05	**0.26**	**0.15**	0.02	**0.10**
	0.05	0.03	**0.10**	**0.09**	0.01	0.00	**0.13**	0.03
Ileum	**0.09**	**0.24**	**0.07**	0.01	**0.19**	**0.28**	0.03	**0.13**
	0.00	0.01	**0.11**	**0.12**	0.02	0.00	**0.16**	0.03
Caecum	**0.12**	**0.09**	**0.13**	0.02	**0.10**	**0.17**	**0.19**	**0.18**
	0.05	0.00	**0.15**	**0.14**	0.05	**0.12**	**0.28**	**0.08**
Caecal tonsil	0.02	0.03	0.00	0.05	**0.07**	**0.30**	0.05	**0.17**
	0.00	0.04	**0.11**	**0.07**	0.02	**0.13**	**0.26**	0.00
Faeces	0.05	**0.46**	0.04	**0.07**	0.01	**0.61**	**0.10**	**0.19**
	0.00	0.04	**0.12**	**1.17**	0.03	0.00	**0.29**	0.03

ESA - ELISA specific absorbance obtained by subtracting the absorbance with SPF tissue from that obtained with test tissue.

* Positive values (>0.06) are shown in bold type.

The VN antibody titres against 717B virus (serotype 8) were 320 to 10240 (Table 6.3). The antibody levels were slightly lower in those birds which were killed during 3 and 4 weeks pi than in the birds which were killed at 6-20 weeks pi.

Table 6.3 **Virus neutralizing (VN) antibody in chickens following oral administration of 717B strain (serotype 8) of AAV and recovery from clinical disease.**

Birds	VN antibody titres at weeks post inoculation							
	3	4	6	8	10	13	16	20
1	320 *	640	5120	2560	1280	640	5120	1280
2	1280	2560	1280	1280	10240	5120	2560	2560
GMT	640	1279	2559	1809	3618	1809	3618	1809

* Reciprocal of the highest dilution of serum which inhibited 100 $TCID_{50}$ of 717B virus.

DISCUSSION

In this study of the pathogenesis of IBH infection in SPF chickens, the level and the pattern of distribution of viral antigens in different organs at various times after the oral administration of a locally-isolated strain of serotype 8 AAV were detected by indirect ELISA and an immunocytochemical procedure, ABC. Persistent shedding of virus was also demonstrated by detecting AAV antigens in several intestinal tissues and faeces for a prolonged period following recovery from clinical IBH.

Acid-resistant viruses, such as adenoviruses, are capable of surviving passage through the stomach and infecting the intestinal epithelium (Fenner et al., 1987). In this study, viral antigens were detected in intestinal tissue 12 hr after the oral administration of virus. The intestinal tract was the first site that viral replication was detected in and high levels of antigen were detected for up to 9 days pi. Extensive damage to the intestinal epithelium with haemorrhage was observed by histological examination (results not shown). Also, after 3 days pi a large amount of cellular debris, which stained positively for viral antigens, was observed in the lumen of the intestine. Not surprisingly, viral antigens were detected in faeces between days 2 and 15 pi. This is similar to the findings of other workers who detected various strains of AAV in faeces from 2-20 days after inoculation (Kohn, 1962; Kawamura et al., 1963; Clemmer and Ichinose, 1968). Although Kawamura et al. (1963) demonstrated maximum titres of virus in trachea and faeces, Kohn (1962) considered the alimentary, rather than the respiratory tract, to be the main site of AAV replication.

Viral antigen was detected in blood plasma from 24 hr pi. It is presumed that virus replicating in the intestinal tract reached the blood stream via draining lymphatics and the thoracic duct(s). Chickens do not have discrete lymph nodes, as do mammals, but an irregular distribution of a rich, accumulative, diffuse lymphoid tissue (analogous to Peyer's patches) occurs along the alimentary tract from pharynx to the cloaca. This is in addition to the most concentrated gut associated lymphoid tissues which occur in the caecal tonsil and bursa of Fabricius (Toivanen et al., 1981; Toivanen and Toivanen, 1983). The chicken also has a system of lymphatic vessels that collect fluid from tissues and pass it first into a pair of thoracic ducts and then into the superior vena cava (Payne, 1971). By 2 days pi, peak levels of viral antigens were detected in blood and at low levels in the liver, pancreas, spleen, bone marrow and thymus by 2-4 days. Presumably, virus reached these sites via the circulation. It is likely that the phagocytic cells in the liver ingested the virus particles which were free in blood plasma and, subsequently, the virus multiplied to high titre in the hepatic cells by 4-7 days pi causing haemorrhage, necrosis and liver damage. This

suggestion is supported by the distribution of viral antigens that was revealed by the immunocytochemistry studies. A second, and higher, peak of viral antigen was detected in blood at 7 days and is probably the result of the release of virus from the damaged hepatic cells. This second, high level viraemia resulted in dissemination into many different organs including spleen, pancreas, thymus, bone marrow and, sometimes, kidney. A similar pattern of primary and secondary viraemias is also a feature of the pathogenesis of mousepox, measles and small pox (Fenner, 1948; Mims and White, 1984). The secondary viraemia in each case follows extensive replication of virus in one or more major body organ.

Low levels of viral antigens detected in oesophagus, proventriculus and trachea presumably reflect limited viral replication following primary exposure to the oral inoculum. Infection of the bursa initially occurred by extension along the intestinal tract and may have subsequently been contributed to by infectious virus from the blood stream. It is also assumed that the gall bladder infection was derived by extension from the liver, since virus was not detected in the gall bladder until 24 hr after virus was first detected in liver. Direct extension of infection from the intestine to the gall bladder may have also occurred in later stages. Cook (1983) demonstrated a wide range of distribution of AAV in different tissues, including blood, but did not demonstrate when a particular organ was first infected, the amount of virus in each organ or the pathway of virus spread throughout the body. Kawamura et al. (1963) also demonstrated viraemia at 4 days pi and a wide spread dissemination of virus in the body following serotype 1 (strain Ote) AAV infection.

The pathogenesis of IBH infection suggested here resembles that described for hepatitis A virus infection of man (White and Fenner, 1986). With hepatitis A infection, a cell-free viraemia also follows initial intestinal replication of the virus and passage, via draining lymphatics and the thoracic duct, to the blood stream. Infection of the liver is then by virus present in the blood. However, a second peak of viraemia has not been recognized with hepatitis A infection (Mims and White, 1984).

ICH virus, in contrast to the AAV associated with IBH infection, first multiplies in lymphoid cells in tonsillar crypts and Peyer's patches and is subsequently carried in infected cells to the local lymph nodes. Virus reaches the blood stream and a cell-associated viraemia spreads virus throughout the body to many organs, including the liver and kidney. ICH virus also replicates in the endothelial cells of blood vessels (Appel, 1987). Similarly, several other viruses such as rubella virus, lymphocytic choriomeningitis virus and cytomegalovirus have a cell-associated viraemic phase (Mims and White, 1984; White and Fenner, 1986).

A number of viruses, such as ND virus and poliovirus, are recognized which may initially replicate in the intestine and then gain access to the blood stream via draining lymphatics (Sabin, 1956; Kohn, 1959; Fenner et al., 1987). Poliovirus, for example, replicates in the lower intestinal tract, particularly in Peyer's patches, and thence passes via the lymphatics, mesenteric lymph nodes and thoracic duct to the blood stream where the resulting viraemia is also cell-free (Sabin, 1956; Mims, 1964). However, this is not the only means of spread of poliovirus and spread to regional nerve ganglia is important in the paralytic disease. A number of Togaviruses, Flaviviruses and Enteroviruses also have a cell-free viraemic phase. However, the two former groups are usually introduced into the host via arthropod vectors (Fenner et al., 1987).

Passage of viruses from the intestinal tract into the blood stream may be extremely rapid. In this present study, IBH virus was detected in blood plasma as early as 24 hr pi. Small particles, such as adenoviruses, may be taken up from the intestinal lumen by absorptive cells which utilize a tubulo-vacuolar apparatus (Worthington and Graney, 1972). This process is rapid and does not depend on the virus infecting and replicating within the cell. Such a process may have contributed to the early appearance of viraemia in these birds.

It is apparent from our results that the peak levels of viral antigens in most tissues were detected between 3 and 7 days pi. Also most deaths occur between 3 and 8 days pi, depending on the route of viral inoculation. Cook (1983) also found that the incubation period for IBH was from 3-8 days. During this period virus spreads to, and rapidly multiplies, in target organs including intestine and liver and produces clinical disease as a result of enteritis and necrotic hepatitis. Coincidentally with the appearance of circulating antibody, levels of viral antigen in the tissues rapidly decrease and the infected birds which have VN antibody after 7 days recover. The VN antibody was first detected in blood at 7 days and increased to high titres (GMT 422) by 13 days pi when viral antigens were undetectable in most organs. From the experiments described here, it appears that there is a direct relationship between appearance of VN antibodies and recovery from infection with IBH virus. This finding would be expected with any such viral infection that has cell-free viraemic phases.

Infectious hepatitis of chickens may be a useful model for similar viral diseases of other species, including man, and for evaluating prophylactic and therapeutic regimens.

Viral antigens were detected by ELISA in different intestinal tissues (duodenum, ileum, caecum and caecal tonsil) and faeces derived from 1 out of 2 birds until 20 weeks after oral administration of IBH virus (serotype 8). This is consistent with the findings of Ahmed

(1971) who was able to isolate adenovirus from the gall bladder and intestine of birds 93 weeks after infection. According to the present findings, viral antigens were also detected from the gall bladder after the recovery of birds from clinical IBH and the level of viral antigens in the gall bladder presumably added to the virus present in the intestine. In view of the resistance of AAV to physical and chemical agents it could be postulated that virus present in the litter was reinfecting the birds, producing the prolonged excretion pattern rather than this being due to persistent infection.

A high level of VN antibody (titres of 320 to 10240) was detected in all of the birds from 3-20 weeks pi. Some individual chickens may have both maternal antibody and still be carrying virus (McFerran, 1981b) in which case it may not be possible to isolate virus in cell culture but viral antigens may still be detected by ELISA. However, it is unknown if some isolates or serotypes can be shed over a prolonged period or whether a carrier state may be established in infected chickens (McFerran, 1981b). To answer the question of whether persistent infection or constant reinfection is the major cause of continued shedding of virus in faeces, it would be necessary to house infected chickens in such a way that contact with infected fomites was prevented. For example, housing on a wire mesh floor to avoid contact with faeces. Because the chickens in the present experiments were housed directly on the solid cage floor, the above results do not conclusively answer this important question. However, the results do show that infection with this virus in a commercial, floor-reared, flock would be quite capable of persisting in the flock for at least 20 weeks. While antibody titres were only measured in 2 chickens at each time interval from 3 to 20 weeks, there is an apparent trend for titres to rise to a peak, fall off to low levels and then rise again (Table 6.4). This pattern is consistent with periodic reinfection. To confirm this it would be necessary to repeat the experiment with a larger number of birds, and preferably to contrast the titres in birds housed on litter with those of birds housed on wire mesh flooring.

SUMMARY

The pathogenesis of IBH was studied following the oral inoculation of a locally-isolated serotype 8 AAV into 2-day-old SPF chickens. Viral antigens were detected in tissues at various times pi by ELISA and by immunocytochemistry. Viral antigens were detected in intestinal epithelium from 12 hr to 13 days pi and in the plasma fraction of blood by 24 hr pi. A biphasic, cell-free viraemia, with peaks at 2 and 7 days pi, was recorded. Viral antigens were first detected in the liver from 2 days and reached peak levels at 6 days pi. The second peak of viral antigens in blood plasma was probably due to release of virus from damaged hepatic cells. Initially, viral antigens in the liver were restricted to cells lining the sinusoids but increasing involvement of hepatocytes occurred with time. Only limited amounts of viral antigens were detected in other tissues. Following the appearance of VN antibodies in serum from 7 days pi, the levels of viral antigens in all tissues decreased to undetectable levels by 15 days pi.

This viral hepatitis of chickens is possibly a useful model for other viral infections where a cell-free viraemic phase is important for the spread of virus from primary sites to target organs, such as the liver.

After recovery from the clinical IBH, virus was still detected in most of the intestinal tissues and faeces until 20 weeks following viral administration.

CHAPTER SEVEN

MORPHOLOGICAL AND ANTIGENIC CHARACTERIZATION OF INCLUSION BODIES IN HEPATOCYTES OF CHICKENS WITH INCLUSION BODY HEPATITIS

INTRODUCTION

Hepatitis associated with INIBs was first identified by Helmboldt and Frazier (1963) in broiler chickens in the United States and since then this condition has been known as IBH. Different types of inclusion bodies have been identified in infected liver tissue depending on their staining characteristics, size, morphology and composition (Kawamura et al., 1964; Maeda et al., 1967; Adair et al., 1979) but their significance is not clearly known. McFerran (1981a) reported that eosinophilic INIBs were consistently found in naturally infected liver and basophilic INIBs in the experimental cases. But basophilic inclusion bodies have also been identified in naturally infected chickens (Itakura et al., 1974; Reece et al., 1986a) and eosinophilic inclusion bodies in those experimentally infected (Winterfield et al., 1973; Cook, 1974). Natural outbreaks of IBH in New Zealand were associated mainly with eosinophilic inclusion bodies (Christensen and Saifuddin, 1989). A list of previous reports of different types of inclusion bodies found in liver tissue of chickens following natural and experimental infections of IBH is shown in Table 7.

This study describes the characteristics of inclusion bodies produced in chickens following inoculation of a locally isolated serotype 8 AAV.

Table 7. **Previous reports of inclusion bodies in the hepatocytes of chickens infected with inclusion body hepatitis virus.**

Natural	Experimental	Reference
Eosinophilic *	NE	Howell et al. (1970)
Basophilic	NE	Young et al. (1972)
Basophilic and eosinophilic	NE	Itakura et al. (1974)
Eosinophilic	NE	Hoffmann et al. (1975)
Eosinophilic and basophilic	NE	McFerran et al. (1976b)
Basophilic	NE	Ivanics and Ratz (1983)
Basophilic, eosinophilic and amphophilic	NE	Bergmann (1978)
Basophilic and eosinophilic	NE	Reece et al. (1986a)
Eosinophilic	Basophilic	MacPherson et al. (1974)
Basophilic	Basophilic	Reece et al. (1987)
NE	Basophilic	McCracken et al. (1976)
NE	Basophilic	Kawamura and Horiuchi (1964)
NE	Basophilic	McDougall and Peter (1974)
NE	Eosinophilic	Fadly and Winterfield (1973)
Eosinophilic	Eosinophilic	Gallina et al. (1973)
NE	Eosinophilic	Winterfield et al. (1973)
NE	Eosinophilic	Cook (1974)

* Results obtained by histological examination following haematoxylin and eosin staining.
NE = Not examined.

MATERIALS AND METHODS

Virus

A strain of serotype 8 AAV isolated from natural outbreaks of IBH in New Zealand (strain 717B) was used in this experiment.

Inclusion bodies in cell culture

CKC monolayer cultures grown on coverslips inside Leighton tubes were infected with virus and at 12, 24 and 36 hr pi were fixed in Bouin's fluid for 15 hr before being stained with HE for microscopic observations.

Inclusion bodies in experimentally infected liver tissue

Liver tissue was collected from each of 2 SPF birds at 3, 4 and 5 days after they had received 500 ul of a suspension of the field isolate of AAV (10^6 $TCID_{50}$) orally at the age of 2 days. Pieces of tissue, not larger than 2 cm^2 x 4 mm, were fixed in 10% buffered formalin at room temperature. After 24 hr, a portion of the tissue was embedded in paraffin and used for histological and immunocytochemical examination. After 10 days, another portion of the tissue was embedded in London Resin (LR)-white (Polaron Equipment Ltd. UK) and used for EM examination. Liver tissue was collected from non-infected, SPF birds and similarly processed for negative controls.

a) HE staining

Three micron-thick sections were cut and stained with HE following standard procedures before being examined by light microscopy. The morphology and staining characteristics of the inclusion bodies were recorded.

b) Immunocytochemistry

Five micron-thick sections were cut and stained using an ABC technique following the procedure described in Chapter 5.

c) Antigenic identity of the inclusion bodies

Five micron-thick sections of the liver tissues collected at 4 days pi, were stained with HE. A few areas in each section containing inclusion bodies were located and the positions of the sections were accurately marked by using scales on the microscope before photomicrographs were taken. The coverslips from the HE stained sections were removed by leaving the slides overnight in xylene. The same sections were then restained with the standardized ABC technique using rabbit antibody to the field isolate of virus. The previously identified areas containing the inclusion bodies on each section were relocated and again photomicrographs were taken.

d) Electron microscopy

Pieces (1-3 mm cube) of formalin-fixed liver tissue collected at 4 days pi, were processed in an automated tissue processor (LYNX el, Biomedical Corporation Ltd. Melbourne, Australia) through a series of ethyl alcohol, uranyl acetate and LR-white [25% alcohol 30 min, 50% alcohol 30 min, 1% uranyl acetate in 75% alcohol 2 hr, absolute alcohol 30 min, absolute alcohol and LR-white (1:1) 30 min and LR-white 2, 12 and 2 hr] at room temperature. Each piece of tissue was individually embedded in LR-white inside a gelatin capsule and allowed to polymerize for 24 hr at 60^{o}C. Seventy nanometer-thick sections were cut (Ultracut E, Reichert-Jung, Austria), and mounted on a copper grid.

The sections were stained with saturated uranyl acetate (in 50% ethyl alcohol) for 6 min and thoroughly washed with 50% ethyl alcohol (3 changes) and distilled water (3 changes). The grid was then placed in a lead citrate solution (0.25%) for 6 min and washed with distilled water (3 changes) before being examined by transmission EM.

Inclusion bodies in cell culture

Polymorphic INIBs were found in infected CKC cultures stained by HE, and were mainly basophilic and surrounded by a clear halo (Fig. 7.1). The nuclear chromatin was marginated at the nuclear membrane and sometimes more than one inclusion body was found in the same nucleus. The nuclei containing inclusion bodies were very much larger than the apparently healthy nuclei. The inclusion bodies at 36 hr pi were comparatively more densely stained than those observed earlier.

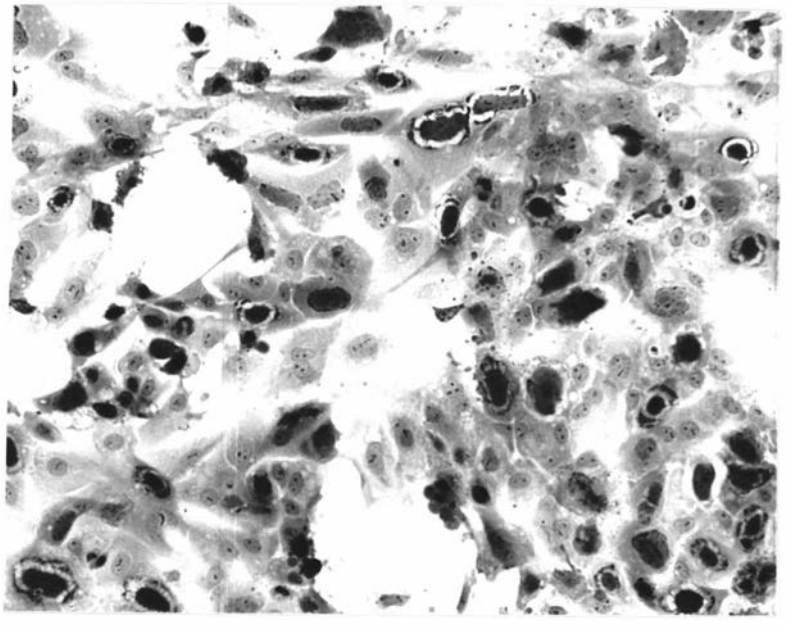

Fig. 7.1 **Basophilic inclusion bodies with clear halo in chicken kidney cells infected with a locally isolated serotype 8 avian adenovirus at 24 hr pi (haematoxylin and eosin, x20).**

Inclusion bodies in livers of experimentally infected birds

a) HE staining

A large number of basophilic INIBs were observed during 4 and 5 days pi in the hepatocytes of clinically sick or moribund birds (Fig. 7.2a). The nuclei were enlarged and each was fully occupied by the inclusion body. A surrounding halo was not prominent with such inclusion bodies. A small number of eosinophilic and amphophilic inclusion bodies which were surrounded by a clear halo were also observed. These nuclei were equally enlarged and the nuclear membrane was always obvious.

b) Immunocytochemistry

A large number of densely stained, dark brown discrete areas were observed in the sections of infected liver tissue which were collected at 4 days pi. The distribution of these areas was somewhat similar to that of the inclusion bodies found in HE stained sections but they were slightly larger in size and greater in number than the basophilic inclusion bodies (Fig. 7.2b). The nuclear structure was not visible in the densely stained areas. A smaller number of cells were stained at 3 days pi than at 4 and 5 days pi. At 5 days pi, the cells were more diffusely stained (i.e. nucleus and cytoplasm) than at 3 and 4 days pi. In non-infected liver tissue, discrete, brown staining was not observed.

c) Antigenic identity of the inclusion bodies

The number of sites stained by the ABC technique was greater than the number of inclusion bodies observed in the same sections stained by HE. A group of inclusion bodies in an HE stained section and their respective sites in the same section following ABC staining are shown in Figures 7.2a and 7.2b. The sites of all of the basophilic INIBs in the HE stained sections coincided with the respective sites which were densely stained following the immunocytochemical procedure. Some of the nuclei which contained eosinophilic inclusion bodies, and a few nuclei without any visible inclusion bodies, were also specifically stained by the ABC technique. These latter nuclei appeared to be larger than the nuclei of most of the other hepatocytes.

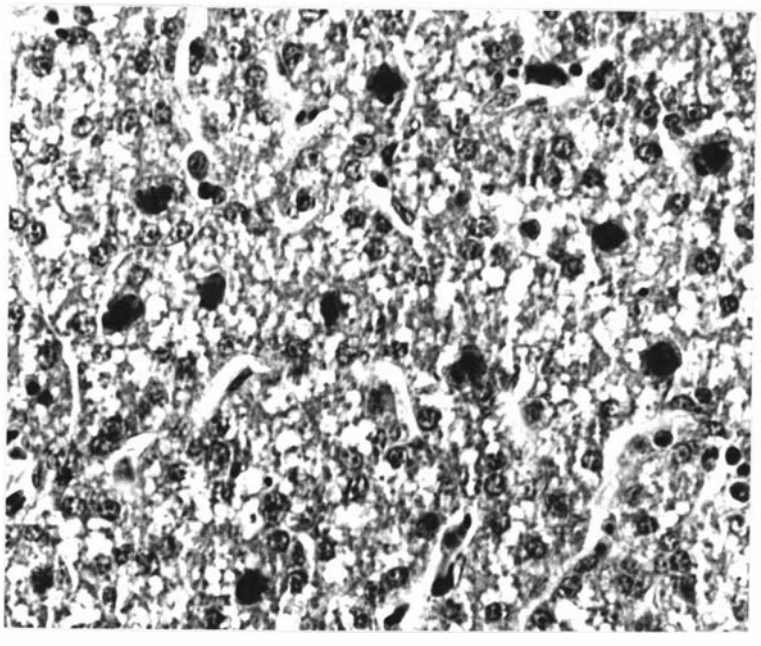

Fig. 7.2a **Basophilic inclusions in the liver tissue derived from an experimentally infected chicken at 4 days pi (haematoxylin and eosin, x20).**

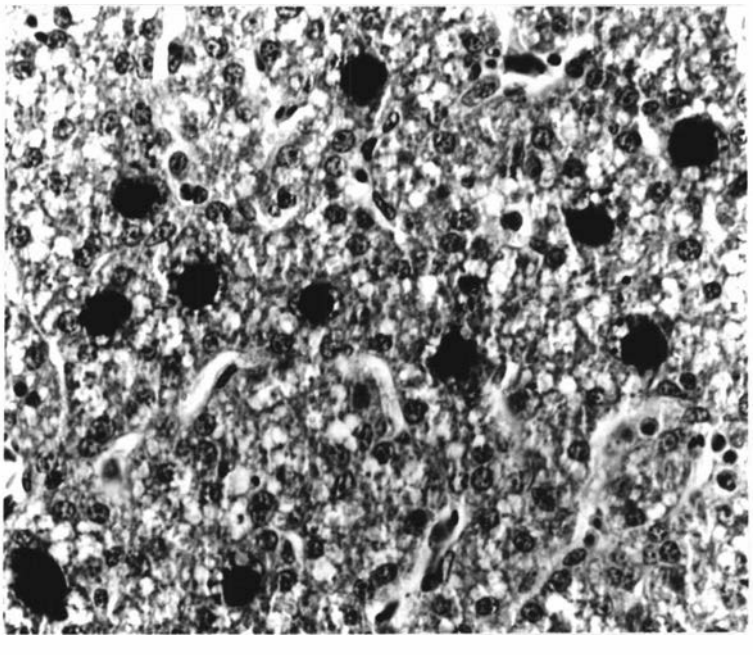

Fig. 7.2b **Same liver section stained by avidin-biotin peroxidase complex procedure (haematoxylin counterstain, x20). Densely stained areas corresponded to the inclusion bodies shown in Fig. 7.2a.**

d) Electron microscopy

Many enlarged nuclei with areas approximately 4 times that of the normal appearing nuclei were observed (Fig. 7.3a). Most of the enlarged nuclei contained many hexagonal particles arranged in a crystalline pattern (Fig. 7.3b). Some of the nuclei were quite enlarged but did not contain any complete virus particles (Fig. 7.4a and b). The nuclear chromatin was distinctly marginated at the nuclear membrane and aggregates of granular material were apparent. A smaller number of a third type of enlarged nucleus was observed (Fig. 7.5). Most of these nuclei contained a dense clump of material, resembling chromatin, and the remainder of the nucleus contained sparsely distributed, granular material.

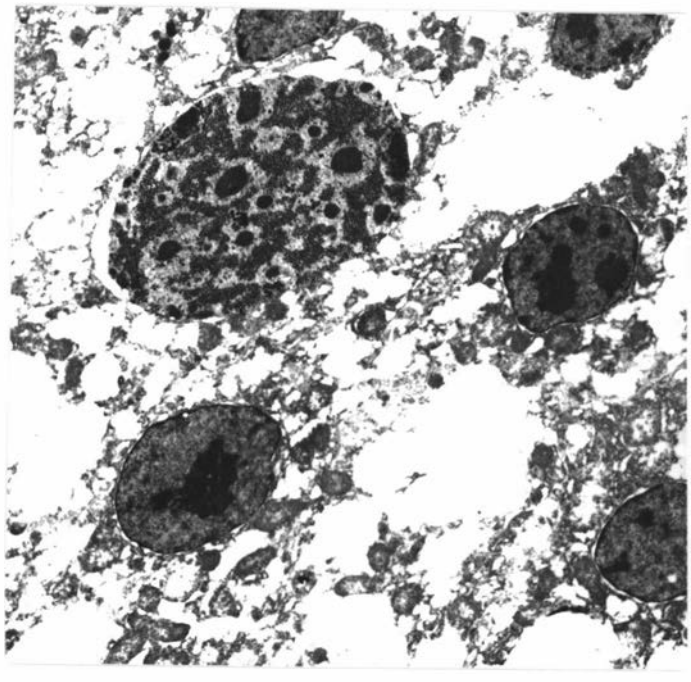

Fig. 7.3a **One enlarged and several apparently normal nuclei in the hepatocytes of a bird infected with IBH virus (EM).**

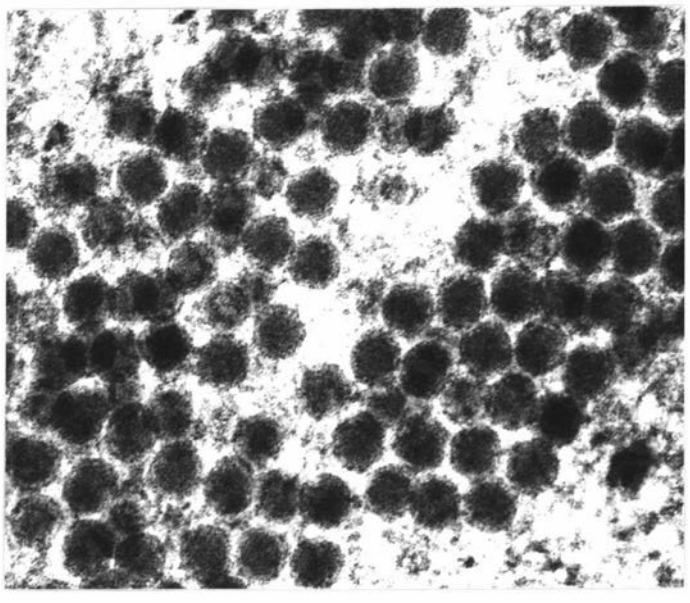

Fig. 7.3b **Hexagonal adenoviral particles arranged in a crystalline array in the enlarged nucleus (EM). This nucleus is more likely to contain a basophilic inclusion.**

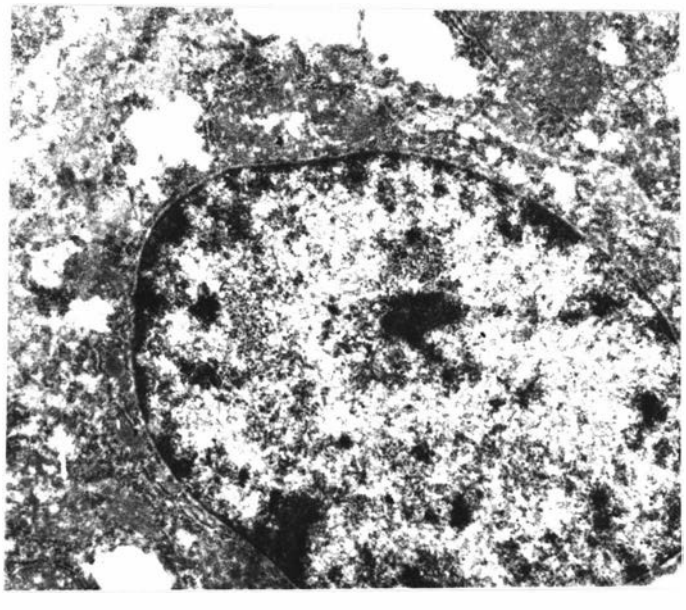

Fig. 7.4a **Margination of nuclear chromatin (arrow) and aggregation of granular materials in the enlarged nucleus but no complete viral particles apparent (EM). Most probably this nucleus contained an eosinophilic type of inclusion.**

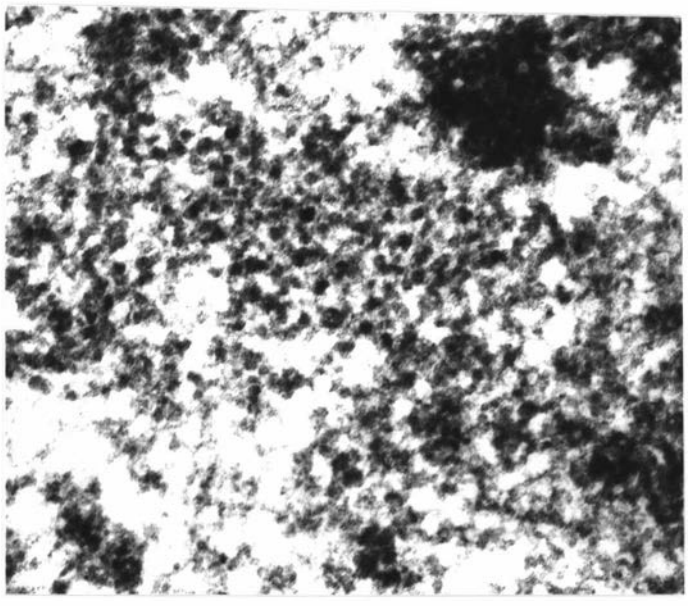

Fig. 7.4b **Higher power view of granular aggregate in the nucleus shown in Fig. 7.4a.**

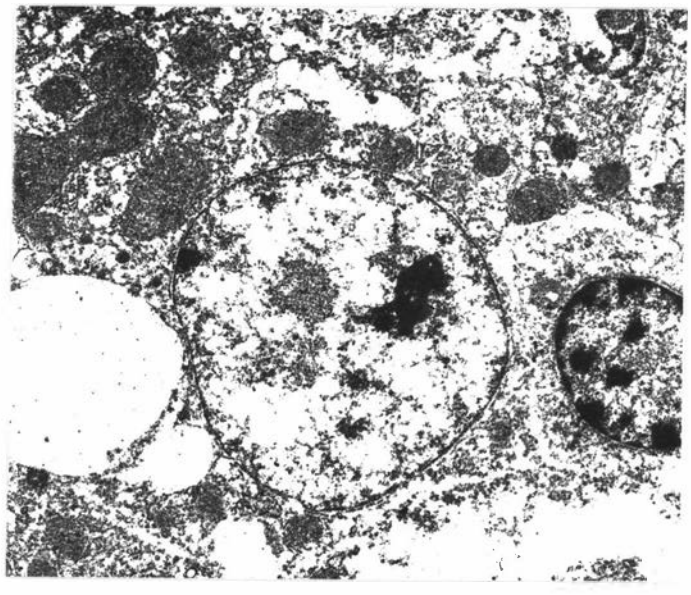

Fig. 7.5 **Enlarged nucleus containing a dense clump of chromatin material (arrow) in the hepatocytes (EM). No viral particles or extensive margination of nuclear chromatin is apparent. This probably represents a third type of nucleus where no obvious inclusion was detected by haematoxylin and eosin staining.**

DISCUSSION

INIBs were observed following HE staining, in CKC by 12 hr pi but by 36 hr they became more obvious. Presumably, viral materials continuously accumulate in the nuclei following replication to form inclusion bodies. Basophilic inclusion bodies predominated in cell cultures, as also reported by Adair (1978). The distinct halo formation surrounding these basophilic inclusion bodies in CKC and the margination of the nuclear chromatin resembles, morphologically, the eosinophilic inclusion bodies observed in the hepatocytes of naturally infected birds by Howell et al. (1970), McFerran et al. (1976b) and MacPherson et al. (1974). Adair (1978) has reported that both eosinophilic inclusion bodies, filling the entire nucleus, as well as centrally located, basophilic inclusion bodies, with clear halos, were present in cell cultures infected with different strains of a single serotype of AAV such as serotype 8.

Large numbers of hepatocytes contained either eosinophilic or basophilic inclusion bodies in their nuclei by 4-5 days pi. The significance of the different types of inclusion bodies is still obscure. In addition to any real differences in the nature of inclusion bodies, it is also necessary to be aware that fixation and staining procedures, which may vary markedly between different laboratories, may affect the staining characteristics of inclusion bodies. Experimentally induced IBH has often been associated with basophilic inclusion bodies in the hepatocytes, whereas field cases more commonly have eosinophilic inclusion bodies (McFerran and Adair, 1977). However, Reece et al. (1986a) reported that basophilic INIBs were commonly found in several naturally infected broiler flocks in Australia. In subsequent experimental infections with AAV isolated from these outbreaks, they also found basophilic inclusion bodies in the hepatocytes (Reece et al., 1987). In natural outbreaks of IBH in New Zealand, mainly eosinophilic inclusion bodies were observed (Christensen and Saifuddin, 1989) whereas, in experimentally infected birds the inclusion bodies were mainly basophilic. Eosinophilic inclusion bodies have also been found in experimentally infected birds (Fadly and Winterfield, 1973; Gallina et al., 1973; Winterfield et al., 1973; Cook, 1974).

The results of the immunocytochemical studies clearly show that cells with basophilic inclusion bodies consistently contain adenoviral antigens. This is consistent with the findings of Itakura et al. (1977) who found that nuclei with basophilic inclusions contained large numbers of viral particles. While many eosinophilic inclusion bodies were stained strongly by the immunocytochemical technique there was a small number which did not stain. Ultrastructural examination of cells with eosinophilic inclusions (Itakura et al., 1977) has revealed fibrillar-granular material, occasionally present as crystals. While some such

cells may contain virus-coded material it was suggested (Itakura et al., 1977) that others represented degenerating nuclear material present in cells dying from the effects of viral infection. It would therefore be expected that the former would stain positively for viral antigens while the latter would not. A third type of cell stained positively in sections of infected liver. These were cells with markedly enlarged nuclei but with no evidence of inclusion body formation in HE stained preparations. It is possible that these represent cells in the very early stages of viral infection, where early viral proteins have been expressed but not all of the later structural proteins. Whether or not such nuclei stain positively would then be dependent on the specificity of the antiserum. An antiserum raised against purified virions would not be expected to react with all early proteins.

Several studies agree that basophilic inclusion bodies generally contain abundant adenovirus-like particles (Itakura et al., 1977; Bergmann, 1978; Ivanics and Ratz, 1983, Reece et al., 1986a) while eosinophilic inclusion bodies generally lack virus particles (Itakura et al., 1977; Bergmann, 1978) and such eosinophilic inclusion bodies probably represent debris remaining after viruses have left the nucleus (Grundmann, 1966). These empty inclusions are considered to be the result of nuclear degeneration and are usually free of DNA. Consequently, the basophilia of nuclei gradually diminishes until the nucleus is filled only by a compact eosinophilic inclusion containing fibrillo-granular material without viral particles. Ivanics and Ratz (1983) proposed that there are 2 forms of IBH characterized by either basophilic or eosinophilic inclusions. Bergmann (1978), however, observed three different types of inclusion bodies, namely, basophilic, eosinophilic and amphophilic in liver tissues from different flocks naturally infected with IBH. Only the basophilic inclusions contained adenovirus particles. Both basophilic and eosinophilic inclusion bodies were reported in birds in the same affected Victorian (Australian) flocks, although both types were rarely observed in the liver of the same bird (Reece et al., 1986a). By contrast, both basophilic and eosinophilic INIBs were observed in the same liver section derived from natural outbreaks of IBH in pigeons (McFerran et al., 1976b). They also found basophilic inclusions, with and without clear haloes, in the same liver sections. Adair (1978) noted that different strains of AAV, even though belonging to the same serotype, can produce different types of inclusion bodies in tissue culture, but it may not be correct to assume that the dynamics of inclusion body formation in cell culture closely resemble that seen in the hepatocytes of infected birds.

Three types of enlarged nuclei could be distinguished by EM. The areas of these nuclei were similar to each other and about four times larger than apparently normal nuclei in the same field. The first type contained a large number of hexagonal particles, resembling adenovirus, in regular arrays. It is believed that these nuclei correspond to those with

basophilic inclusions seen by light microscopy. The second type was characterized by margination of chromatin at the nuclear membrane and aggregations of granular material. This type seemed most likely to correspond to nuclei with eosinophilic inclusion bodies. The third type, which did not have distinct margination of chromatin but often contained a dense clump of material, presumably chromatin, as well as finely dispersed granular material, possibly correspond to those nuclei which stained positively in the immunohistochemical procedure but did not have an obvious inclusion body. It is not known if these represent cells very early in infection or end stage cells after viral replication is complete and the progeny virions have left the cell.

SUMMARY

The characteristics of inclusion bodies produced in chickens following inoculation of a locally isolated serotype 8 AAV were studied by light and electron microscopy and by immunocytochemical staining. Predominantly basophilic, and a smaller number of eosinophilic and amphophilic inclusions, were demonstrated by HE staining. AAV antigens were detected by the immunocytochemical technique in all the basophilic and most of the eosinophilic inclusion bodies and also in a few nuclei where inclusion bodies were not obvious. Clusters of adenoviral particles were identified by EM examination in a number of markedly enlarged nuclei in formalin-fixed and resin-embedded liver tissues.

CHAPTER EIGHT

GENERAL DISCUSSION

In 1987, outbreaks of disease, resulting in up to 30% mortality, occurred in 3-7-week-old broiler flocks in New Zealand. The clinical features included sudden rise in mortality rates, decrease in food intake, reluctance to move, prostration and eventual death. Post mortem examination revealed diffuse haemorrhage, necrosis and degeneration of the liver associated with INIBs in the hepatocytes, pallor of the bone marrow, anaemia, jaundice and atrophy of bursa and thymus with lymphocytic depletion. On the basis of the histopathological findings, particularly the changes in the liver, the disease was believed to be IBH. Occasionally the outbreaks were associated with necrotic enteritis and *Clostridium sp.* was identified in the intestinal tissues of affected birds. However, treatment with oxytetracycline, furaltadone and furazolidone had no effect on the course of the disease in flocks in which the signs were of the haemmorrhagic-anaemic and hepatitis type. CAA was also suspected to be involved in some of the outbreaks.

Attempts were made to isolate viruses from the tissues of affected birds using CKC and MDCC-MSB1 cell line cultures and avian embryos. Also serum samples from several clinically affected and normal broiler and breeder flocks were collected for serological investigation. Adenovirus was isolated from liver, bursa and intestinal tissues collected from several clinically affected broiler flocks. Viruses were identified as AAV by their relative stability in chloroform and at various temperatures, characteristic morphology revealed by EM examination and antigenic relationships shown by cross-neutralization tests using 12 prototype strains of AAV. No other agents, including CAA, were isolated in these samples. The isolation of adenoviruses was not surprising as they have frequently been isolated from normal as well as diseased chickens (Yates and Fry, 1957; Green et al., 1976). Since the first report of IBH in 1963 (Helmboldt and Frazier, 1963) in broiler flocks in the United States, there has been speculation about the aetiological role of the AAVs in IBH in chickens. It has been suggested that AAVs can only cause clinical IBH if the birds are concurrently exposed to one of the immunosuppressive agents or factors (Rosenberger et al., 1973; Fadly et al., 1976; Winterfield, 1984). The IBD virus, which causes profound immunosuppression in chickens, is often considered responsible for many outbreaks of IBH. However, New Zealand's freedom from this commonly suspected immunosuppressive agent provided the necessary impetus to carry out the next steps of this study. Important questions included the possible origin of these AAVs and whether they were capable of

producing IBH in commercial broiler chickens in their own right.

A mild form of IBH was reported in New Zealand in 1977 (Bains and Watson, 1977), so one possibility was that, due to changes in the viruses or the immune status of the birds, IBH had re-emerged as a much more severe disease. Also a number of outbreaks of IBH in broiler flocks in Australia, unrelated to prior infection with IBD virus, had been reported in 1985. A further possibility was that the virus responsible for those outbreaks might have crossed over to New Zealand. This raised further questions. How could such transmission happen and are the New Zealand viruses closely related to the Australian isolates?

Most of the viral isolates recovered from the severely affected flocks were identified by VN tests as serotype 8 AAV and VN antibodies to serotype 8 were demonstrated in the broiler and breeder flocks which had recovered from the disease. It was interesting to note that serotype 8 AAVs were also predominantly involved with the outbreaks of IBH in the Australian flocks. Antibody to IBD virus, which was commonly demonstrated in Australian flocks, was not detected in any of the flocks tested in New Zealand. In this situation, the next question was how to determine whether Australian and New Zealand isolates of serotype 8 viruses are similar? It was decided to analyse these viruses at the genomic level using restriction endonucleases. The DNAs of two New Zealand isolates, 717B and 716W and three Australian isolates, QLD, VRI and WA, all recovered from severe outbreaks of IBH, and the reference strain (HVI) of serotype 8 AAV were analysed by the restriction endonuclease fingerprinting technique. The New Zealand isolates were found to be similar to each other and obviously different from the Australian isolates in the number and position of the genomic fragments generated by EcoRI and BamHI digestion. The Australian isolates were also found to be similar to each other while the reference strain HVI was somewhat similar to the New Zealand isolates. Evidence here suggests that the New Zealand viruses were not recently derived from Australia. DNA sequencing of both virulent and nonvirulent strains of AAV is necessary to determine why serotype 8 viruses are not always associated with clinical IBH.

To determine whether New Zealand isolates of AAV from field outbreaks of IBH were capable of producing IBH, one isolate (717B) was administered orally to 2-day-old conventionally raised broiler chickens. Fatal hepatitis resembling IBH was reproduced in 30% of the birds by 3-8 days after administration of virus (Saifuddin and Wilks, 1990). Large numbers of basophilic and occasional eosinophilic INIBs were identified by histological examination in the hepatocytes of clinically affected birds. Severe growth depression was recorded in the survivors which was similar to the findings of Cook (1983) and Grimes and King (1977b). The same serotype of virus was recovered in SPF CKC from

liver suspensions of all the birds which died after experimental infection.

Once it was known that the New Zealand isolate 717B was highly pathogenic in its own right, research was continued to explore some other important areas such as the pathogenesis and certain epidemiological aspects of IBH caused by this strain of AAV.

As it is often not possible to titrate the amount of infectious virus in tissues of infected birds there was a need to develop an assay which permitted ready detection and quantification of virus in a range of tissues at various times after infection. An ELISA to detect viral antigens seemed most likely to satisfy this need. An highly sensitive ELISA was developed to detect and quantify AAV in various tissues including blood (Saifuddin and Wilks, 1990). A positive EA value was obtained with an infected liver suspension containing less than 100 $TCID_{50}$ of infectious virus per gram. Positive reactions were also obtained with material infected with any serotype of AAV but never with uninfected materials. This assay was therefore suitable, not only for the study of pathogenesis, but also for laboratory diagnosis of IBH irrespective of the serotypes involved, and for surveying commercial and SPF flocks.

However, an ELISA does not reveal the precise site of viral replication within a particular tissue so it has limitations for studies of viral pathogenesis. An immunocytochemical technique utilizing ABC was considered suitable for monitoring the effects of host-virus interactions at the cellular level in various tissues. The ABC technique was developed to localize AAV antigens in paraffin embedded infected tissue sections. A clearly distinguishable colour reaction was obtained with infected tissues which contained levels of viral antigens similar to the minimum level detected by ELISA. Both in the ELISA and the ABC techniques, a group-specific antigen common to the 12 serotypes of AAV was demonstrated.

Very little is known about the pathogenesis of IBH infection following exposure to virus by a natural route. The pathogenesis of IBH was studied in SPF chickens and the level and the pattern of viral spread in body organs was detected by an ELISA and an immunocyto-chemical procedure at various times after oral administration of the 717B isolate of AAV. The intestinal epithelium was found to be the first site of viral replication and a high level of viral antigens was detected in the intestinal tract by 24 hr and subsequently in liver by 2 days pi. Appearance of viral antigens in the liver followed a high titred, cell free viraemic phase. It is proposed that the phagocytic cells in the liver ingested the virus particles which were free in blood plasma and, subsequently, the virus multiplied to a high titre in the hepatic cells causing haemorrhage, necrosis and liver damage. A second, and higher peak of viral antigens was detected in blood at 7 days. This is believed to be the result of release

of virus from the damaged hepatic cells. By 2 days pi, viral antigens were visualized by immunocytochemistry in a large number of intestinal epithelial cells and in cells lining the liver sinusoids and, by 3 days pi, in the nuclei of a small number of hepatocytes close to the sinusoids. By 4-6 days pi, viral antigens were extensively distributed into both the cytoplasm and nuclei of a large number of hepatocytes. By 9 days pi, viral antigens were not detected in the liver tissue of the birds which had survived the infection. Viral antigens were also visualized in other organs corresponding to the levels detected by ELISA. It was found that the pathogenesis of IBH infection somewhat resembled that previously described for hepatitis A virus infection in man (White and Fenner, 1986). On the other hand, ICH virus first multiplies in tonsillar crypts and Peyer's patches, is subsequently carried to the local lymph nodes from which a cell associated viraemia spreads virus throughout the body organs including liver (Appel, 1987).

Several further questions were addressed by using the ELISA and immunocytochemical techniques. Do these viruses cause persistent infection and for how long can they be detected in chicken tissues after recovery from the clinical disease? Until 20 weeks, different intestinal tissues (duodenum, ileum, caecum and caecal tonsil) and faeces were tested by ELISA for the detection of viral antigens following oral administration of 717B virus. Low levels of viral antigens were detected in all of the tissues and faeces of about 50% of the birds until 20 weeks pi although quite high levels of VN antibodies were present in these birds. From this result, it was difficult to decide whether the virus present in the litter was reinfecting the birds or a carrier state was established in infected chickens. To answer this question of whether persistent infection or constant reinfection is the major cause of continued viral shedding in faeces it would be necessary to house the infected chickens in such a way that contact with infected fomites was prevented.

Because viral shedding could be detected for at least 20 weeks after infection it was decided to study the possibility that virus could be directly passed from the parents to the progeny via eggs. To answer this, 10 eggs from each of 6 sheds of broiler breeder chickens, which had been naturally exposed to the virus, were tested by ELISA for the presence of viral antigens in the yolk and albumen components. Low levels of viral antigens were detected in egg yolk and egg albumen of 26% and 8% respectively of the eggs tested. Serum samples from birds in these breeder sheds contained high levels of VN antibodies. This is the first time AAV antigens have been detected directly in egg materials. This direct detection of viral antigens in eggs strongly supports the hypothesis that transovarian transmission of IBH occurs in chickens if infection is present in breeder flocks.

Although in many previous reports it has been suggested that immunosuppression due to

infection with IBD virus is necessary for IBH to occur there have been indications that some strains of IBH virus may themselves be immunosuppressive. The involvement of lymphoid organs such as bursa, thymus and spleen in both natural and experimental infections with IBH virus were therefore examined. The question was, why does lymphocytic depletion occur consistently in IBH infection even when there is no obvious association with any other identifiable immunosuppressive agent or factor? Does IBH virus cause immunosuppression by itself? The effects of infection with IBH virus on various lymphoid organs were closely examined by histology, ELISA and immunocytochemical procedures following natural and experimental infections. Obvious lymphoid depletion was demonstrated in bursa, thymus and spleen by histological examination. Viral antigens were detected by ELISA in most of the lymphoid organs including bursa, thymus, spleen, caecal tonsil and bone marrow at various times pi. The sites of viral replication were also visualized by the ABC technique in the lymphoid aggregates of all of the lymphoid organs except bone marrow. Besides the morphological and antigenic evidence of viral involvement and damage to the lymphoid organs, there was evidence of a decreased ability of the birds which were infected with IBH virus to make antibodies against an unrelated antigen (SRBC).

While the pathogenicity of AAVs has often been questioned in the absence of concurrent involvement of an immunosuppressive factor, it now appears that some strains of AAV are capable of producing clinical IBH and also of causing immunosuppression in chickens due to their direct effect on various lymphoid organs.

The significance of the differences between the inclusion bodies identified in infected liver tissue following natural and experimental infections of IBH is incompletely understood. The differences in terms of their staining characteristics, morphology and internal composition were studied by light and electron microscopy and their antigenic composition by immunocytochemistry. Most of the obviously enlarged nuclei contained large basophilic inclusion bodies following experimental infection. A small number of equally enlarged nuclei containing eosinophilic and amphophilic inclusion bodies which were surrounded by a clear halo were also observed. Densely stained AAV antigens were detected in all of the basophilic, some of the eosinophilic and amphophilic inclusion bodies and occasionally in some enlarged nuclei which did not contain inclusion bodies. By EM examination three types of markedly enlarged nuclei were observed in the liver tissue collected from experimentally infected birds. One type of enlarged nucleus frequently contained a large number of hexagonal viral particles. These nuclei are consistent with those containing basophilic inclusion bodies as described by others (Itakura et al., 1977). The two other types did not contain viral particles. The nuclear chromatin was marginated at the nuclear membrane and aggregates of granular material were apparent in one group and a dense

clump of material resembling nuclear chromatin was observed in the third type of nuclei. It is not clear whether the second and third types of nuclei represent cells very early in infection which contain incomplete virion materials or the cells where viral replication is complete and the mature virion particles have already left the cells.

Future research which builds upon this work could include comparison of the pathogenesis of virulent and nonvirulent strains of AAV; whether protection against virulent strains follows vaccination with a nonvirulent strain; and location by nucleotide sequencing of the DNA sequence(s) associated with virulence of particular strains of AAVs.

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APPENDIX I

BUFFERS AND SOLUTIONS USED FOR CELL CULTURE AND VIRAL IDENTIFICATION

I.A Eagle's minimum essential medium (MEM, pH 7.4)

MEM powder *	10.00 g
$NaHCO_3$	1.00 g
Deionised water	to 1 litre

Sterilize by filtration.

I.B Antibiotic-Trypsin-Versene (ATV, pH 7.4)

Trypsin	0.50 g
Versene (EDTA, tetrasodium salt)	0.20 g
NaCl	8.00 g
KCl	0.40 g
Dextrose	1.00 g
$NaHCO_3$	0.58 g
Penicillin	$2x10^5$ IU
Streptomycin	0.10 g
Phenol red	0.02 g
Deionised water	to 1 litre

Sterilize by filtration.

I.C Phosphate buffered saline (PBS, 0.01 M, pH 7.4)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Deionised water	to 1 litre

Sterilize by autoclave.

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* Flow Laboratories, UK. # 10-101

I.D **Penicillin, Streptomycin, Kanamycin (PSK)**

Penicillin	1 mega vial
Streptomycin	1.00 g
Kanamycin	1.00 g
PBS	to 100 ml

Sterilize by filtration.

I.E **Trypan blue**

Trypan blue powder	0.20 g
PBS	100 ml

Make 1.8 ml aliquot in bijou bottle.

I.F **Tissue culture plate staining solution**

Crystal violet stock -

Crystal violet	15 g
95% ethyl alcohol	100 ml

Working solution -

Crystal violet stock	5 ml
Glutaraldehyde (25%) *	10 ml
Distilled water	85 ml

I.G **Preparation of copper grid for electron microscopy**

Bovine serum albumin (BSA, 0.05%)	10 sec
Viral sample	40-70 sec
Distilled water wash	10 sec
Phosphotungstic acid (PTA 2%, pH 7.4)	50-70 sec

Air dry before keep in the grid holder.

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* SERVA, Heidelberg, New York.

APPENDIX II

BUFFERS AND SOLUTIONS USED FOR ELISA

II.A **Carbonate-bicarbonate buffer (Coating buffer 0.05 M, pH 9.6)**

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.20 g
Deionised water	to 1 litre

(can be used up to 2 months).

II.B **PBS-Tween 20 (washing buffer, 0.02 M, pH 7.4)**

NaCl	8.00 g
KH_2PO_4	0.20 g
$Na_2HPO_4, 12H_2O$	2.90 g
KCl	0.20 g
Tween 20 *	0.50 ml
NaN_3	0.20 g
Deionised water	to 1 litre

(can be used up to 2 weeks).

II.C **PBS-Tween-Bovine serum albumin (PBS-Tween-BSA, dilution buffer)**

PBS-Tween 20	100 ml
BSA	2.00 g

Use freshly prepared.

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* BDH Chemicals Ltd. England.

II.D **Citrate-phosphate buffer (Substrate buffer, 0.1 M, pH 5.0)**

Citric acid	10.50 g
Na_2HPO_4	14.20 g
Deionised water	to 500 ml

(can be used up to 2 months).

II.E **Orthophenylenediamine (OPD, substrate solution)**

OPD tetrahydrochloride *	10 mg
Citrate-phosphate buffer	20 ml
Hydrogen peroxide (30% w/v)	12 ul

Prepare immediately before use (keep out light).

* Merck, Dermstadt, West Germany.

APPENDIX III

BUFFER AND SOLUTION USED FOR IMMUNOCYTOCHEMISTRY

III.A **Phosphate buffered saline (PBS 0.01 M, pH 7.4)**

KH_2PO_4	1.09 g
Na_2HPO_4	1.14 g
NaCl	9.00 g
Deionised water	to 1 litre

III.B **Diaminobenzidine (DAB, substrate solution)**

DAB tetrahydrochloride *	5 mg
Hydrogen peroxide (30% w/v)	12 ul
Deionised water	10 ml

* Sigma Chemical Co. USA.

APPENDIX IV

BUFFERS USED FOR DNA WORK

IV.A **Tris-EDTA (TE) buffer (0.1 M Tris-0.01 M EDTA, pH 8.5)**

Tris-aminomethane (Tris base) *	12.10 g
EDTA-disodium salt	3.72 g
Deionised water	to 1 litre

Sterilize by autoclave (used for dialysis of virus).

IV.B **TE buffer (10 mM-Tris-1 mM EDTA, pH 7.5)**

Tris base (1 M)	10 ul
EDTA-disodium salt (200 mM)	5 ul
Deionised water	1 ml

Used for DNA suspension.

IV.C **Tris-acetate/EDTA (TAE) electrophoresis buffer (pH 7.8, 10x)**

Tris base	48.40 g
EDTA-disodium salt	7.40 g
Sodium acetate, anhydrous	16.40 g
Glacial acetic acid	17 ml
Deionised water	to 1 litre

Sterilize by autoclave.

Used for undigested DNAs (high MW).

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* Boehringer Mannheim, West Germany.

IV.D **Tris-borate/EDTA (TBE) electrophoresis buffer (pH 8.3, 10x)**

Tris base	121.10 g
Boric acid (anhydrous) *	61.80 g
EDTA-disodium salt	7.40 g
Deionised water	to 1 litre

Sterilize by autoclave.

Used for DNAs digested with restriction enzyme.

IV.E **Sample loading buffer (10x)**

Glycerol	5 ml
EDTA-disodium salt	0.37 g
Sodium dodecyl sulfate **	0.10 g
Bromophenol blue (BPB, dye)	0.01 g
Deionised water	to 10 ml

Keep at room temperature.

IV.F **Restriction enzyme digestion buffer (High salt buffer, 10x)**

a) **Buffer used for EcoRI digestion (pH 7.5)**

NaCl (1 M)	100 ul (conc. 100 mM)
Tris HCl (1 M)	50 ul (conc. 50 mM)
$MgCl_2$ (1 M)	10 ul (conc. 10 mM)
Dithioerythritol (DTE, 1 M) ***	1 ul (conc. 1 mM)
Deionised water	839 ul (to make 1000 ul)

Store at -20^{o}C.

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* AnalaR, BDH Chemical Ltd. England.

** SERVA, Heidelberg, New York.

*** Boehringer Mannheim, West Germany.

ai) **Dithioerythritol (DTE, 1 M)**

DTE	15.43 g
Deionised water	to 100 ml

Store at -20^{o}C.

b) **Buffer used for BamHI digestion (pH 8.0)**

NaCl (1 M)	100 ul (conc. 100 mM)
Tris HCl (1 M)	10 ul (conc. 10 mM)
$MgCl_2$ (1 M)	5 ul (conc. 5 mM)
2-Mercaptoethanol (1 M) *	1 ul (conc. 1 mM)
Deionised water	884 ul (to make 1000 ul)

bi) **Mercaptoethanol (1 M)**

2-Mercaptoethanol	7.81 g
Deionised water	to 100 ml

Store at -20^{o}C.

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* Boehringer Mannheim, West Germany.

APPENDIX V

V.A Relationship between ELISA absorbance (EA) and infectivity of AAV in cell culture

Dilution	10% SPF liver suspension plus cell culture virus		10% infected liver suspension	
	Log_{10} $TCID_{50}$	EA	Log_{10} $TCID_{50}$	EA
1:10	7.60	2.45	5.30	2.58
1:50	6.90	2.18	4.60	2.25
1:100	6.60	2.02	4.30	2.14
1:200	6.30	1.76	4.00	1.89
1:400	6.00	1.47	3.69	1.62
1:800	5.70	1.15	3.40	1.19
1:1600	5.40	0.78	3.10	0.79
1:3200	5.10	0.47	2.80	0.46
1:6400	4.80	0.32	2.49	0.34
1:12800	**4.49** *	**0.23**	2.19	0.24
1:25600	4.19	0.21	**1.89** *	**0.22**
1:51200	3.89	0.18	1.59	0.18
1:102400	3.59	0.17	1.30	0.16
1:204800	3.29	0.15	1.00	0.15
1:409600	2.99	0.15	0.60	0.15

* Minimum level of infective virus detected by ELISA per gram of infected tissue.

V.B **ELISA absorbance (EA) of egg yolk and egg albumin**

Chicken sheds	EA values for egg yolk (SPF mean plus 3 SD = 0.22)									
1.	0.16	0.11	0.18	0.17	**0.23** *	0.19	**0.27**	0.20	0.16	0.13
2.	0.15	**0.23**	0.14	0.19	0.17	**0.28**	0.10	0.15	**0.27**	0.19
3.	**0.24**	0.17	0.13	**0.26**	**0.28**	0.19	0.21	**0.29**	0.14	0.18
4.	0.13	0.18	0.17	0.20	0.12	**0.24**	0.11	0.14	**0.25**	0.19
5.	0.15	0.19	**0.48**	0.21	0.18	0.11	0.17	0.14	0.14	0.15
6.	**0.26**	0.16	0.19	0.20	**0.23**	**0.25**	0.18	**0.26**	0.18	0.15
	EA values of egg albumin (SPF mean plus 3 SD = 0.12)									
1.	0.08	0.10	0.08	0.05	0.06	0.09	0.11	0.10	0.07	0.05
2.	0.07	0.06	**0.15**	0.10	0.11	0.05	0.08	0.06	0.07	0.08
3.	**0.15**	0.04	0.09	0.05	**0.13**	0.06	0.09	0.08	0.10	0.11
4.	0.11	0.07	0.04	0.08	0.08	0.06	0.05	0.10	0.09	0.06
5.	0.09	0.05	**0.21**	0.09	0.09	0.07	0.04	0.07	0.06	0.10
6.	0.08	0.07	0.10	0.05	**0.14**	0.11	0.09	0.06	0.10	0.05

* Positive values are in bold mode.

APPENDIX VI

PATHOGENESIS OF INCLUSION BODY HEPATITIS

VI.A **Detection of viral antigens in blood plasma collected from SPF birds following oral inoculation of 717B strain (serotype 8) of AAV at the age of 2 days.**

Birds	ESA * values at days post inoculation											
	0.5	1	2	3	4	5	6	7	9	11	13	15
3401	0.04	0.36	**0.65 ****	0.48	0.14	0.18	0.46	**0.61**	0.33	0.04	0.01	0.00
3402	0.02	0.24	0.49	**0.50**	0.18	0.32	0.37	**0.46**	0.21	0.00	0.01	0.00
3403	0.03	0.29	**0.47**	0.41	0.10	0.17	0.28	**0.49**	0.31	0.04	0.02	0.01
3404	0.04	0.22	**0.34**	0.27	0.22	0.20	**0.45**	0.43	0.37	0.05	0.03	0.00
3405	0.05	0.04	0.05	0.09	0.15	0.21	0.25	**0.51**	0.23	0.00	0.02	0.00
Mean	0.04	0.23	**0.40**	0.35	0.16	0.22	0.36	**0.50**	0.29	0.03	0.02	0.00

* ELISA specific absorbance obtained by subtracting the absorbance with SPF blood plasma from that obtained with test blood plasma from infected bird.

** Peak values are presented in bold mode.

VI.B **Detection of adenoviral antigens in various tissues of chickens following oral administration of 717B strain (serotype 8) of AAV**

Tissues	Mean ESA * values at days post inoculation											
	0.5	1	2	3	4	5	6	7	9	11	13	15
Duodenum	0.11	0.41	1.10	1.41	1.51	1.45	**1.63****	1.39	1.06	0.14	0.08	0.02
Ileum	0.48	1.00	1.33	1.71	**1.79**	1.69	1.72	1.74	1.35	0.38	0.09	0.04
Caecum	0.55	0.95	1.37	1.63	**1.93**	1.79	1.78	1.80	1.47	0.40	0.08	0.04
Caecal tonsil	0.60	1.08	1.63	1.90	**1.97**	1.91	1.93	1.96	1.41	0.36	0.10	0.03
Colon	0.00	0.40	1.26	1.55	1.81	1.60	**1.90**	1.80	0.90	0.12	0.07	0.00
Cloaca	0.03	0.04	0.34	0.56	0.66	**1.22**	1.05	0.48	0.18	0.06	0.03	0.04
Oesophagus	0.01	0.03	**0.23**	0.13	0.12	0.05	0.03	0.02	0.00	0.01	0.02	0.01
Proventriculus	0.03	0.03	0.06	0.08	**0.15**	0.11	0.04	0.01	0.00	0.00	0.00	0.00
Trachea	0.00	0.01	0.06	0.14	**0.22**	0.15	0.05	0.04	0.03	0.04	0.02	0.00
Liver	0.03	0.04	0.06	0.25	0.48	0.66	**0.70**	0.65	0.23	0.05	0.03	0.02
Gall bladder	0.01	0.01	0.03	0.09	0.31	0.36	**0.41**	0.31	0.26	0.20	0.06	0.02
Pancreas	0.00	0.01	0.00	0.12	0.42	**0.52**	0.45	0.13	0.04	0.02	0.00	0.00
Spleen	0.00	0.00	0.02	0.04	0.11	0.19	**0.26**	0.17	0.04	0.02	0.01	0.00
Kidney	0.00	0.01	0.01	0.00	0.01	0.07	**0.09**	0.01	0.00	0.01	0.00	0.00
Thymus	0.00	0.01	0.02	0.08	**0.10**	0.09	0.07	0.02	0.02	0.00	0.00	0.00
Bursa	0.00	0.01	0.10	0.16	0.23	**0.33**	0.30	0.17	0.09	0.04	0.03	0.02
Bone marrow	0.00	0.00	0.01	0.05	**0.15**	0.11	0.09	0.03	0.01	0.02	0.00	0.00
Lung	0.00	0.00	0.00	0.03	**0.04**	0.02	0.01	0.00	0.00	0.00	0.00	0.00
Muscle	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00
Brain	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pharynx	0.02	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* ELISA specific absorbance (ESA) value obtained by subtracting the absorbance with SPF tissue from that obtained with test tissue.

** Peak values shown in bold type.

VI.C Detection of adenoviral antigens in duodenum (SPF mean = 0.14)

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.19*	0.40	0.99	1.37	1.63	1.51	1.69	1.75	1.30	0.40	0.25	0.18
0.17	0.74	1.55	1.69	2.00	1.26	1.82	1.57	0.76	0.19	0.19	0.16
0.18	0.59	1.87	1.32	2.14	1.77	2.13	1.70	1.14	0.26	0.28	0.13
0.31	0.66	1.12	1.44	1.48	1.68	1.86	1.43	1.25	0.38	0.17	0.17
0.40	0.36	0.67	1.93	1.00	1.73	1.35	1.20	1.55	0.17	0.21	0.16
0.25**	0.55	1.24	1.55	1.65	1.59	1.77	1.53	1.20	0.28	0.22	0.16
0.11***	0.41	1.10	1.41	1.51	1.45	1.63	1.39	1.06	0.14	0.08	0.02

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.D Detection of adenoviral antigens in ileum (SPF mean = 0.16)

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.83*	1.31	1.60	2.10	1.88	1.69	1.70	1.61	1.29	0.61	0.23	0.18
0.17	0.78	1.22	1.59	2.37	2.01	1.99	2.31	1.72	0.54	0.17	0.28
0.97	1.54	1.57	1.92	2.24	1.76	1.63	1.84	1.35	0.73	0.38	0.11
1.03	0.98	1.71	2.00	1.91	1.81	2.09	1.66	2.11	0.48	0.25	0.24
0.20	1.19	1.35	1.74	1.35	1.98	1.99	2.08	1.08	0.34	0.22	0.19
0.64**	1.16	1.49	1.87	1.95	1.85	1.88	1.90	1.51	0.54	0.25	0.20
0.48***	1.00	1.33	1.71	1.79	1.69	1.72	1.74	1.35	0.38	0.09	0.04

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.E **Detection of adenoviral antigens in colon (SPF mean = 0.15)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.11*	0.61	1.22	1.79	2.05	1.46	2.14	1.82	1.11	0.26	0.22	0.19
0.17	0.42	1.53	1.35	1.98	1.78	2.09	2.20	1.28	0.20	0.32	0.15
0.14	0.47	1.45	1.85	1.73	1.83	1.93	2.16	0.83	0.26	0.21	0.11
0.18	0.58	1.60	1.91	2.20	1.95	2.18	1.77	0.76	0.38	0.13	0.16
0.15	0.67	1.25	1.60	1.84	1.73	1.91	1.80	1.27	0.25	0.22	0.14
0.15**	0.55	1.41	1.70	1.96	1.75	2.05	1.95	1.05	0.27	0.22	0.15
0.00***	0.40	1.26	1.55	1.81	1.60	1.90	1.80	0.90	0.12	0.07	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.F **Detection of adenoviral antigens in cloaca (SPF mean = 0.13)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.16*	0.22	0.57	0.67	0.43	0.98	1.73	0.34	0.39	0.21	0.18	0.14
0.19	0.13	0.39	0.98	1.25	1.89	0.88	0.56	0.45	0.14	0.11	0.17
0.12	0.17	0.26	0.42	0.89	1.64	1.59	1.13	0.23	0.19	0.15	0.18
0.15	0.14	0.68	0.53	0.56	0.83	0.74	0.39	0.18	0.26	0.16	0.15
0.18	0.19	0.45	0.85	0.82	1.41	0.96	0.63	0.30	0.15	0.20	0.21
0.16**	0.17	0.47	0.69	0.79	1.35	1.18	0.61	0.31	0.19	0.16	0.17
0.03***	0.04	0.34	0.56	0.66	1.22	1.05	0.48	0.18	0.06	0.03	0.04

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.G **Detection of adenoviral antigens in caecum (SPF mean = 0.15)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.19*	1.11	1.78	2.08	2.13	1.67	1.65	1.89	1.46	0.41	0.15	0.17
0.97	0.92	1.30	1.29	1.96	1.49	2.22	2.15	1.57	0.46	0.22	0.17
0.84	1.38	1.92	2.11	2.32	2.24	1.74	1.46	1.78	0.57	0.23	0.23
0.73	1.20	1.44	1.64	2.03	1.88	1.90	1.92	1.92	0.60	0.24	0.22
0.77	0.89	1.16	1.78	1.96	2.42	2.14	2.33	1.37	0.71	0.31	0.16
0.70**	1.10	1.52	1.78	2.08	1.94	1.93	1.95	1.62	0.55	0.23	0.19
0.55***	0.95	1.37	1.63	1.93	1.79	1.78	1.80	1.47	0.40	0.08	0.04

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.H **Detection of adenoviral antigens in caecal tonsil (SPF mean = 0.15)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.82*	1.11	2.30	2.11	2.25	1.85	2.26	1.86	1.42	0.50	0.35	0.17
0.18	1.54	1.33	2.24	2.20	2.06	2.17	2.03	1.73	0.61	0.23	0.15
0.84	1.23	1.84	1.86	1.91	2.10	1.92	2.19	1.47	0.47	0.23	0.24
1.00	1.20	1.40	2.00	2.11	1.99	2.09	2.30	1.28	0.67	0.18	0.11
0.91	1.07	2.03	2.04	2.13	2.30	1.96	2.17	1.90	0.30	0.26	0.23
0.75**	1.23	1.78	2.05	2.12	2.06	2.08	2.11	1.56	0.51	0.25	0.18
0.60***	1.08	1.63	1.90	1.97	1.91	1.93	1.96	1.41	0.36	0.10	0.03

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.I **Detection of adenoviral antigens in oesophagus (SPF mean = 0.12)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.16*	0.20	0.31	0.24	0.18	0.18	0.16	0.15	0.13	0.17	0.12	0.08
0.09	0.15	0.50	0.31	0.27	0.11	0.19	0.12	0.11	0.14	0.16	0.16
0.11	0.12	0.29	0.23	0.32	0.15	0.13	0.10	0.08	0.12	0.17	0.14
0.18	0.18	0.37	0.19	0.22	0.20	0.14	0.15	0.12	0.10	0.13	0.15
0.11	0.10	0.28	0.28	0.21	0.21	0.13	0.18	0.16	0.12	0.12	0.12
0.13**	0.15	0.35	0.25	0.24	0.17	0.15	0.14	0.12	0.13	0.14	0.13
0.01***	0.03	0.23	0.13	0.12	0.05	0.03	0.02	0.00	0.01	0.02	0.01

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.J **Detection of adenoviral antigens in proventriculus (SPF mean = 0.18)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.18*	0.19	0.26	0.27	0.32	0.28	0.22	0.20	0.17	0.18	0.17	0.17
0.21	0.25	0.23	0.25	0.35	0.30	0.19	0.17	0.18	0.16	0.20	0.17
0.24	0.17	0.25	0.26	0.31	0.27	0.24	0.18	0.15	0.18	0.19	0.19
0.20	0.19	0.20	0.25	0.31	0.28	0.23	0.18	0.19	0.16	0.15	0.18
0.22	0.25	0.26	0.27	0.36	0.32	0.22	0.22	0.21	0.17	0.19	0.19
0.21**	0.21	0.24	0.26	0.33	0.29	0.22	0.19	0.18	0.17	0.18	0.18
0.03***	0.03	0.06	0.08	0.15	0.11	0.04	0.01	0.00	0.00	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.K **Detection of adenoviral antigens in trachea (SPF mean = 0.10)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.08*	0.11	0.14	0.22	0.32	0.24	0.14	0.12	0.08	0.10	0.15	0.10
0.09	0.12	0.12	0.28	0.30	0.29	0.11	0.09	0.13	0.10	0.09	0.12
0.10	0.10	0.15	0.20	0.34	0.23	0.15	0.15	0.14	0.17	0.07	0.07
0.13	0.08	0.19	0.26	0.28	0.26	0.17	0.17	0.16	0.15	0.13	0.13
0.10	0.14	0.20	0.24	0.36	0.23	0.18	0.17	0.14	0.18	0.16	0.08
0.10**	0.11	0.16	0.24	0.32	0.25	0.15	0.14	0.13	0.14	0.12	0.10
0.00***	0.01	0.06	0.14	0.22	0.15	0.05	0.04	0.03	0.04	0.02	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI. L **Detection of adenoviral antigens in liver (SPF mean = 0.12)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.15*	0.12	0.25	0.19	0.33	0.72	0.69	0.61	0.56	0.17	0.14	0.13
0.18	0.20	0.13	0.43	0.68	0.83	1.75	1.09	0.39	0.15	0.17	0.14
0.12	0.18	0.21	0.38	1.12	1.39	0.72	0.78	0.23	0.18	0.14	0.17
0.16	0.14	0.17	0.49	0.46	0.61	0.58	0.44	0.27	0.12	0.18	0.15
0.14	0.16	0.14	0.36	0.41	0.35	0.36	0.93	0.30	0.23	0.12	0.11
0.15**	0.16	0.18	0.37	0.60	0.78	0.82	0.77	0.35	0.17	0.15	0.14
0.03***	0.04	0.06	0.25	0.48	0.66	0.70	0.65	0.23	0.05	0.03	0.02

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.M **Detection of adenoviral antigens in gall bladder (SPF mean = 0.14)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.16*	0.12	0.19	0.26	0.45	0.53	0.54	0.50	0.46	0.36	0.21	0.15
0.16	0.17	0.20	0.21	0.42	0.47	0.61	0.46	0.39	0.36	0.17	0.18
0.15	0.14	0.17	0.19	0.46	0.42	0.53	0.44	0.34	0.35	0.23	0.13
0.13	0.18	0.15	0.22	0.42	0.49	0.57	0.45	0.45	0.31	0.19	0.16
0.15	0.14	0.14	0.27	0.50	0.59	0.50	0.40	0.36	0.32	0.20	0.18
0.15**	0.15	0.17	0.23	0.45	0.50	0.55	0.45	0.40	0.34	0.20	0.16
0.01***	0.01	0.03	0.09	0.31	0.36	0.41	0.31	0.26	0.20	0.06	0.02

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.N **Detection of adenoviral antigens in pancreas (SPF mean = 0.10)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.06*	0.08	0.07	0.25	0.53	0.59	0.59	0.23	0.15	0.09	0.07	0.02
0.09	0.12	0.12	0.20	0.50	0.67	0.55	0.26	0.15	0.13	0.05	0.05
0.05	0.09	0.07	0.22	0.57	0.60	0.49	0.21	0.11	0.07	0.06	0.09
0.11	0.12	0.10	0.21	0.55	0.63	0.53	0.22	0.18	0.15	0.10	0.07
0.09	0.14	0.14	0.22	0.45	0.61	0.59	0.23	0.11	0.16	0.07	0.07
0.0**	0.11	0.10	0.22	0.52	0.62	0.55	0.23	0.14	0.12	0.07	0.06
0.00***	0.01	0.00	0.12	0.42	0.52	0.45	0.13	0.04	0.02	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.O **Detection of adenoviral antigens in spleen (SPF mean = 0.16)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.17*	0.19	0.18	0.16	0.28	0.32	0.41	0.23	0.19	0.15	0.16	0.15
0.13	0.15	0.14	0.21	0.19	0.55	0.32	0.43	0.16	0.22	0.22	0.13
0.14	0.16	0.22	0.15	0.22	0.24	0.64	0.52	0.17	0.16	0.14	0.17
0.20	0.13	0.20	0.25	0.35	0.38	0.45	0.18	0.22	0.20	0.18	0.20
0.16	0.17	0.16	0.23	0.31	0.26	0.28	0.29	0.26	0.17	0.15	0.15
0.16**	0.16	0.18	0.20	0.27	0.35	0.42	0.33	0.20	0.18	0.17	0.16
0.00***	0.00	0.02	0.04	0.11	0.19	0.26	0.17	0.04	0.02	0.01	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.P **Detection of adenoviral antigens in kidney (SPF mean = 0.11)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.14*	0.12	0.15	0.12	0.16	0.19	0.21	0.13	0.14	0.09	0.12	0.05
0.11	0.11	0.06	0.15	0.11	0.23	0.25	0.13	0.11	0.13	0.06	0.10
0.10	0.14	0.11	0.11	0.12	0.16	0.16	0.12	0.08	0.12	0.09	0.07
0.10	0.08	0.12	0.09	0.10	0.14	0.19	0.10	0.08	0.15	0.11	0.14
0.10	0:15	0.16	0.08	0.11	0.18	0.19	0.12	0.14	0.11	0.17	0.14
0.11**	0.12	0.12	0.11	0.12	0.18	0.20	0.12	0.11	0.12	0.11	0.10
0.00***	0.01	0.01	0.00	0.01	0.07	0.09	0.01	0.00	0.01	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.Q **Detection of adenoviral antigens in thymus (SPF mean = 0.15)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.17*	0.14	0.17	0.19	0.20	0.18	0.19	0.19	0.20	0.17	0.17	0.13
0.12	0.18	0.20	0.35	0.23	0.35	0.13	0.19	0.13	0.14	0.09	0.17
0.16	0.13	0.16	0.14	0.26	0.28	0.31	0.11	0.14	0.16	0.16	0.13
0.20	0.19	0.19	0.20	0.39	0.14	0.27	0.15	0.16	0.10	0.15	0.15
0.10	0.16	0.13	0.27	0.17	0.25	0.20	0.21	0.22	0.18	0.18	0.17
0.15**	0.16	0.17	0.23	0.25	0.24	0.22	0.17	0.17	0.15	0.15	0.15
0.00***	0.01	0.02	0.08	0.10	0.09	0.07	0.02	0.02	0.00	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.R **Detection of adenoviral antigens in bursa (SPF mean = 0.12)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.11*	0.14	0.26	0.31	0.34	0.44	0.47	0.30	0.25	0.19	0.18	0.15
0.09	0.08	0.20	0.26	0.36	0.46	0.35	0.28	0.20	0.14	0.16	0.17
0.12	0.14	0.21	0.27	0.37	0.49	0.41	0.26	0.16	0.16	0.12	0.09
0.15	0.12	0.23	0.32	0.38	0.39	0.51	0.26	0.19	0.14	0.10	0.13
0.13	0.17	0.20	0.24	0.30	0.47	0.36	0.35	0.25	0.17	0.19	0.16
0.12**	0.13	0.22	0.28	0.35	0.45	0.42	0.29	0.21	0.16	0.15	0.14
0.00***	0.01	0.10	0.16	0.23	0.33	0.30	0.17	0.09	0.04	0.03	0.02

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.S **Detection of adenoviral antigens in bone marrow (SPF mean = 0.12)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.06*	0.12	0.16	0.19	0.29	0.20	0.23	0.19	0.14	0.17	0.13	0.15
0.13	0.08	0.13	0.18	0.23	0.26	0.18	0.17	0.16	0.15	0.14	0.07
0.11	0.14	0.11	0.20	0.21	0.25	0.24	0.15	0.09	0.12	0.11	0.14
0.07	0.08	0.14	0.16	0.36	0.25	0.22	0.11	0.10	0.18	0.11	0.10
0.13	0.13	0.11	0.12	0.26	0.19	0.18	0.13	0.16	0.08	0.11	0.14
0.10**	0.11	0.13	0.17	0.27	0.23	0.21	0.15	0.13	0.14	0.12	0.12
0.00***	0.00	0.01	0.05	0.15	0.11	0.09	0.03	0.01	0.02	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.T **Detection of adenoviral antigens in lung (SPF mean = 0.12)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.10*	0.13	0.14	0.16	0.18	0.17	0.11	0.10	0.15	0.12	0.14	0.10
0.11	0.11	0.10	0.19	0.11	0.10	0.14	0.08	0.09	0.08	0.13	0.09
0.12	0.09	0.17	0.14	0.17	0.11	0.12	0.11	0.09	0.16	0.16	0.12
0.12	0.13	0.10	0.10	0.15	0.18	0.16	0.15	0.13	0.12	0.08	0.11
0.15	0.14	0.09	0.16	0.19	0.14	0.12	0.16	0.14	0.07	0.09	0.13
0.12**	0.12	0.12	0.15	0.16	0.14	0.13	0.12	0.12	0.11	0.12	0.11
0.00***	0.00	0.00	0.03	0.04	0.02	0.01	0.00	0.00	0.00	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.U **Detection of adenoviral antigens in brain (SPF mean = 0.08)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.07*	0.08	0.04	0.07	0.05	0.05	0.03	0.06	0.06	0.09	0.09	0.06
0.05	0.06	0.07	0.08	0.08	0.07	0.08	0.07	0.08	0.06	0.09	0.03
0.09	0.08	0.03	0.05	0.11	0.09	0.06	0.08	0.07	0.07	0.07	0.09
0.04	0.07	0.11	0.07	0.09	0.09	0.05	0.06	0.06	0.08	0.06	0.07
0.10	0.06	0.05	0.08	0.07	0.05	0.08	0.03	0.08	0.10	0.04	0.10
0.07**	0.07	0.06	0.07	0.08	0.07	0.06	0.06	0.07	0.08	0.07	0.07
0.00***	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.V **Detection of adenoviral antigens in pharynx (SPF mean = 0.07)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.12*	0.05	0.10	0.07	0.05	0.04	0.07	0.05	0.05	0.09	0.05	0.03
0.06	0.09	0.08	0.08	0.08	0.05	0.09	0.04	0.05	0.07	0.06	0.06
0.09	0.06	0.09	0.05	0.07	0.06	0.09	0.07	0.05	0.10	0.03	0.04
0.08	0.08	0.05	0.04	0.06	0.04	0.04	0.09	0.04	0.08	0.07	0.04
0.10	0.12	0.08	0.06	0.09	0.06	0.06	0.05	0.06	0.06	0.04	0.08
0.09**	0.08	0.08	0.06	0.07	0.05	0.07	0.06	0.05	0.08	0.05	0.05
0.02***	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.W **Detection of adenoviral antigens in muscle (SPF mean = 0.06)**

Days post inoculation											
0·5	1	2	3	4	5	6	7	9	11	13	15
0.05*	0.07	0.06	0.06	0.04	0.07	0.09	0.07	0.05	0.04	0.08	0.07
0.04	0.07	0.04	0.03	0.05	0.11	0.04	0.04	0.07	0.05	0.04	0.04
0.07	0.05	0.08	0.07	0.05	0.08	0.05	0.05	0.06	0.06	0.07	0.05
0.03	0.05	0.05	0.03	0.04	0.04	0.10	0.12	0.08	0.05	0.05	0.05
0.06	0.06	0.07	0.06	0.07	0.05	0.07	0.05	0.04	0.05	0.06	0.04
0.05**	0.06	0.06	0.05	0.05	0.07	0.07	0.07	0.06	0.05	0.06	0.05
0.00***	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.02	0.00	0.00

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.X **Detection of adenoviral antigens in faeces (SPF mean = 0.15)**

Days post inoculation											
0.5	1	2	3	4	5	6	7	9	11	13	15
0.16*	0.17	0.17	0.45	0.41	1.13	0.97	0.41	0.36	0.32	0.28	0.27
0.15	0.11	0.23	0.36	0.40	1.10	0.98	0.46	0.33	0.27	0.23	0.31
0.10	0.21	0.25	0.38	0.47	1.18	0.86	0.39	0.31	0.24	0.25	0.26
0.15	0.16	0.27	0.40	0.45	1.25	1.00	0.42	0.26	0.28	0.26	0.32
0.19	0.15	0.18	0.46	0.52	1.09	0.84	0.42	0.29	0.34	0.33	0.29
0.15**	0.16	0.22	0.41	0.45	1.15	0.93	0.42	0.31	0.29	0.27	0.29
0.00***	0.01	0.07	0.26	0.30	1.00	0.78	0.27	0.16	0.14	0.12	0.14

* ELISA absorbance (EA) ** Mean EA *** Mean ESA

VI.Y **Seroconversion detected by virus neutralization (VN) test in SPF chickens following oral administration of a locally isolated 717B strain (serotype 8) of avian adenovirus.**

Days post inoculation											
0	1	2	3	4	5	6	7	9	11	13	15
- *	-	-	-	-	-	-	40	20	160	640	2560
-	-	-	-	-	-	-	20	160	150	320	1280
-	-	-	-	-	-	-	10	320	320	320	640
-	-	-	-	-	-	-	-	320	160	640	1280
-	-	-	-	-	-	-	40	40	320	320	1280
- **	-	-	-	-	-	-	13	106	211	422	1280

* Reciprocal of the highest dilution of serum which completely inhibited the cytopathic effect caused by virus (- represents VN titre <5).

** Geometric mean titre of the 5 birds.