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MOLECULAR AND PATHOLOGIC CHARACTERIZATION OF CANADIAN AVIAN REOVIRUS FIELD ISOLATES

A thesis
submitted to the Graduate Faculty
in Partial Fulfilment of the Requirements
for the Degree of
Master of Science
in the Department of Pathology and Microbiology
Faculty of Veterinary Medicine
University of Prince Edward Island

Yatri Drastini
Charlottetown, P.E.I.
June, 1992

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ABSTRACT

Fourteen isolates of avian reovirus previously grown in chicken embryo liver cells were adapted to replicate in an African green monkey (Vero) cell line. The growth curves of five viruses studied in Vero cells showed them to be highly cell-associated. Different procedures were examined for releasing the cell-associated virus following propagation in Vero cells, including several freeze-thaw cycles, treatment with sterile distilled deionized water (ddH_2O), freon extraction, and trypsin treatment. It was observed that treatment of virus infected cultures with ddH_2O was the most effective, and trypsin treatment was the least effective procedure for dissociation of virus from cells. Treatment of virus-infected cultures with ddH_2O is a simple and effective procedure which can be used where large amounts of virus are required for experimental purposes.

Experiments were undertaken to characterize the trypsin sensitivity of 14 avian reoviruses in relation to their genotypes, serotypes, and pathogenicity in day-old chicks in an attempt to develop a rapid method for differentiation of pathogenic from non-pathogenic virus strains. The migration patterns of purified viral RNA of the 14 viruses in sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed heterogeneity in the 10 double-stranded RNA genome segments between isolates. Two main genotypes were recognized on the basis of the migration patterns of the M2 gene. This grouping seemed to correlate with sensitivity of the viruses to trypsin in that the M2 gene of 5 of 6 viruses sensitive to trypsin migrated more slowly, i.e., was larger, than that of 7 of 8 trypsin resistant viruses. Analysis of the serological relationships of 8 selected viruses identified three distinct serotypes, one of which had at least four subtypes. Unlike the mammalian reoviruses, trypsin sensitivity of these avian reoviruses was found to be highly variable within and between the serotypes. Comparison of five avian reoviruses, 3 resistant and 2 sensitive to trypsin, for pathogenicity in day-old chicks following oral inoculation showed the trypsin resistant strains to be more pathogenic. Mortality, hepatic necrosis and tenosynovitis occurred only in the groups of chickens inoculated with trypsin resistant viruses. These viruses also persisted longer in heart and hock joint tissues of more chickens than the trypsin sensitive viruses. However, since no other pathological manifestations of avian reovirus infection were observed in inoculated birds beside the mortality, hepatic necrosis and tenosynovitis, it could not be confirmed if trypsin sensitivy *per se* would serve as a rapid test for identification of pathogenic avian reoviruses.

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ABBREVIATIONS

AGP	= agar gel precipitin
BHK	= baby hamster kidney
BSA	= bovine serum albumin
C	= cutaneous
CAM	= chorioallantoic membrane
CE	= chicken embryo
CEF	= chicken embryo fibroblast
CEK	= chicken embryo kidney
CELi	= chicken embryo liver
CELu	= chicken embryo lung
CK	= chicken kidney
CL	= chicken lung
CO_2	= carbon dioxide
CPE	= cytopathic effect
CrFK	= Crandell feline kidney
ddH_2O	= distilled deionized water
dsRNA	= double-stranded RNA
D	= day
DEPC	= diethylpyrocarbonate
DMSO	= dimethyl sulfoxide
DTT	= dithiothreitol
et. al.	= et alii (L and others)
EMEM	= Eagle's minimum essential medium
FA	= fluorescent antibody
FBS	= fetal bovine serum
FC	= Fahey-Crawley or Crawley
FP	= foot pad
Freon	= 1, 1, 2-trichlorotrifluoro-ethane
GBK	= Georgia bovine kidney
GM	= growth medium
IM	= intramuscular
IN	= intranasal
IT	= intratracheal
I.U.	= international unit
k1	= ascending slope
k2	= descending slope
k_A	= the means of sample 1
k_B	= the means of sample 2
KCl	= potassium chloride
kD	= kilodalton
KH_2PO_4	= monopotassium acid phosphate
L	= large
M	= medium
M	= molar
MDBK	= Madin-Darby bovine kidney
MDCK	= Madin-Darby canine kidney
mg	= milligram
mM	= millimolar

MM = maintenance medium
NCS = newborn calf serum
nm = nanometer
μCi = microcurie
μg = microgram
μl = microliter
NaCl = sodium chloride
Na_2HPO_4 = disodium acid phosphate
Na acetate = sodium acetate
NLIN = nonlinear regression
NS = non-structural
O = oral
p = probability
PBS = phosphate buffered saline
PFU = plaque forming units
pH = the symbol relating the hydrogen ion (H^+) concentration.
PI = post inoculation
PK = porcine kidney
$[^{32}P]pCp$ = phosphorus-32
R = resistant
RBM = rabbit bone-marrow
RK = rabbit kidney
RNA = ribonucleic acid
S = sensitive or small
SDS-PAGE = sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SE = standard error
SPF = specific pathogen free
S_{kA} = standard errors of the means of samples 1
S_{kB} = standard errors of the means of samples 2
$TCID_{50}$ = median Tissue Culture Infectious Dose
Ts/VA = Tenosynovitis/Viral arthritis
UK = United Kingdom
USA = United States of America
V = voltage
Vero = African green monkey kidney
VN = virus-neutralization
Vol. = volume
W = week

GENERAL INTRODUCTION

Reoviruses belong to the family Reoviridae which has an extremely wide host range among animals and plants (Samuel, 1988). Members of the Reoviridae family found in vertebrates form five genera: Orthoreovirus, Rotavirus, Orbivirus, Coltivirus, and Aquareovirus (Francki et. al., 1991). The members of the orthoreovirus genus are distinguishable into two distinct groups: those of avian origin, and those of mammalian origin (Kawamura and Tsubahara, 1966). Both groups of viruses have a genome consisting of 10 segments of double-stranded RNA (dsRNA), separable into three size classes, which are enclosed within a double protein capsid shell 70 to 80 nm in diameter with a similar protein composition and distribution (Spandidos and Graham, 1976; Schnitzer et. al., 1982; Joklik, 1983). However, avian reoviruses, unlike their mammalian counterparts, exhibit a wide heterogeneity in their neutralizing antigens (Robertson and Wilcox, 1986), possess a different group-specific antigen (Petek et. al., 1967), cause no haemagglutination (Glass et. al., 1973), have been associated with disease in their natural hosts (Kibenge and Wilcox, 1983), and cause syncytium formation both in infected cell culture and in susceptible chickens (Wilcox and Compans, 1982; Kibenge and Wilcox, 1983). In addition, all avian reoviruses are reported to be resistant to trypsin (Kawamura et. al., 1965; Petek et. al., 1967; Jones et. al., 1975)

whereas among the three serotypes of mammalian reovirus, serotype 3 is trypsin-sensitive while serotypes 1 and 2 are resistant (Joklik, 1983).

At least 11 serotypes of avian reovirus, with extensive cross-reactivity among the heterologous types, have been identified among strains isolated in Japan, UK, Germany, and the USA (Wood et. al., 1981). Robertson and Wilcox (1984) also found considerable antigenic heterogeneity among Australian avian reovirus isolates, but assigned the 10 selected strains examined to 3 subtypes rather than distinct serotypes. Most avian reovirus serotypes have been associated with a variety of pathologic conditions in chickens worldwide, and some have also been isolated from normal chickens (Robertson and Wilcox, 1986) and diseased chickens infected with other viruses and bacteria (Olson, 1984). These viruses are associated with enteric disorders, growth abnormalities, leg deformities and respiratory disease. However, correlative relationships between the serotypes and specific diseases have not yet been demonstrated. Moreover, avian reoviruses, like the mammalian reoviruses, exhibit marked polymorphism in genomic segment electrophoretic migration patterns (genotypes) among isolates of the same serotype as well as among different serotypes (Hrdy et. al., 1979; Gouvea and Schnitzer, 1982; Lozano et. al., 1992).

Avian reoviruses can be readily grown in the laboratory in primary cultures of cells of avian origin (Guneratne et.

al., 1982; Barta et. al., 1984). However, among the established mammalian cell lines tested, only Vero cells have been reported to support replication of certain strains of avian reovirus (Barta et. al., 1984; Wilcox et. al., 1985; Nwajei et. al., 1988; Jones and Afaleq, 1990). The avian reoviruses in the present study were adapted to Vero cells which, in addition to being readily available, allowed for consistent titration of infectious virus because of the uniform nature of cells of the continuous cell line. However, the only report on the nature of avian reovirus replication in Vero cells found the virus to be highly cell-associated, requiring several freeze-thaw cycles to release virus from infected cells (Wilcox et. al., 1985). Since that study examined only one avian reovirus strain (RAM-1) because other strains could not replicate in Vero cells, it was decided to determine the growth curves of additional avian reoviruses in Vero cells. Furthermore, several procedures were evaluated for recovery of virus grown in Vero cells.

Since the original report by Fahey and Crawley (1954), avian reoviruses have been isolated from different clinical conditions in Canada by several workers (Rekik et. al., 1990). The virus is endemic in most poultry farms and has become of significant economic importance to require the vaccination of broiler breeders, although disease outbreaks occur despite vaccination. The present studies were designed to increase our understanding of the significance of the biological

heterogeneity among avian reoviruses occurring in Canada. The main need for the poultry industry in Canada, as elsewhere, is a rapid method of identification of avian reovirus in clinical specimens and differentiation of pathogenic from non-pathogenic virus strains. A trypsin sensitive avian reovirus was recently reported; experimental infections of chickens with this isolate indicated it had a different pathogenesis from that for trypsin resistant strains (Kibenge et. al., 1985; Alfaleq and Jones, 1991). The trypsin sensitive avian reovirus was shown to persist in the intestinal tract for a shorter time than usual, indicating that the resulting viremia and virus dissemination to other tissues would also be severely limited unless very large doses of virus were ingested (Alfaleq and Jones, 1991). It is hypothesized that the trypsin sensitivity assay may be used to differentiate between pathogenic and non-pathogenic avian reovirus strains.

CHAPTER 1.

REVIEW OF LITERATURE

1.1 Pathology of avian reovirus infections in chickens

1.1.1 General pathogenesis

In Canada avian reovirus was first isolated from chickens suffering from chronic respiratory disease (Fahey and Crawley, 1954). This initial isolate was originally termed the Fahey-Crawley (FC) agent, and was later characterized as a member of the reovirus group (Petek et. al., 1967). Subsequently, other isolations of avian reoviruses were made from a variety of clinical conditions in chickens including cloacal pasting (Deshmukh and Pomeroy, 1969a), tenosynovitis/viral arthritis (Glass et. al., 1973; Jones et. al., 1975), hydropericardium (Spradbrow and Bains, 1974; Jones, 1976), and a runting syndrome/malabsorption syndrome (Van der Heide et. al., 1981; Page et. al., 1982; Robertson et. al., 1986). Avian reoviruses have also been isolated from clinical conditions in other av an spps, such as turkeys affected with infectious synovitis (Olson, 1984; Levisohn et. al., 1980), tenosynovitis (Page et. al.,

1982; Afaleq et. al., 1991) and diarrhoea and conjunctivitis (McFerran et. al., 1976); from ducks exhibiting diarrhoea (Kaschula, 1950; Malkinson et. al., 1981); from pigeons with diarrhoea (McFerran et. al., 1976); from grey parrots with enteritis (Meulemans et. al., 1983); from wedge-tail eagle with tenosynovitis (Aquila andax)(Jones and Guneratne, 1984); and quails suffering from enteritis (Ritter et. al., 1986). However, the role of avian reoviruses in disease has not been clearly established. In addition to their association with the above disease conditions, avian reoviruses have been isolated from clinically normal chickens (Robertson et. al., 1984), and from cell cultures prepared from apparently healthy chicken kidneys (Mustafa-Babjee and Spradbrow, 1971).

To cause disease, a virus must enter a host by specifically adsorbing to target cells, replicate inside these host cells with the resultant viremia disseminating virus within the host in characteristic pathways, causing damage to host tissues (Joklik, 1983). Reoviruses generally infect via the respiratory and enteric tracts of animals, hence the name "REO" virus (Sabin, 1959), the acronym standing for "respiratory enteric orphan". Initial replication of avian reovirus occurs in the mucosa of the gastrointestinal and respiratory tracts (Menendez et. al., 1975; Ellis et. al., 1983). In case of

the gastrointestinal tracts, the virus comes in contact with trypsin, other proteolytic enzymes and bile salts. That interaction may be important in determining the virulence of the virus. If the virus survives within the lumen environment, it enters intestinal epithelial cells, causing local inflammation, or enters the systemic circulation (Kauffman, 1983) with resultant tissue damage at distant sites.

The pathogenesis of reovirus infection has been studied in detail with mammalian reoviruses. Mammalian reovirus type 1 crosses the mucosal barrier via M cells at the Peyer's patches (Tyler and Fields, 1990). The virus then spreads to the mesenteric lymph nodes and spleen (Kauffman, 1983), or is trapped by hepatic Kupffer cells and is then excreted in the bile (Tyler and Field, 1990). The latter mechanism may also be an important route by which a virus producing systemic disease re-enters the intestinal tract for excretion via faeces (Rubin et. al., 1986).

The pathogenesis of avian reovirus infection is similar to that described for mammalian reoviruses. Following oral inoculation of 1-day-old specific pathogen-free (SPF) light hybrid chicks with strain R2 of avian reovirus (Jones et. al., 1975), the virus was re-isolated from the pancreas, oesophagus, ileum, caecal tonsils, and rectum at 1 day post inoculation

(PI)(Kibenge et. al., 1985). Maximum virus titers were found in the liver at 3 days PI, and declined by 7 days PI. Virus could be re-isolated from the heart at 10 days PI and in the hock joint at 14 days PI. Jones et. al. (1989) using the same virus strain reported that the virus entered and replicated primarily in the intestinal epithelium and bursa of Fabricius within 12 hours PI, spread to most tissues within 1 to 2 days PI, and finally localized in joint tissues by 4 days PI.

The mortality caused by the virus is higher in 1-day to 1-week-old chickens than in 2 weeks of age or older chickens. Also, the surviving 1-day to 1 week-old chickens frequently developed a persistent virus infection (Toivanen, 1987).

Several factors, including virus strain, dose, route of inoculation (Wood and Thornton, 1981; Islam et. al., 1988), breed of chicken (Jones and Kibenge, 1984), immune status (Carboni et. al., 1975; Wood and Thornton, 1981; Jones and Georgiou, 1984a; Tang et. al., 1987; Kibenge et. al., 1987) and other infections (MacKenzie and Bains, 1977; Kibenge et. al., 1982; Hill et. al., 1989) and non-infectious factors have been shown to influence the course of experimental reovirus infection in chickens (Table 1).

1.1.2 Tenosynovitis/Viral arthritis

Avian tenosynovitis/viral arthritis occurs worldwide. The disease was first reported in England in 1967 (Dalton and Henry, 1967). It has since been reported in Netherlands (Krasselt and Voute, 1969), Italy (Rossi et. al., 1969), France (Meulemans et. al., 1980), Yugoslavia (Velner et. al., 1980), U.S.A. (Olson and Solomon, 1968; Johnson and van der Heide, 1971; Glass et. al., 1973; van der Heide et. al., 1974), Australia (Mackenzie and Bains, 1976, 1977; Kibenge et. al., 1982), Japan (Itakura et. al., 1976 and 1977), Canada (Ide and DeWitt, 1979), and Egypt (Tantawi et. al., 1984).

The term avian tenosynovitis was originally used to describe an inflammation of the tendon sheaths and tendons caused by Mycoplasma synoviae (Dalton and Henry , 1967), whereas the reovirus-associated disease was referred to as viral arthritis (Olson, 1973). Currently, both terms are used to describe the reovirus-associated disease (Kibenge and Wilcox, 1983).

Tenosynovitis or viral arthritis lesions have been found in various degrees in affected birds. The severity of the lesions depends on the breed of chickens (Jones and Kibenge, 1984), the age of chickens at the time of infection (Carboni et. al., 1975; Wood and Thornton, 1981; Jones and Georgiou, 1984a), route of virus

Table 1. Experimental infection with reoviruses in chickens

Experimental Diseases	Virus strains	Chicken breed and ages used light / heavy	Route of infection	Incubation period	Reference
Ts/VA[a]	WVU 1675	-	FP IM IV	4 D 11-30 D 11 D	Olson, 1959
Ts/VA	WVU 1675	2-20 W	FP	6 D	Kerr & Olson, 1964
Ts/VA	WVU 1675	2 W	FP	285 D	Olson $ Kerr, 1967
Ts/VA	WVU 2937	3 W	FP IV IT C	2 D 5-7 D 9 D 13-21 D	Olson & Solomon,1968
Ts/VA	WVU 1675	-	FP	4 D	Kerr & Olson, 1969
Ts/VA	FC	1 D	FP IN	3 D <43 D	Olson & Weiss, 1972
Ts/VA	UM 1-203	1-2 D	FP IP, IM	3 D 3-13 D	Johnson, 1972
Ts/VA	FC	2 W	IN	15 D	Olson & Khan, 1972

Experimental Diseases	Virus strains	Chicken breed and ages used light / heavy	Route of infection	Incubation period	Reference
Ts/VA	Texas	2 D	O, SC, FP	-	Glass et.al. 1973
Ts/VA	S1133	1 D	O, C SC, IP	<7.5 W <5 W	van der Heide, 1974
Ts/VA	-	-	SC, IN, O, C	<29 D	van der Heide, 1975
Ts/VA	9 strains	2 W	FP	7 D	Sahu & Olson 1975
Ts/VA	R1	4 D	FP C	<14 D 7 W	Jones et.al., 1975
Ts/VA	5 strains	1-15 D	FP	7-8 D	Carboni et.al., 1975
Ts/VA	UM 1-203	1 D	FP	1-2 D	Mandelli et.al., 1978
Ts/VA	R1	1 D	FP C	7 D 5-7 D	Jones & Onunkwo,1978
Ts/VA	R1	1 D	FP	5-7 D	Bradbury & Onunkwo,1978
Ts/VA	M126, M67	3 W	FP	3 D	MacDonald et.al., 1978

Experimental Diseases	Virus strains	Chicken breed and ages used light / heavy	Route of infection	Incubation period	Reference
Ts/VA	4 strains	2 W	FP	5 D	Sahu et.al., 1979
Ts/VA	R2	1 D	FP O	7 D 3 W	Jones et.al., 1980
Ts/VA	A, B, S1133	3 W	FP	3 D	Levisohn & Weisman,1980
Ts/VA	Turkey	1 D	FP	3 D	van der Heide, 1980
Ts/VA	S1133	1 D 2 W	FP O	3 W 7 W	Wood & Thornton, 1981
Ts/VA	S1133	12 W	O IP SC	115 D	Marquardt et.al., 1983
Ts/VA	R2	1 D / 1 D	FP, O	3 W 7 W 12 W	Jones & Kibenge, 1984
Ts/VA	R2	1 D	O	2 W 5 W 8 W 10 W	Jones & Georgiou, 1984b

Experimental Diseases	Virus strains	Chicken breed and ages used light / heavy	Route of infection	Incubation period	Reference
Ts/VA	R2	1 D	O	1, 3, 5, 7 10, 12, 14, 16, 18, 20 D	Kibenge et.al., 1985
Ts/VA	W3-492	1 D	O	2, 3, 4, 5 W	Kibenge & Dhillon,1986
Ts/VA	W3-492	1 D	O	3, 7, 14 D	Kibenge & Dhillon,1987
Ts/VA	176	1 D	O	1, 2, 3, 4, 5, 6, 7 D	Tang et.al., 1987
Ts/VA	-	-	-	-	Islam et.al., 1988
Ts/VA	176	1 D	FP, O	1, 2, 3, 4, 5, 10, 15, 20 W	Hill et.al., 1989
Ts/VA	R2	1 D	O	2, 6, 12 H 1, 2, 3, 4, 5 7, 9, 12 D	Jones et.al., 1989
Ts/VA	R2 R11 R19	1 D	FP	1, 3 W	Alfaleq & Jones, 1989
Respiratory disese	-	1-52 W	Egg inoculation	-	McFerran et.al., 1971

Experimental Diseases	Virus strains	Chicken breed and ages used light / heavy	Route of infection	Incubation period	Reference
Respiratory disease	-	2 W	FP	1, 2, 4 W	Hussian et.al., 1981
Enteric disease	81-68	1 D	O	3, 5, 7, 14 21, 28 D	Nersessian et.al., 1981
Enteric disease	2035 2408	5 D	O	2, 4, 6, 8, 10, 14, 21 D	Guy et.al., 1988
Malabsorp-tion	-	1 D	O	1-7 D	Decaesste cker et.al., 1986
Malabsorp-tion	-	1 D	O	7, 14 D	Kouwenhoven et.al., 1988

[a] Ts/VA denotes Tenosynovitis/Viral arthritis

inoculation (Wood and Thornton, 1981; Islam et. al., 1988), and presence of secondary bacterial infections (MacKenzie and Bains, 1977; Kibenge et. al., 1982; Hill et. al., 1989). Among three breeds of chicken examined, the commercial (non-SPF) Ross I broilers were more sensitive to an arthrotropic reovirus infection than SPF light-hybrids and commercial (non-SPF) White Leghorn egg layers (Jones and Kibenge, 1984). With regard to the age, the younger chickens were more susceptible to infection (Carboni et. al., 1975; Wood and Thornton, 1981; Jones and Georgiou, 1984a; Tang et. al., 1987). Four routes of infection, foot-pad, intra-articular, oral, and contact exposure produced tenosynovitis, with the severest lesions occuring after foot-pad and intra-articular routes (Wood and Thornton, 1981; Islam et. al., 1988). It has been suggested that the tenosynovitis outbreaks are caused by a primary subclinical virus infection, with Staphylococcus aureus as a secondary infection responsible for the development of clinical signs (MacKenzie and Bains, 1977; Kibenge et. al., 1982; Hill et. al., 1989). However, bacteria were not present in all clinical outbreaks of tenosynovitis (Kibenge et. al., 1982). The sex of broiler chicks inoculated subcutaneously at 1 day of age with avian reovirus strain 81-176 from hock joint had no affect on the mortality rate (Montgomery et. al., 1986).

The clinical response to infection following oral inoculation of 1-day-old SPF light-hybrid chicks included

depression, prostration (Jones and Georgiou, 1984a) and anorexia at 2 days PI (Tang et. al., 1987), but by 8 days PI the chicks appeared normal. Sometimes no clinical signs were observed until 3 (Kibenge et. al., 1985) to 5 weeks PI (Kibenge and Dhillon, 1986). The tenosynovitis lesion was a unilateral swelling on the plantar aspect of the leg, below the hock joint by 3 to 4 weeks PI (Jones and Georgiou, 1984a), which decreased by 8 weeks PI. Mortality of chicks caused by the virus began 4 days PI (Tang et. al., 1987; Alfaleq and Jones, 1991) and continued until 10 days PI (Kibenge and Dhillon, 1986). Mortality was due mainly to severe hepatitis characterized by congestion, hemorrhages and yellow necrotic areas.

The growth rates observed by measuring the body weight were found to be significantly lower in inoculated chickens than those of the corresponding control group within 5 weeks PI (Kibenge and Dhillon, 1987). However, another experiment in which a different strain of avian reovirus was used showed no significant differences between the weights of infected and the control groups within 2 to 6 weeks PI (Jones and Kibenge, 1984).

The gross lesions in the affected chickens include a tendon swelling below the hock at 3 weeks PI and above the hock by 6 weeks PI. Rupture of digital flexor tendons has been observed at 6 weeks PI, and fusion of the tendons at 9 weeks PI (Jones and Georgiou, 1984a). A yellowish-brown gelatinous

exudate between tendons in the swollen legs, varying degrees of thickening and fusion of tendons, and pitted erosions of the articular cartilage of the hock joints were observed by 12 weeks PI (Jones and Kibenge, 1984; Jones and Georgiou, 1984b).

1.1.3 Respiratory disease

Avian reovirus was originally isolated from chickens suffering from respiratory disease (Fahey and Crawley, 1954). The virus has also been isolated from a laying flock concurrently infected with infectious bronchitis virus (McFerran et. al., 1971), and from geese with a respiratory disease (Csontos and Miklovich-Kis Csatari, 1967). However, respiratory disease has not yet been reproduced by experimental inoculation of chickens with avian reovirus (Table 1). Intranasal inoculation of chicks with a trypsin-sensitive avian reovirus was effective in causing viral arthritis, and therefore it may be that the respiratory route of infection is of greater significance with reoviruses of this type (Alfaleq and Jones, 1991).

1.1.4 Enteric disease

Avian reoviruses associated with enteric disease have been isolated from chickens suffering from severe cloacal pasting (Deshmukh and Pomeroy, 1969a), from turkeys with

depression, anorexia, 30% mortality (Simmons et. al., 1972) or infectious enteritis (Gershowitz and Wooley, 1973), and from quails experiencing severe enteritis (Ritter et. al., 1986; Guy et. al., 1987)

In experimentally infected chickens, there were neither clinical signs nor microscopic intestinal lesions following oral inoculation of 5-day old SPF chickens (Guy et. al., 1988). One of two strains used in that study caused significantly depressed weight gains in virus inoculated chickens compared with the uninoculated controls up to 8 days PI, while the other strains did not affect weight gain. Virus was recovered from faeces by 2 to 8 days PI, and from liver and spleen beginning on day 4 PI (Guy et. al., 1988).

1.1.5 Runting syndrome

This disease syndrome was initially reported in chickens in the Netherlands (Kouwenhoven et. al., 1978a). It was then described in England (Bracewell and Wyeth, 1981), USA (Page et. al., 1982) and Australia (Pass et. al., 1982; Reece et. al., 1984). The terms infectious stunting and runting syndrome were initially used by Bracewell and Wyeth (1981). Other names that have been used to refer to the same condition include infectious proventriculitis, osteoporosis (Kouwenhoven et. al., 1978b), helicopter disease, bristle bone disease, femoral head necrosis, pale bird syndrome (van der Heide et. al.,

1981), and malabsorption syndrome (Page et. al., 1982).

Runting syndrome in chickens is characterized by stunted growth, elevated feed conversion ratios, poor feathering, leg weakness, decreased weight gain (Kouwenhoven et. al., 1978b; Bracewell and Wyeth, 1981; Page et. al., 1982), diarrhoea (Vertommen et. al., 1980), femoral head fractures, and osteoporosis (van der Heide et. al., 1981).

Infected chickens showed a reduction in growth rates ranging from 5 to 20% in 1-week-old chickens, and signs of lameness and poor feather development in 2-week-old chickens (Kouwenhoven et. al., 1978a). It was also determined that the most prominent lesions were proventriculitis and a catarrhal enteritis. In another observation in chickens, the growth rates were reduced between one and two weeks of age (Bracewell and Wyeth, 1981). The feathering defects and leg weakness were also observed up to 5 weeks of age. One-day old commercial broiler chickens inoculated orally with a malabsorption-syndrome-suspected-virus exhibited decreased weight gains, elevated feed conversion ratios, feathering defects, lameness, poor pigmentation of the shanks, and poorly digested food in the intestinal tracts. Histopathological lesions included proventriculitis, myocarditis, catarrhal enteritis, bursal atrophy and pancreatic atropy (Page et. al., 1982). However, other studies suggested that the reoviruses isolated from affected chickens were not necessarily associated with the syndrome since the isolation rate of reovirus from normal 3-

week-old chickens was at least equivalent to that obtained from age-matched chickens with the runting/stunting syndrome (Robertson et. al., 1984).

1.1.6 Immunosuppression

Several workers have studied the effect of avian reoviruses on the immune system of their hosts, but there is no report that designates avian reovirus as an immunosuppresive agent. In the bursa of Fabricius of birds infected with a tenosynovitis-producing avian reovirus strain WVU 1675, lymphoid cells were decreased as early as 7 days PI (Kerr and Olson, 1969). The bursa of Fabricius was also reported to be atrophied in field cases of reovirus-associated malabsorption (Page et. al., 1982). Avian reovirus strain 81-5 isolated from chicken with malabsorption syndrome decreased the bursa of Fabricius and increased the spleen organ-to-body weight ratio up to 3 weeks PI following subcutaneous inoculation with $10^{4.5}$ PFU/0.1 ml, and altered the spleen weight alone at 1 week PI following the same inoculation with a lower dose of $10^{2.5}$ PFU/0.1 ml (Montgomery et. al., 1985). But this virus strain had no effect when $10^{4.2}$ PFU/0.1 ml was given orally. Avian reovirus strain WVU 1675 also caused hematological effects (Kerr and Olson, 1969). Four antigenically similar avian reoviruses were divided into two groups (Rosenberger, 1983; Rinehart, 1984). One group

consisting of two non-pathogenic viruses was found in lymphoid tissues other than thymus, and caused no gross or microscopic changes. The second group of two pathogenic viruses persisted in the bursa of Fabricius and/or thymus over 24 days PI, and caused atrophy of the lymphoid follicles in the bursa of Fabricius and depletion of lymphoid cells in both the bursa of Fabricius and thymus.

Avian reoviruses interfered with the development of immunity to coccidia and Marek's disease virus (Rinehart et. al., 1983; van der Heide et. al., 1983; Ruff et. al., 1985), and impaired the T-cell function and the phagocytic ability of circulating monocytes (Montgomery et. al., 1986). Chickens dually infected with Eimeria mitis and avian reovirus strain 2035 exhibited synergistic depression of weight gain (Ruff and Rosenberger, 1985). Dual infection with Cryptosporidium baileyi and reovirus strain 2035 resulted in significant weight gain depression over a 21-day period PI. Chickens inoculated with only reovirus strain 2035 showed no effect on weight gain during the same period, and C. baileyi infection significantly depressed weight gain only 8 to 14 day PI (Guy et. al., 1988). However, reovirus infection in the dually infected SPF chickens did not modify the microscopic changes caused by C. baileyi (Guy et. al., 1988).

1.1.7 Reoviruses in avian species other than chickens

Avian reoviruses have been isolated from ducks (Kaschula, 1950; Malkinson et. al., 1981), turkeys (Kawamura et. al., 1965; Alfaleq et. al., 1989), pigeons (McFerran et. al., 1976; Vindevogel et. al., 1982), pet birds (Meulemans et. al., 1983; Panigrahy, 1986), wedge-tail eagle (Jones and Guneratne, 1984), and quails (Ritter et. al., 1986). Most of these avian species exhibited enteritis, and some were suffering from tenosynovitis/arthritis.

Turkeys inoculated with an enteric avian reovirus originally isolated from turkey showed that virus distribution in most organs occurred by 3 to 7 days PI (Nersessian et. al., 1985). Viremia occurred by 7 days PI. The virus was recovered from tendons at 3 to 7 days PI and 28 days PI (Nersessian et. al., 1985). In addition, turkeys seem to be much more resistant than chickens to the induction of arthritis/ tenosynovitis by reoviruses from either species (Alfaleq and Jones, 1989).

No experimental infections of avian reovirus in other avian species have been reported.

1.2 Cultivation of avian reoviruses

1.2.1 Isolation and propagation in primary cell cultures

Primary cell cultures which have been used for the isolation and propagation of avian reoviruses include the whole chicken embryo (CE)(Deshmukh and Pomeroy, 1969a); chicken embryo lung (CELu)(Petek et. al., 1967), fibroblast (CEF)(Lee et. al., 1973), kidney (CEK)(Glass et. al., 1973), and liver (CELi)(McFerran et. al., 1976); chicken kidney (CK) (Kawamura et. al., 1965), lung (CL), and testicular cell cultures (Sahu and Olson, 1975); turkey kidney cells (Fujisaki et. al., 1969); and duck embryo fibroblast cell cultures (Lee et. al., 1973). Of these, CELi cell cultures have been reported to be the most sensitive for the cultivation of avian reoviruses (Guneratne et. al., 1982; and Barta et. al., 1984).

The cytopathic effect (CPE) in cell cultures infected with avian reoviruses is characterized by the formation of multinucleated cells (syncytia) which detach from the monolayers leaving small holes in the cell monolayer (Deshmukh and Pomeroy, 1969a; Robertson and Wilcox, 1986). In CE cell cultures, CPE was first detected on the ninth, seventh, and fourth passages of avian reovirus strains Type 24, Type 25 and Type 59, respectively (Deshmukh and Pomeroy, 1969a). The CPE of

these viruses appeared at 5 to 7 days PI, became numerous and larger upon further incubation, until the entire monolayer was destroyed by 9 days PI. However, the maximum virus titer was less than 10^2 PFU/ml. Strain R1 of avian reovirus when prepared in CELu, CEK and CELi cell cultures, produced titers of 6.2, 6.2 and 7.0 $\log_{10}$ $TCID_{50}$ by the third passage, which increased to 8.2, 7.2 and 8.4 $\log_{10}$ $TCID_{50}$, respectively, by the twenty-fifth passage, while the virus propagated in CEF produced a maximum titer of only 6.0 $\log_{10}$ $TCID_{50}$/ml (Guneratne et. al., 1982). In addition to the higher titer of the virus, CELi cultures also produced larger plaques than CEK. More blind passages (5 to 7) were required before CPE was observed in virus isolation attempts using CL, CK, and testicular cell cultures with avian reovirus strains Type 24, Type 25, Type 59, FC, WVU 1464-29H, WVU 2937, WVU 2986, and WVU 71-212 (Guneratne et. al., 1982).

1.2.2 Adaptation to continuous cell lines

Several mammalian cell lines have been used to grow avian reoviruses, including those of bovine origin [Madin Darby bovine kidney (MDBK), and Georgia bovine kidney (GBK)], canine origin [Madin Darby canine kidney (MDCK)], human origin (Chand C, HEp2 and HeLa), mouse origin (L929), monkey origin [LLC-MK, and African green monkey

kidney (Vero)], feline origin [Crandell feline kidney (CrFK)], baby hamster kidney (BHK), rabbit kidney (RK), and porcine kidney (PK)(Sahu and Olson, 1975; Barta et. al., 1984). Mammalian cell lines have advantages of being convenient for use, and with no risk of contamination with vertically transmitted avian reoviruses in contrast to avian cell cultures.

Avian reovirus strain RAM-1 passaged 14 times in CK cells followed by 4 to 5 freeze-thaw cycles produced CPE in Vero cells within 48 h of the first passage; whereas 5 other strains passaged 8 to 10 times in CK cells could not be adapted to grow in Vero cells (Wilcox et. al., 1985). Another study found that all 22 avian reovirus strains passaged several times in avian embryonic cell cultures, produced CPE in Vero cells after 1 to 3 passages (Nwajei et. al., 1988). The CPE produced in Vero cells is characterised by focal areas of cell fusion. The virus was so highly cell-associated that it required up to 10 days PI to obtain maximum CPE (Wilcox et. al., 1985). In addition, infected cells had to be frozen and thawed at least 4 times before the virus could be passaged successfully. In contrast, in chicken cell cultures, avian reoviruses required only 4 days incubation to produce CPE and one freeze-thaw cycle to release virus from infected cells (Robertson and Wilcox, 1984). In other studies, avian reovirus strains produced

CPE on Vero cell monolayers after an incubation period of 4 to 5 days (Barta et. al., 1984) or 5 to 6 days (Sahu and Olson, 1975).

Avian reovirus strain WVU 2937 produced CPE after three blind passages on GBK, CrFK and BHK cells, five passages on RK cells, and ten passages on PK cells. No CPE was produced on rabbit bone-marrow (RBM)(Barta et. al., 1984). In CELi cell cultures the virus produced CPE on the first passage, 40 h after inoculation. Reovirus strain S1133 penetrated and uncoated in mouse L cells, but there was no viral genome replication or viral progeny formation (Spandidos and Graham, 1976). Subsequent investigations reported that avian reovirus strain S1133 could replicate in mouse L cells at pH 6.4 and 7.2, but not at pH 8.2 (Mallo et. al., 1991a). These authors suggested that the pH of the medium mainly affected viral transcription, with little or no influence on viral mRNA translation. Vero cells were found to be unsuitable for the isolation of avian reoviruses from field materials, and it was suggested that with Vero-adapted reovirus strains, Vero cells might be used in neutralisation tests and in the production of live attenuated reovirus vaccines (Nwajei et. al., 1988).

1.3 Molecular structure of avian reoviruses

1.3.1 Electron microscopy

The morphology structure of avian reovirus has been studied by electron microscopy in chickens (Kawamura et. al., 1965; Hieronymus et. al.,1983), turkeys (Simmons et. al., 1972; Nersessian et. al., 1986), and quails (Ritter et. al., 1986).

By a transmission electron microscope, the virus particle from infected chickens showed a double capsid and icosahedral symmetry (Simmons et. al., 1972; Hieronymus et. al., 1983). Another observation of a reovirus isolate from the hock joint of a chicken showed that the particle was hexagonal to spherical (Glass et. al., 1972). The outer capsid consists of 92 capsomers (Kawamura et. al., 1965). In mammalian reoviruses the outer shell is sensitive to digestion by chymotrypsin, but the cores are completely resistant (Shatkin and Sipe, 1968a; Smith et. al., 1969). Also, the cores can be disrupted by high concentrations of sodium dodecyl sulfate (Joklik, 1983). The diameter of the avian reovirus particle ranges from 70 to 82 nm (Kawamura et. al., 1965), 60 nm (Dutta and Pomeroy, 1967), 58 to 64 nm (Koide, 1970), 56 to 61 nm (Deshmukh et. al., 1971), and 68 to 72 nm (Hieronymus et. al., 1983).

1.3.2 Avian reovirus genome

The avian reovirus genome was initially determined to consist of RNA by using DNA inhibitors (Kawamura et. al., 1965; Hieronymus et. al., 1983), and susceptibility to DNase or RNase (Deshmukh and Pomeroy, 1969; Spandidos and Graham, 1976) digestion. The genome was then showed to consist of double-stranded (ds)RNA (Glass et. al., 1973; Sekiguchi et. al., 1968; Spandidos and Graham, 1976), and its segments were also separated into three size classes: three large (L1, L2 and L3), three medium (M1, M2 and M3), and four small segments (S1, S2, S3 and S4) in sucrose gradients (Spandidos and Graham, 1976), or by analysis of the migration patterns of the viral genome using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)(Rekik et. al., 1990). The approximate molecular weight range of each genomic segment group is 2.5 to 2.7 X 10^6, 1.3 to 1.8 X 10^6, and 0.71 to 1.2 X 10^6 daltons for L, M and S segments, respectively (Spandidos and Graham, 1976; Gouvea and Schnitzer, 1982; Lozano et. al., 1992). The migration patterns of dsRNA segments of avian reovirus genomes are heterogenous within the same serotype as well as among different serotypes (Shatkin et. al., 1968; Rekik et. al., 1990). The migration patterns of genome segments of avian reoviruses isolated from turkeys and chickens were

found to be heterogenous, particulary in the M and S segments, while those of canary and cockatiel isolates were not (Lozano et. al., 1992).

1.3.3 Viral proteins of avian reovirus

Characterization of viral proteins of avian reoviruses has been done (Spandidos and Graham, 1976; Schnitzer et. al., 1982), but assignment of the specific protein products to individual gene segments has not been reported yet (Robertson and Wilcox, 1986). In mammalian reoviruses, each genome segment is transcribed into a ssRNA molecule by a dsRNA-dependent-RNA polymerase within the reovirus core, and is then translated into a single viral coded polypeptide in the cytoplasm of infected cells (Joklik, 1981)(Table 2). The three large genome segments (L1, L2 and L3) encode 3 large proteins (λ_3, λ_2 and λ_1, respectively), the three medium segments (M1, M2 and M3) encode μ_2, μ_1/μ_{1C} and a non structural (μNS) protein, respectively, and S1, S2, S3 and S4 segments encode σ_1 and σ_{1s}, σ_2, σNs and σ3, respectively (Tyler and Fields, 1990).

Similarly to the dsRNA genome segments, the viral proteins from purified avian reovirus particles also resolve into three size classes on SDS-PAGE: 3 large ($\lambda_{1/A}$, $\lambda_{2/B}$ and $\lambda_{3/C}$), 2 medium ($\mu_{1/A}$ and $\mu_{2/B}$), and 3 small

Table 2. The gene coding assignments of avian and mammalian reoviruses

Mammalian reovirus			Avian reovirus		
Genome Segment	Viral Protein	Molecular Weight (kD)	Genome Segment	Viral Protein	Molecular Weight (kD)
L1	λ3	135	L1	A	145
L2	λ2	140	L2	B	130
L3	λ1	155	L3	C	115
M1	μ2	70	M1	A	72
M2	μ1/μ1C	80/72	M2	B	70
M3	μNs	75	M3		
S1	σ1	12	S1	A	39
S2	σ2	38	S2	B	36
S3	σNs	36	S3	C	32
S4	σ3	34	S4		
				NS[a]	75
				NS	34
				TC[b]	34

[a] NS denotes non-structural viral protein.
[b] TS denotes top component viral protein.

bands ($\sigma_{1/A}$, $\sigma_{2/B}$ and $\sigma_{3/C}$) with the molecular weights of 115 to 145 K, 70 to 85 K, and 32 to 40, respectively (Spandidos and Graham, 1976; Schnitzer et. al., 1982)(Table 2). In mammalian reoviruses, the outer capsid consists of three proteins: σ_1, σ_3, and μ1C (Tyler and Fields, 1990). The σ_1 protein is the primary determinant of reovirus pathogenesis since it functions as the virus attachment protein, as hemagglutinin, and as the antigen for inducing neutralizing antibody production and cell mediated immune responses (Tyler and Fields, 1990). The μ1C protein encoded by the M2 gene segment functions as a determinant of transcriptase activation following protease treatment in vitro. This protein confers resistance of the outer capsid to protease digestion in vivo, and functions to modulate virulence within a serotype (Tyler and Fields, 1990). Biologic functions of the σ_3 protein include affinity for dsRNA, zinc metalloprotein, inhibition of cellular RNA and protein synthesis, establishment of persistent infection, and regulation of viral transcription and translation (Tyler and Fields, 1990).

The mammalian reovirus cores contain three major proteins (λ_1, λ_2 and σ_2) and two minor proteins (λ_3 and μ_2). The λ_1, λ_2 and λ_3 were suggested to contain transcriptase activities (Tyler and Fields, 1990).

Besides the viral proteins above, there are three

nonstructural proteins (σNS, μNS and σ_{1s}). σNS is encoded by the S3 gene segment and functions to condense the ssRNA into precursor subviral particles in preparation for dsRNA synthesis (Gomatos et. al., 1981). This protein may play other important roles early in reovirus replication (Cross and Fields, 1972). The μNS protein encoded by the M3 gene segment is presumed to form part of the viral transcriptase complex (Morgan and Kingsbury, 1980). σ_{1s} which is encoded by the S1 gene is thought to play a role in the virus replication cycle (Ernst and Shatkin, 1985).

1.3.4 Physicochemical properties

The physicochemical properties of avian reoviruses reported include resistance to trypsin (Kawamura et. al., 1965; Alfaleq et. al., 1991), sodium deoxycholate (Kawamura et. al., 1965), Zephiran (Deshmukh and Pomeroy, 1969), 2% formaldehyde at 4°C (Meulemans et. al., 1982), and the lipid solvents ether and chloroform (Glass et. al., 1973; Nersessian et. al., 1986); sensitivity to 2% phenol, 100% ethanol, tincture of iodine (Petek et. al., 1967), Environ-D, mercury bichloride (Deshmukh and Pomeroy, 1969b). The density of virions has been reported to be 1.36 to 1.37 g/ml in caesium chloride gradients (Spandidos and Graham, 1976; Schnitzer et. al., 1982).

The stability of avian reoviruses to pH and temperatures vary. Avian reovirus strains Type 24, Type 25, and Type 59 isolated from enteritis in chickens were stable at pH 3.0 for 30 min (Deshmukh and Pomeroy, 1969b). Glass et. al. (1973) have reported the reovirus isolated from chicken hock joints to be resistant to pH 3.0 and 9.0 for 4 h. The avian reovirus strains UMI-203 and S1133 isolated from enteritis were partially sensitive to pH 3.0 and 12.0 for 3 h (Carboni et. al., 1975). Avian reovirus strains BC-3 and BC-7 obtained from turkeys suffering from enteritis were reported to be stable at pH 3.0 and 7.0 for 3 to 5 h (Gershowitz et. al., 1973), while four other turkey reovirus isolates were also stable at pH 3.0 for 4 h (Nersessian, 1986). The viruses isolated from chickens with enteritis were stable at room temperature for more than 2 months, at 4°C for more than 3 months (Dutta and Pomeroy, 1967), and at 50°C for only 1 h (Deshmukh and Pomeroy, 1969b). The viruses recovered from chickens suffering from malabsorption syndrome and tenosynovitis were stable at 56°C for 1 to 6 h (van der Heide and Kalbac, 1975; and Hieronymus, 1983), but other researchers have reported the viruses to be partially inactivated at this temperature within 10 to 30 min (Dutta and Pomeroy 1967; Mustaffa-Babjee et. al., 1973). The turkey reovirus isolates were stable at 56°C for from 30 min to 6 h

(Nersessian et. al., 1985).

It has been reported that avian reoviruses isolated from chickens are resistant to trypsin (Kawamura et. al., 1965; Jones et. al., 1975). The infectivity of an arthrotropic reovirus strain R1 of chickens (Jones and Guneratne, 1984) in CELi cell monolayers was also unaff ected after treatment with 0.01% trypsin for 30 min , but that of strain TR1 isolated from a turkey with arthritis was reduced indicating this strain to be trypsin sensitive (Alfaleq and Jones, 1991).

1.4 Antigenic characteristics of avian reoviruses

1.4.1 Common precipitin antigens

The common antigens among avian reoviruses have been detected by a direct fluorescent antibody (FA)(Kawamura and Tsubahara, 1966; Menendez et. al., 1975), an indirect FA (Ide, 1982), complement fixation (Kawamura and Tsubahara, 1966) and the agar gel precipitin (AGP) tests (Kawamura and Tsubahara, 1966; Wood et. al., 1980). Up to four precipitin lines, and therefore at least four common precipitin antigens, have been detected in the AGP test (Olson and Weiss, 1972).

1.4.2 Neutralizing antigens

The serum neutralization test has been used in embryonated eggs inoculated via the CAM route (Olson and Weiss, 1972), in CK (Kawamura et. al., 1965), and in CEK cells (Hieronymus et. al., 1983). Kawamura et. al. (1965) grouped 77 Japanese strains into 5 serotypes. Types 24, 25 and 59 were classified into 2 serotypes, in which, Type 59 was antigenically different from the other two (Deshmukh and Pomeroy, 1969a; Sahu and Olson, 1975). The WVU 2937 and FC strains were grouped as one serotype based on one-way serum neutralization tests conducted by CAM inoculation of embryonated eggs (Olson and Weiss, 1972). However with a cross-neutralization test using a constant virus-varying serum plaque reduction test in CK cell cultures, WVU 2937 was of a different antigenic group from the FC strain (Sahu and Olson, 1975). A similar observation had been made by Munro and Wooley (1973) using neutralization kinetics. Later, Sahu et. al. (1979) classified strains WVU 2937, Texas, UMI 203 and S1133 as subtypes of the same serotype. Six strains (R1, U.comn. S1133, EK 2286, Kaleta 122, Kosters, and Kaleta 795) have been grouped into 3 serotypes (Wood et. al., 1980). Another six strains were also placed into 3 serotypes: S1133, C08 and type 45; type 81-5 and 43A; and type 82-9 (Hieronymus, 1983). Robertson and Wilcox

classified 10 Australian avian reovirus strains into 3 subtypes. Overall, at least 11 serotypes of avian reoviruses have been demonstrated by serum neutralization tests (Wood et. al., 1980).

1.5 Research objectives

The work reported in this thesis was designed to increase our understanding of the significance of the biological heterogeneity among avian reoviruses in Canada. The main need for the poultry industry in Canada, as elsewhere, is a rapid method of identification of avian reovirus in clinical specimens and differentiation of virus strains causing disease.

The objectives of this study were:

1. to determine the growth curves of additional avian reoviruses in Vero cells.
2. to evaluate different procedures for recovery of virus grown in Vero cells.
3. to determine the relationship between trypsin sensitivity of avian reoviruses and their genotypes, serotypes, and pathogenicity in day-old chickens.
4. to determine if a trypsin sensitivity assay may serve as a rapid test for identification of pathogenic avian reoviruses

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell cultures, media, and solutions

2.1.1. Source of fertile specific pathogen free (SPF) eggs

SPF eggs were obtained from the Health of Animals Laboratory, Sackville, New Brunswick, Canada. The eggs were used for preparing chicken embryo liver cells, and to hatch chickens for use in experimental infections with avian reoviruses. The eggs were incubated at 37°C in a humidified commercial incubator (Humidaire Incubator Co., New Madison, Ohio) and rotated twice daily.

2.1.2. Chicken embryo liver (CELi) cells

Chicken embryo liver (CELi) cells were prepared from 13 to 15 day-old SPF chicken embryos. The eggs were opened by removing the shell around the air sac. Embryos were placed into a petri dish. Each embryo was opened to dissect out both lobes of the liver. Livers obtained were macerated by passing them through a 10 ml syringe into a side arm flask. Phosphate buffered saline (PBS) 'A' solution (0.02 mM NaCl, 0.0003 mM KCl, 0.001 mM Na_2HPO_4,

0.0002 mM KH_2PO_4, 100 I.U. penicillin, 100 μg streptomycin, and 25 units nystatin) was added and stirred with a magnet stirrer at medium speed for 5 min to wash the tissues. The tissues were allowed to settle and the supernatant was discarded. Ten to 15 ml of 0.025% prewarmed trypsin 10 X solution (porcine trypsin, Sigma Chemical Company, St. Louis) was added to the tissues, and was stirred for 5 min. The supernatant containing liver cells was then collected into a centrifuge tube with 2 ml of newborn calf serum (NCS). Fresh warm 0.025% trypsin solution was added and the trypsinization procedure was repeated 5 times. The cell suspension from a total of six harvests was centrifuged at 300 g for 2 min. The cells were pooled by resuspending them in cold growth medium (GM) consisting of Eagle's minimum essential medium (EMEM) with 10% heat inactivated FBS, 100 I.U. penicillin/ml, 100 μg streptomycin/ml, and 25 units nystatin/ml. The cell suspension was filtered through 4 X thickness of cotton gauze, and was then centrifuged to determine the packed cell volume. Cells were resuspended 1:550 in GM, plated out, and incubated at 37°C in a humidified 5% CO_2 atmosphere. Growth medium was changed after 24 h and the cell monolayers were used after 48 h.

2.1.3. African green monkey kidney (Vero) cells

The Vero cell line (American Type Culture Collection, Rockville, Maryland) was stored in aliquots in liquid nitrogen. For use, one vial containing 1 ml of cell suspension was thawed and resuspended in a total of 25 ml GM, and was then incubated at a 37°C in a humidified 5% CO_2 atmosphere. GM was changed after 24 h to remove residual dimethyl sulfoxide (DMSO), and for use, confluent monolayers were split 1:7 at 3 to 7 day intervals.

2.2 Viruses and viral cultivation

2.2.1. Virus strains

Fourteen avian reovirus strains from the Health of Animals Laboratory, Sackville, New Brunswick, Canada, were used in these studies. Four were reference strains belonging to different serotypes (FC, Type 24, Type 59, and WVU 2937), and ten were local isolates of unknown antigenic types (Table 3). The viruses were isolated in CELi cell cultures and were then passaged 2 to 8 times in Vero cells.

Table 3: Source of avian reovirus

Virus strains	Disease condition[a]	Avian spps	Reference
FC	Respiratory	Chicken	Fahey and Crawley, 1954
Type 59	Enteric	Chicken	Deshmukh and Pomeroy, 1969
Type 24	Enteric	Chicken	Deshmukh and Pomeroy, 1969
WVU 2937	Viral arthritis	Chicken	Olson and Solomon, 1968
SK25a	Mortality	Chicken	HAL[b], Sackville, 1975
SK58a	Viral arthritis	UK[c]	HAL, Sackville, 1977
SK103a	UK	Birds	HAL, Sackville, 1978
SK73a	UK	Chicken	HAL, Sackville, 1979
SK98a	UK	Chicken	HAL, Sackville, 1979
SK114a	UK	Chicken	HAL, Sackville 1980
SK125	Chlamydia	Cockatiel	HAL, Sackville, 1980
SK131	Reovirus	UK	HAL, Sackville, 1981
SK138a	Reovirus	UK	HAL, Sackville, 1983
SK164	Viral arthritis	Chicken	HAL, Sackville, 1988

[a] Disease condition suspected.
[b] The Health of Animals Laboratory, Sackville, New Brunswick, Canada.
[c] UK denotes not known.

2.2.2. Virus isolation in CELi cells

All virus samples were passaged once in primary CELi cell monolayers on 24-multiwell tissue culture plates that had been prepared as described in section 2.1.2. For virus inoculation, the GM on the monolayers was removed, and one drop of virus sample was added to each well.The plate was incubated in a humidified 5% CO_2 atmosphere at 37°C for 1 h to allow adsorption of virus to cells. One ml of maintenance medium (MM) was added to each well, and the plates were incubated and microscopically examined daily for 7 days for the appearance of cytopathic effects (CPE). Maintenance medium was the same as GM with FBS reduced to 2%.

2.2.3. Adaptation of avian reovirus to Vero cells

Attempts to adapt virus strains to Vero cells were performed by inoculating 0.1 ml of virus suspension onto confluent monolayers of Vero cells in 25 cm^2 tissue culture flasks, and adsorbing for 1 h at 37°C in humidified 5% CO_2 atmosphere. The inoculum was then replaced with 8 ml of MM, and the flasks were further incubated at 37°C. When the CPE appeared, cell cultures were frozen and thawed three times, and clarified at 800 g for 20 min to remove cell debris. The virus-containing

supernatant was either diluted 1:10 and used as inoculum for further passage or was stored at -70°C as stock viruses.

2.2.4. Viral growth curves in Vero cells

The virus strains used were FC, SK73a, SK103a, SK125 and SK138a. Thirty two 25 cm^2 tissue culture flasks with monolayers of Vero cells were inoculated with 10^5 $TCID_{50}$ in 0.1 ml of medium, considered sufficient to produce a "one-step" growth curve. After an adsorption period of 1 h at 37°C in a humidified 5% CO_2 atmosphere, the infected monolayers were washed once with warm PBS 'A', and 8 ml MM was then added to each flask and incubated. At each interval up to 144 h two flasks were removed and three fractions designated extracellular, cell-associated, and total viruses were harvested. To harvest extracellular virus, medium in one of the flasks was removed and clarified at 800 g for 20 min, and the supernatant was stored at -70°C until assayed for virus infectivity. Two ml of MM were added to the infected monolayer to harvest the cell-associated virus. The extracellular and cell-associated samples, as well as the flask to assay for total virus were frozen and thawed three times prior to titration for virus infectivity. The assay for the growth curve of one selected virus was

repeated three times.

2.2.5. Harvesting of virus grown in Vero cells

The following 8 methods were investigated for recovery of virus grown in Vero cells: 1,1,2-trichlorotrifluoro-ethane (Freon) extraction, treatment with sterile distilled deionized water (ddH_2O), trypsin treatment, and freeze-thawing 3, 5, 7, 10 and 15 times. Avian reovirus strain SK125 was used to examine the efficiency of these methods for releasing cell-associated virus. For this, eight 25 cm^2 tissue culture flasks with confluent Vero cell monolayers were each inoculated with 0.1 ml of virus and were regularly examined for CPE until approximately 75% of the monolayer contained CPE and before cell detachment from the substrate occurred, usually 48 h post-inoculation (PI). The medium of one flask was then discarded, and was replaced with 2 ml of ddH_2O. The flask was stored at -70°C and was later assayed for virus infectivity. A second flask was frozen and thawed three times, and centrifuged at 800 g for 20 min. The cell lysate was extracted once with Freon (J.T. Baker Chemical Co., Phillipsburg, New Jersey), and the aqueous phase was stored at -70°C until when assayed for virus infectivity. The medium of a third flask was discarded, and the infected cell monolayer was treated

with 1 ml of 0.25% trypsin at 37°C for 10 min. Following trypsinization, 2 ml of GM was added and the flask was stored at -70°C and later assayed for virus infectivity. The fourth, fifth, sixth, seventh and eighth flasks were frozen and thawed 3, 5, 7, 10 and 15 times, respectively, and following centrifugation at 800 g for 20 min to remove cellular debris, the supernatants were titrated to determine virus infectivity. Flasks treated with sterile ddH_2O or with trypsin were also frozen and thawed three times prior to titration of the viral supernatants. Each assay was repeated three times.

2.3. Trypsin sensitivity assays

To 0.1 ml of virus was added 50 μl of 2.5% trypsin (i.e. 0.83% overall trypsin concentration). The treated and control untreated virus samples were incubated at 37°C. After 45 min of incubation, the trypsinization was stopped with 0.1 ml of GM. Each assay was repeated three times and all samples were stored at -70°C prior to titration for virus infectivity.

2.4. Titration of virus

The serial 10-fold dilutions of each virus sample

were prepared in EMEM, and each dilution was transferred to four wells of a 96-well microtiter tissue culture plate using a 50-μl dropper. An equal volume of GM was added to the last vertical column on each plate to serve as cell control. Each well then received 50 μl of a fresh Vero cell suspension. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 7 days and were then examined microscopically prior to fixation and staining with 1% crystal violet in 70% ethanol. The virus titer was determined from the CPE using the Karber method (Lennette et al., 1979).

2.5. Gel electrophoresis of avian reovirus RNA

2.5.1. Extraction and preparation of viral double-stranded RNA

Each virus was grown in Vero cells in a 75-cm^2 tissue culture flask. At 48 h PI, when approximately 75% of the monolayer contained CPE and before cell detachment occurred, the medium was discarded and virus was harvested by treatment of the virus-infected cell monolayers with sterile ddH_2O as described above. Virus was pelleted by ultracentrifugation at 130,000 g for 4 h at 4°C. Each pellet was resuspended in sterile ddH_2O and the viral double-stranded (ds) RNA was released from

virion using 0.5% SDS-proteinase K (20 mg/ml) treatment at 37°C for 30 min. The nucleic acid was extracted by phenol:chloroform (25:24) treatment, and was precipitated in 0.1 Vol of 3M Na acetate and 2.5 Vol. of 100% cold ethanol.

Duplicate viral nucleic acid pellets were resuspended in diethylpyrocarbonate (DEPC) treated H_2O to a concentration of 50 μl. The viral dsRNA segments were labeled at the 3'-termini with [^{32}P]pCp (Amersham Canada Limited, Oakville, Ontario) and T4 RNA ligase (Pharmacia Canada Limited, Baie d'Urfe, Quebec) using standard techniques (Sambrook et. al., 1989). Briefly, 5 μl of sample RNA were mixed with 1 μl of T4 RNA ligase in a reaction buffer consisting of 1 M N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 10 mM adenosine triphosphate (ATP), and 10 X bovine serum albumin (BSA) to a reaction volume of 50 μl. One microliter of 10 μCi pCp P^{32} was added and the solution was incubated at 15°C for 16 h. The ^{32}P-end-labeled dsRNAs were then resolved by electrophoresis.

2.5.2. Polyacrylamide gel electrophoresis (PAGE)

The nucleic acid pellets were dissolved in sample reducing buffer consisting of 0.125 M Tris-HCl, 20% of

glycerol, and 0.002% of bromophenol blue (Laemmli, 1970) and heated in a boiling water bath for 2 min prior to gel electrophoresis. The viral dsRNA segments were resolved on discontinuous PAGE by using a 10% stacking gel and a 7.5% resolving gel. Electrophoresis was performed at a constant voltage of 100 v for 20 h at room temperature. Gels were silver stained using a commercial silver staining kit (BioRad, Mississauga, Ontario).

The ^{32}P-end-labeled dsRNAs were also resolved by electrophoresis on 7.5% resolving gels and were detected using autoradiography. For this, the gel was dried and exposed to X-ray film (Kodak X-OMAT, Picker International, Dartmouth, Nova Scotia) at -70°C for 1 to 4 days.

2.6 Virus neutralization of avian reovirus

2.6.1 Production of avian reovirus antisera in Guinea pigs

Eight male guinea pigs (200 g average body weight), housed and treated according to Canadian Council on Animal Care (CCAC) guidelines, were inoculated subcutaneously in the neck with 0.4 ml of avian reovirus strains FC, SK73a, SK125, SK98a, SK114a, Type 24, Type 59

or WVU 2937 emulsified (1:1) in Freund's complete adjuvant (Difco Laboratories, Detroit Michigan, USA). This was followed at 4 weeks with an intraperitoneal inoculation of 1 ml of the same virus alone. Antiserum was collected 7 days later, and was used in the virus-neutralization test. Before used to product the antisera, the guinea pigs were bled to check a preinoculation.

2.6.2 Virus-neutralization (VN) tests

In the VN tests, all viruses were separately used as antigens. The VN tests were carried out in 96-well microtiter tissue culture plates using 50 μl two-fold dilutions of serum against 100 $TCID_{50}$ of 50 μl, and incubated for 1 h at 37°C before addition of 50 μl of fresh Vero cells in medium to each well. Plates were examined as described for virus titration above (section 2.4) after a further 7 days incubation in humidified 5% CO_2 atmosphere at 37°C. The neutralization titer was interpreted as the reciprocal of the highest dilution of serum inhibiting CPE (Jones and Kibenge, 1984). The degree of antigenic relatedness of two viruses was calculated by applying the following formula of Archetti and Horsfall (1950) to the results of cross-neutralization tests.

$$r = \sqrt{\frac{\text{titer strain I antiserum II X titer strain II antiserum}}{\text{titer strain II antiserum I X titer strain I antiserum}}}$$

The criterion suggested by Wadey and Faragher (1981) for recognizing different serotypes of infectious bronchitis virus was used to group the avian reoviruses in the present study. Thus viruses were considered to be the same serotype if the r value was greater than 50% and intermediate (subtype) if the r value was 20% to 50%.

2.7 Experimental infection of chickens with avian reoviruses

2.7.1 Source of 1 day old chicks and management of experimental chickens

One-day-old egg-layer type chicks were hatched from embryonated SPF eggs from the Health of Animals Laboratory, Sackville, New Brunswick, Canada. Chickens were reared up to 6 weeks of age according to CCAC guidelines. The chickens were fed with a commercial starter ration throughout, and water was provided ad libitum.

2.7.2 Experimental design and sampling procedure

Groups of 16 one-day-old chicks were inoculated orally with 10^5 $TCID_{50}$ of virus in 0.4 ml of EMEM using strains SK125, SK98a, SK138a, SK103a, and SK73a. One group was left uninoculated to serve as control. All groups were reared separately and the experiment was continued for 6 weeks PI. Four chicks from each group were weighed and swabbed via the cloaca for virus isolation, and were killed by cervical dislocation at 1, 2, 3, and 6 weeks PI. Tissues of killed or dead chicks from infection were taken from the liver, spleen, heart, lung, kidney, bursa of Fabricius, caecal tonsil, duodenum, and hock joints for virus isolation and for histopathology.

2.7.3 Preparation of tissue samples for virus isolation

Portions of liver, heart, joint tissues, and cloacal swabs were individually homogenized to a 10% suspension in EMEM, containing 1 mg/ml streptomycin, 1000 I.U./ml penicillin, and 250 units/ml nystatin. All suspensions were clarified at 800 g for 20 min and 0.2 ml of the supernatant was inoculated onto two wells of Vero cell monolayers in a 24-multiwell tissue culture plate. Cultures were examined daily for the presence of CPE, and

up to three passages were conducted at 10-day intervals before a sample was recorded as negative.

2.7.4 Histopathology

Immediately following euthanasia, a complete postmortem was done on each chicken. Pieces of heart and liver were fixed in 10% buffered neutral formalin solution for at least 72 h. The skin was removed from the hind limbs and the left tibiotarsal joint was placed in a Cal-Ex II decalcifying-fixation solution (Fisher Scientific, Orangeburg, N.Y.) for at least five days. Once decalcified, joints were cut sagittally at the midline. All tissues were processed by routine techniques. Joints were embedded in paraffin so that whole joint section, tendons and ligaments could be examined. Tissues were sectioned at 5 μm, mounted on glass slides and stained with hematoxylin and eosin (Luna, 1968). The tissues were exposed to a transmitted-light photomicroscope III (Zeiss, Oberkochen) with a TMX 135-36 film (Eastman Kodak Company, Rochester, New York).

2.8 Statistics

The viral growth curves were analyzed by a non-linear regression procedure with the Gompertz model

(Draper et. al., 1981) in the SAS statistical package to compare among the extracellular, cell-associated, and total virus growth curves. The data on harvesting of virus grown in Vero cells, and from the trypsin sensitivity assays were analyzed by analysis of variance (Glantz, 1987). Body weights were analyzed using the "Student's" t-test (Glantz, 1987).

CHAPTER 3. RESULTS

3.1 Avian reovirus growth curves in Vero cells

Figures 1 to 5 show the growth curves of avian reovirus strains FC, SK125, SK73a, SK103a, and SK98a adapted to Vero cells by 3, 5, 4, 5, and 6 passages, respectively. The CPE, characterized by formation of multinucleated cells (synctia), was first detected in strain SK125 and SK98a at 14 h PI, and in the other three strains at 22 h PI. In four of five strains studied, the growth curve of cell-associated virus resembled that of total virus. The titers of total virus and cell-associated virus peaked at 30 to 96 h PI; whereas the maximum titers of extracellular virus were steadily rising throughout the 144 h observation periods.

The growth curve of SK73a was analyzed by the Nonlinear regression (NLIN) procedure with the Gompertz model in the SAS statistical package in order to determine if the differences between the extracellular virus curve and the cell-associated, and total virus curves were statistically significant (Fig. 6). However, this cell-associated character of avian reovirus replication in Vero cells could not be confirmed by statistical analysis. For this analysis, the amount of virus, A, at time t was modelled using an adaptation of the Gompertz model for growth curves (Draper et. al., 1981).

$$A = a\exp[- b_1 e^{-k_1 t} - b_2 e^{-k_2 t}]$$

In this model, k_1 indicates the magnitude of the ascending slope and k_2 the magnitude of the descending slope. For the extracellular virus curve, the estimated value of the descending slope (k2) is slightly less than 0 (Table 4) indicating a slight dip in the second half of the curve (Fig. 6). However, this coefficient was not statistically significant. The coefficient for the cell-associated virus curve was statistically significant. The growth curve of the total virus drops down more than that of the extracellular virus, but the k2 is not significantly different from 0. Thus the k2 of the total virus is between that of the extracellular and the cell-associated virus curves.

To determine whether the k2 of the total virus curve resembled that of the extracellular or the cell-associated virus curve, the following formula for the t test was used:

$$t = \frac{k_A - k_B}{\sqrt{S_{k_A}^2 + S_{k_B}^2}}$$

Where k_A and k_B represent the means of samples 1 and 2,

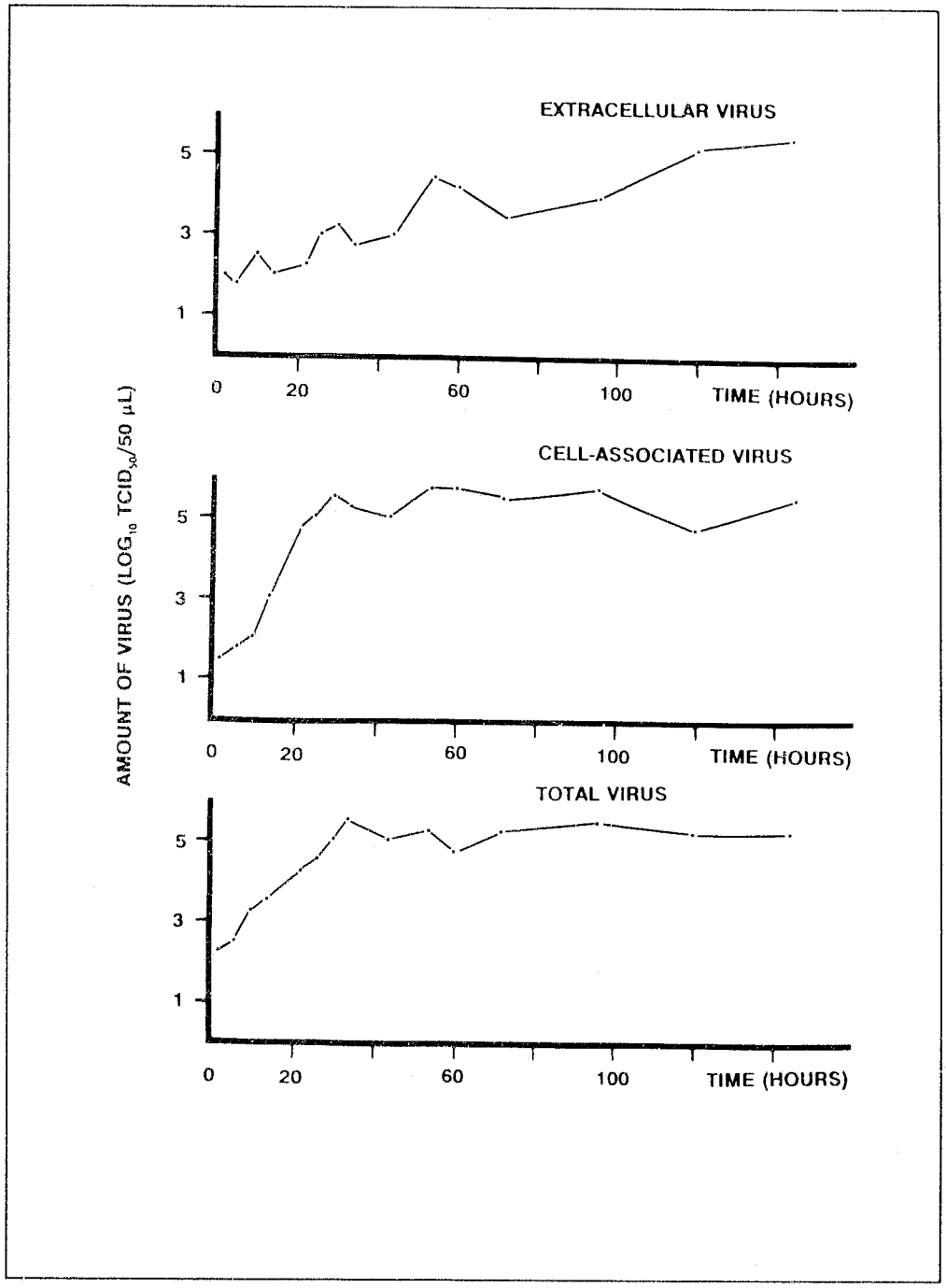

Figure 1. Growth curve of avian reovirus strain FC.

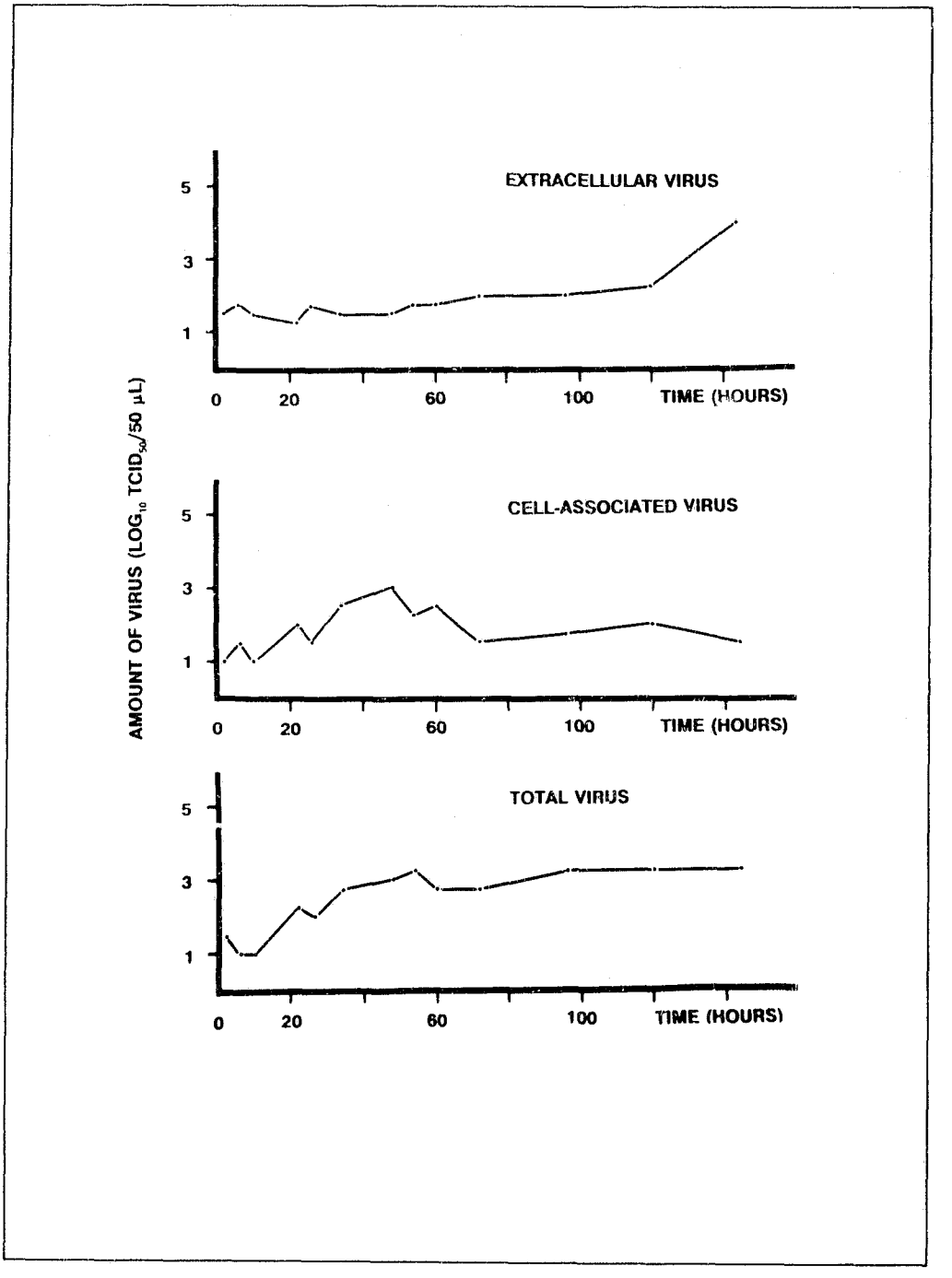

Figure 2. Growth curve of avian reovirus strain SK125.

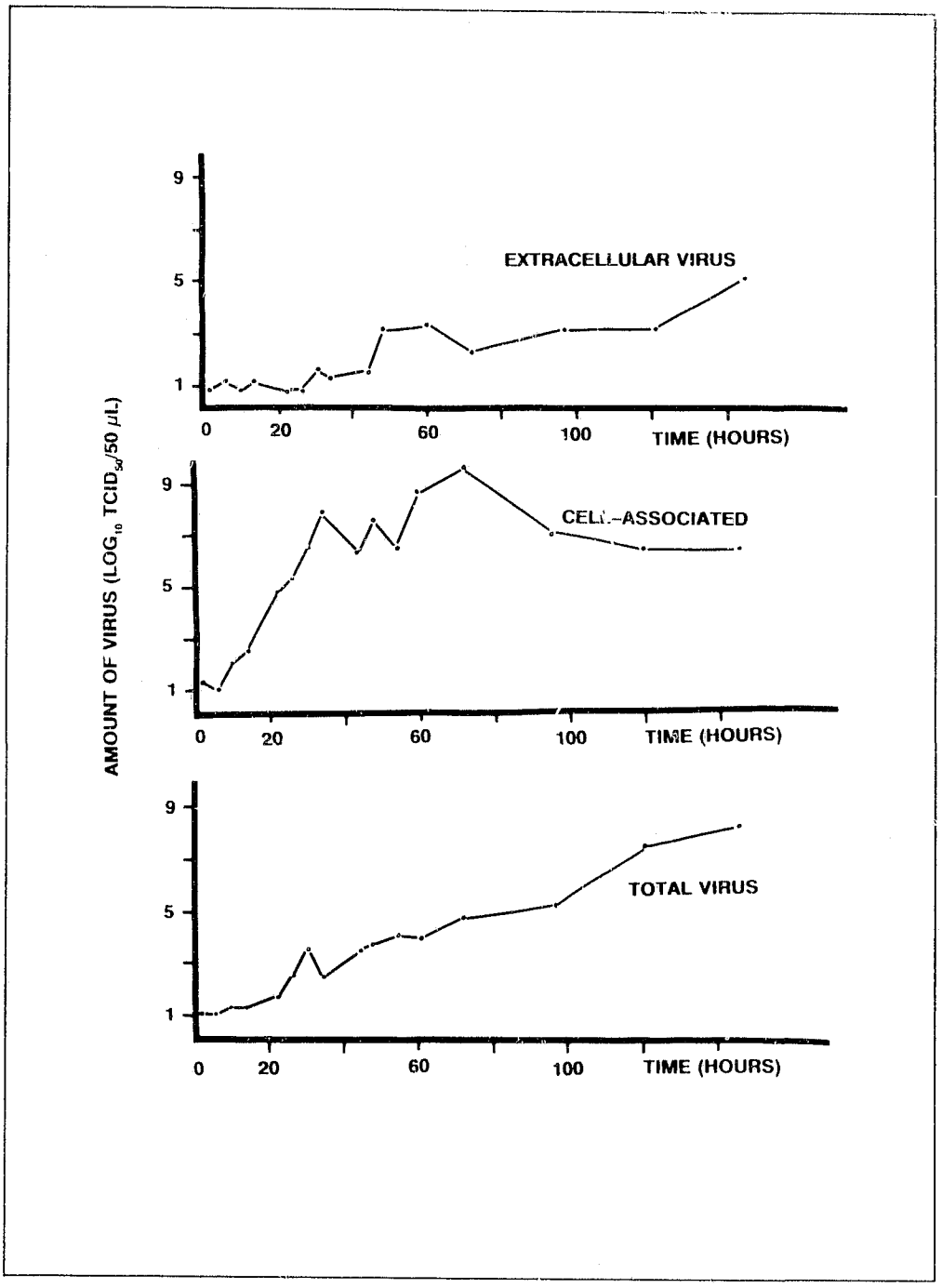

Figure 3. Growth curve of avian reovirus strain SK103a.

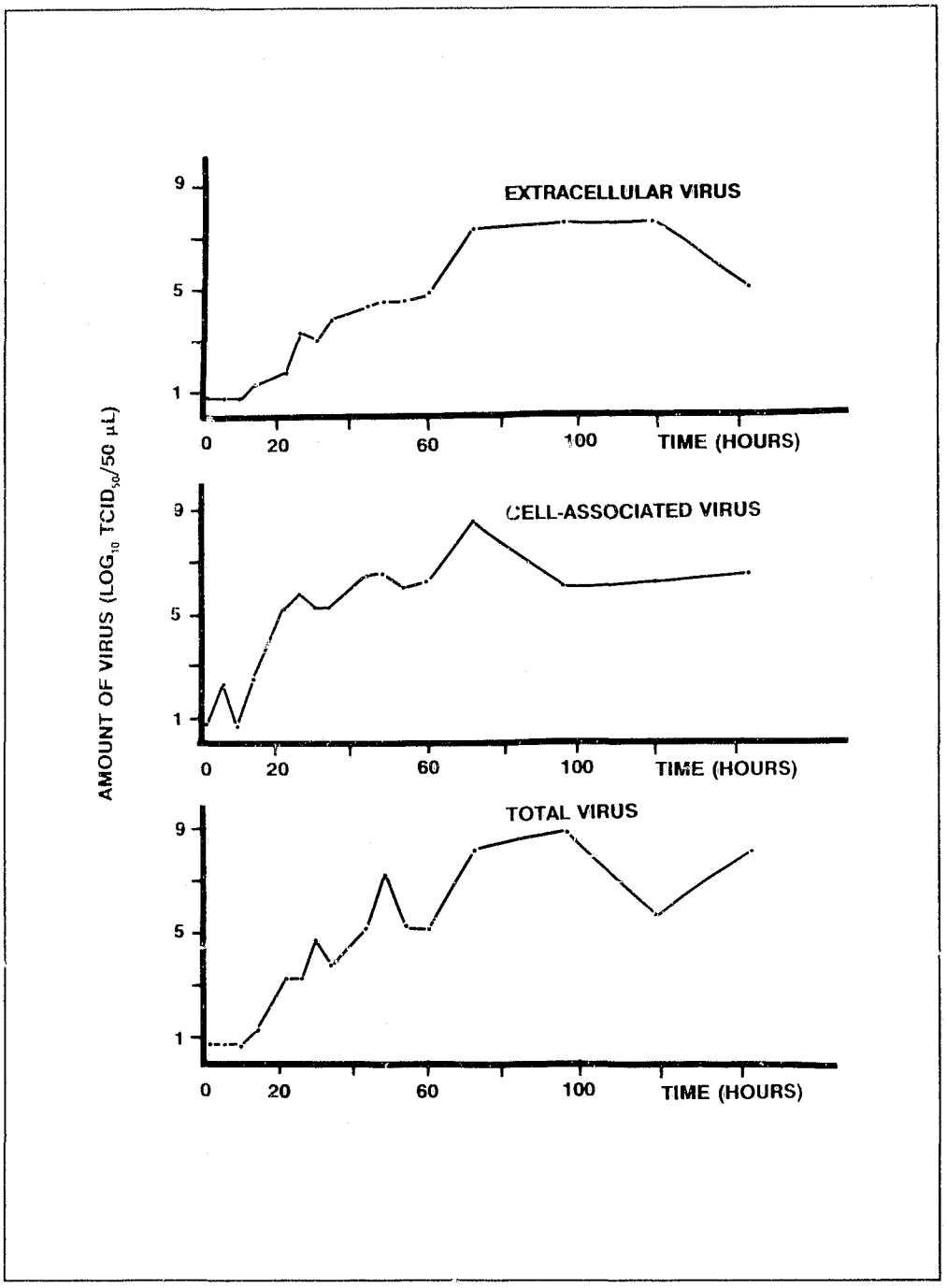

Figure 4. Growth curve of avian reovirus strain SK98a.

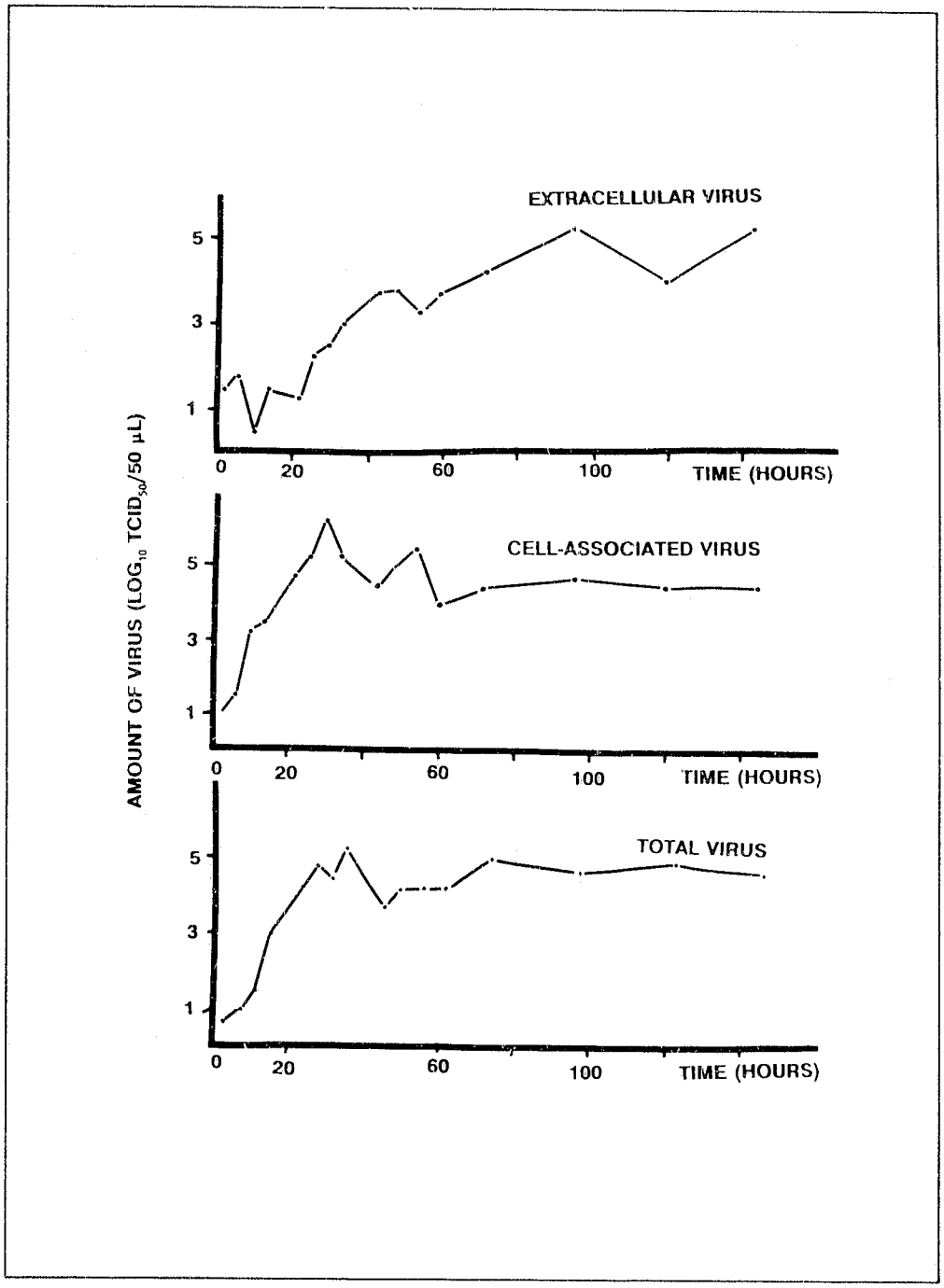

Figure 5. Growth curve of avian reovirus strain SK73a.

Table 4. Analysis of avian reovirus strain SK73a by the Nonlinear regression (NLIN) procedure in the SAS package.

Parameters	Virus harvest fraction	Estimates	Standard Error	Confidence Interval	
				Lower	Upper
k1[a]	Extra-cellular	0.022	0.008	0.004	0.038
	Cell-associated	0.046	0.029	-0.016	0.108
	Total	0.063	0.032	-0.005	0.130
k2[b]	Extra-cellular	-0.001	0.001	-0.002	0.000
	Cell-associated	-0.003	0.001	-0.006	0.000
	Total	-0.002	0.001	-0.005	0.000

[a] SE = Standard Error
[b] k1 = the ascending slope
[c] k2 = the descending slope

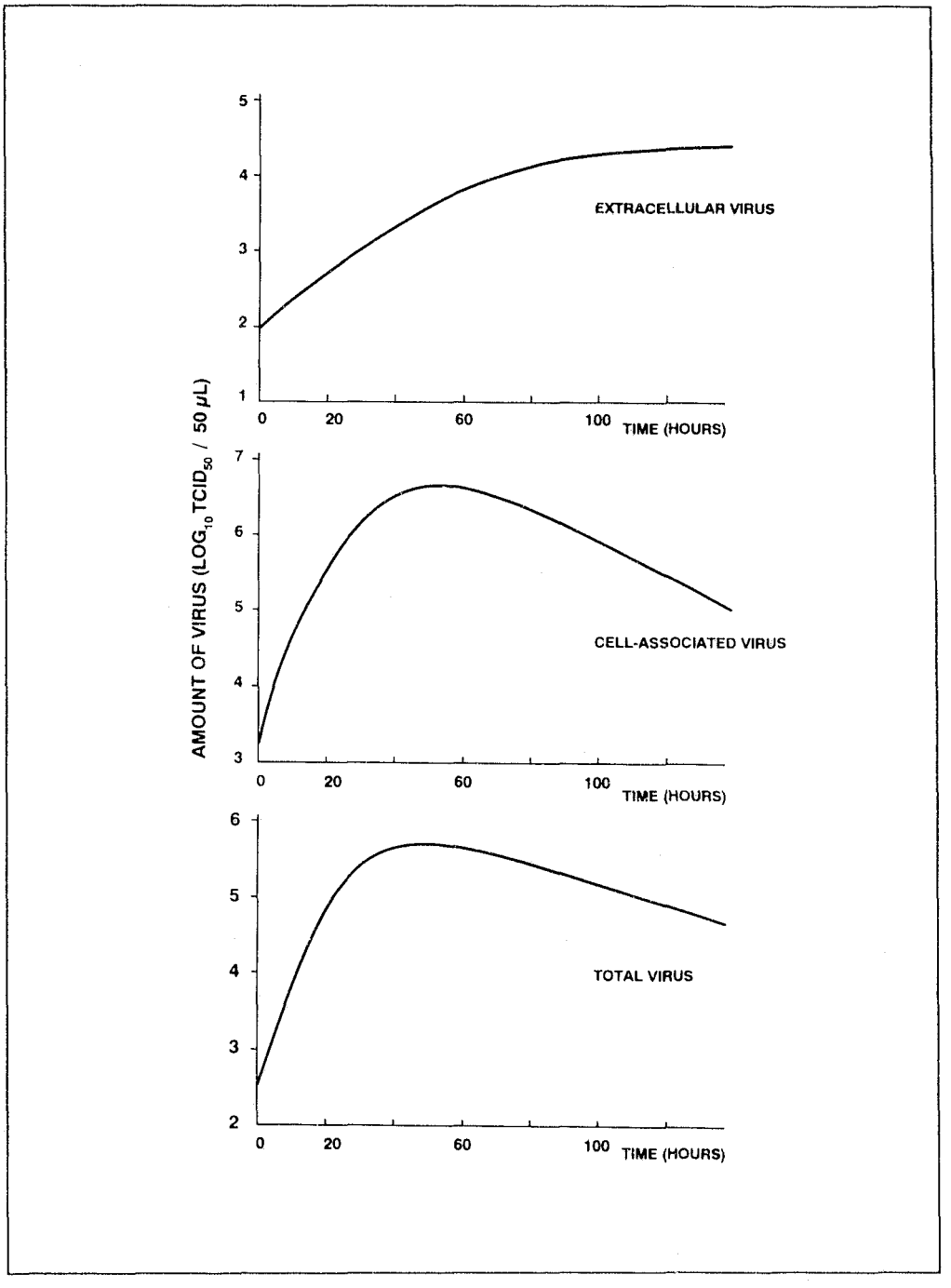

Figure 6. Growth curve of SK73a analyzed by the Nonlinear regression (NLIN)

The height: α; the ascending slope: k1; the descending slope: k2 (see Table 4).

respectively; while s_{kA}^2 and s_{kB}^2 represent the standard errors of the means of samples 1 and 2, respectively.
The t value for the k2 of the total virus curve and the extracellular virus curve is 0.919 ($P>0.05$), while the t of the k2 of the total virus curve and the cell-associated virus curve is 0.51807 ($P>0.05$). The fact that neither of these values is statistically significant ($P>0.05$) indicates that the amount of total virus declines at the same rate as the amounts of cell-associated and extracellular virus.

3.2 Harvesting of Vero-propagated virus by different treatments

Table 5 shows the effect of different treatments on dissociation of SK125 strain from Vero cells following propagation. Treatment with sterile ddH_2O yielded the highest amount of virus. Freezing and thawing the cell lysate 5 times, similar to the procedure used by Wilcox et. al. (1985) to adapt the RAM-1 avian reovirus strain to Vero cells, was intermediate, while trypsin treatment released the least amount of virus (Table 5).

3.3 Sensitivity of avian reoviruses to trypsin

Table 6 shows the effect of trypsin treatment of different avian reovirus strains. There was a wide variation

in the degree of sensitivity to trypsin. A drop in mean titer of 10^2 or more $TCID_{50}$/ml between treated and untreated virus samples was considered significant virus inactivation, and 6 viruses showing such a drop were judged sensitive; reovirus strain Type 24 being the most sensitive to trypsin treatment. Eight other avian reovirus strains were resistant to trypsin treatment. These results were reproducible over a wide period of incubations at 37°C, ranging from 15 to 60 min (data not shown).

3.4 Electrophoretic migration patterns of avian reovirus genomes

The migration patterns of the 14 viruses on discontinuos PAGE showed heterogeneity in the 10 RNA genome segments between isolates (Fig. 7). Four electropherotypes were recognized on the basis of the migration patterns of the S1 gene. Two main electropherotypes were recognized on the basis of the migration patterns of the M2 gene. The M2 genome segment of six strains migrated more slowly than that of the remaining 8 strains (Fig. 8).

Table 5. The effect of different treatments on dissociation of avian reovirus[a] from Vero cells following propagation.

Treatments	Viral titer[b]
Distilled deionized water	5.78 ± 0.61
Freon extraction	4.80 ± 0.63
Trypsin	3.55 ± 0.63
3 X freeze-thaw cycles	4.63 ± 0.36
5 X freeze-thaw cycles	4.05 ± 0.38
7 X freeze-thaw cycles	3.55 ± 0.76
10 X freeze-thaw cycles	3.97 ± 0.46
15 X freeze-thaw cycles	4.13 ± 0.60

[a] Avian reovirus strain SK125.
[b] Mean titer of virus expressed as negative $\log_{10}TCID_{50}$/ml ± standard error of mean (SEM).

Table 6. Effect of trypsin treatment on different avian reovirus strains

Avian Reovirus strain	Treated[a]	Untreated[a]	Degree of Sensitivity[b]
Type 24	3.30 ± 0.29	6.05 ± 0.14	2.75 ± 0.14
SK125	3.80 ± 0.52	6.30 ± 0.14	2.50 ± 0.43
SK73a	3.55 ± 0.00	5.80 ± 0.14	2.25 ± 0.14
SK58a	2.88 ± 0.36	4.97 ± 0.44	2.08 ± 0.30
SK164	4.47 ± 0.30	6.47 ± 0.30	2.00 ± 0.14
SK114a	4.72 ± 0.08	6.63 ± 0.46	1.92 ± 0.42
Type 59	4.22 ± 0.17	5.97 ± 0.17	1.75 ± 0.29
SK25a	4.13 ± 0.08	5.80 ± 0.14	1.67 ± 0.08
SK98a	3.97 ± 0.08	5.63 ± 0.08	1.67 ± 0.08
WVU2937	4.05 ± 0.14	5.63 ± 0.08	1.58 ± 0.08
SK103a	3.63 ± 0.22	5.13 ± 0.30	1.50 ± 0.25
SK131	3.30 ± 0.25	4.55 ± 0.43	1.25 ± 0.25
FC	4.97 ± 0.51	6.22 ± 0.30	1.25 ± 0.25
SK138a	3.72 ± 0.51	4.80 ± 1.01	1.08 ± 0.55

[a] Mean titer of virus expressed as negative $\log_{10}TCID_{50}$/ml ± standard error of mean.

[b] Degree of sensitivity is expressed as difference in mean virus titer (± standard error of mean) between virus treated and untreated with trypsin. Viruses with a degree of sensitivity ≥ 2.0 were considered sensitive to trypsin

3.5 Serological relationships of avian reoviruses

The neutralization titers of the Guinea pig antisera against the 14 avian reoviruses are shown in Table 7. The geometric mean of the homologous titers varied from 2.2 $\log_{10}$ to 3.4 $\log_{10}$ while the heterologous titers ranged from <1.0 $\log_{10}$ to 3.4 $\log_{10}$. Antisera to virus strains Type 24 and SK114a had neutralizing titers against all the 14 viruses tested while virus strains SK125 and SK25a were neutralized to some degree by all 8 antisera (Table 7) indicating considerable heterogeneity of neutralizing antigens among the virus strains examined. On the basis of the r values in Table 8, the viruses could be grouped into three distinct serotypes represented by strains FC, Type 59, and SK125. The FC serotype which contained SK73a also cross-reacted with Type 24, and SK98a by 41% and 29%, respectively. Since SK98a, WVU2937, and SK114a also show considerable cross-reactions, they were all considered subtypes of the FC serotype (Table 8).

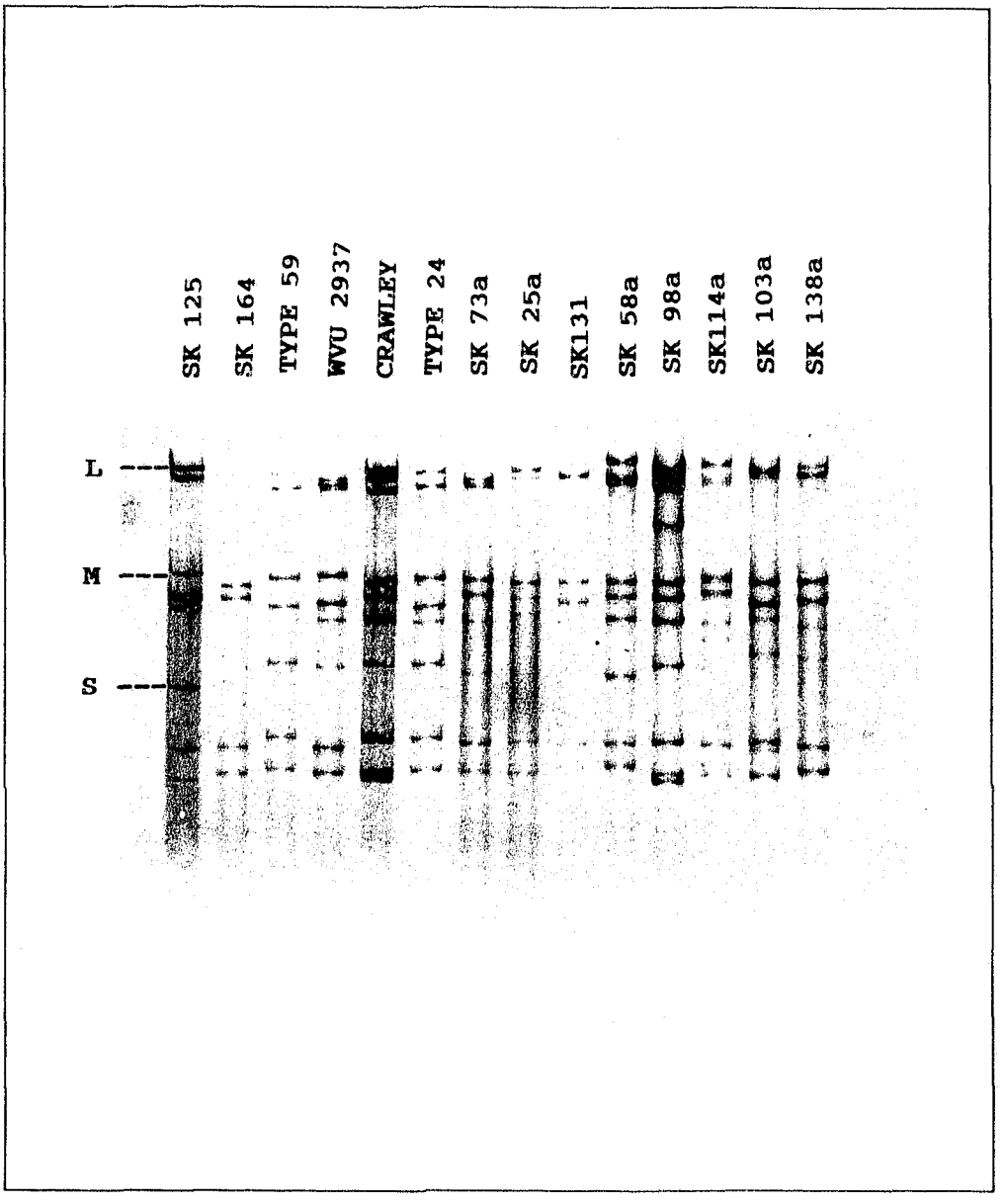

Figure 7. Comparison of migration patterns of genome segments of 14 avian reoviruses.

Discontinuous PAGE by using a 7.5% resolving gel. L: 3 large segments; M: 3 medium segments; S: 4 small segments.

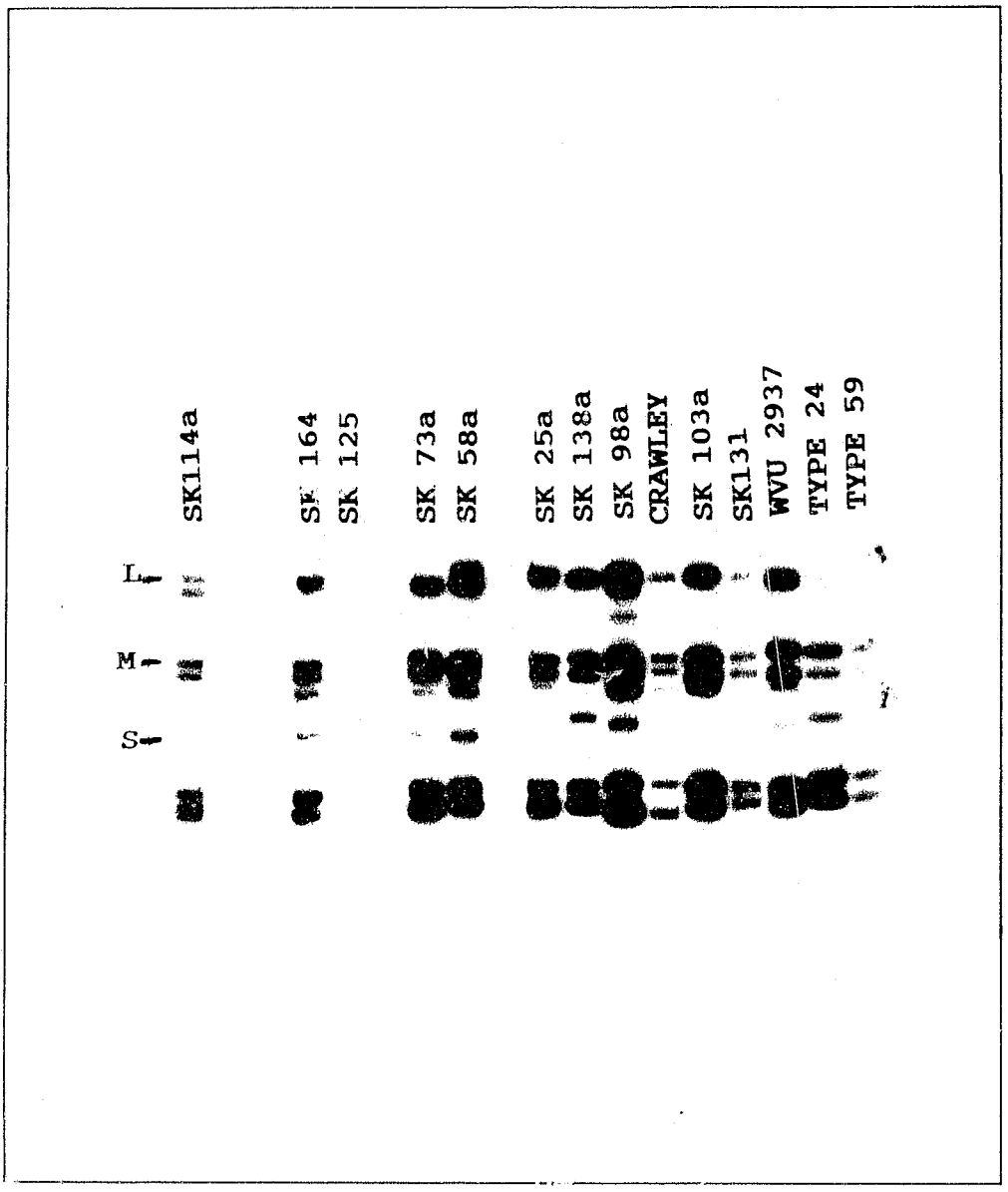

Figure 8. Comparison of migration patterns of ^{32}P-end -labeled genome segments of avian reoviruses

The viruses are arranged on the basis of the migration of the M2 gene segment, from the slowest to the fastest moving M2 relative to the M1 gene segment.
L: 3 large segments; M: 3 medium segments; S: small segments.

3.6 Avian reovirus experimental infections

3.6.1 Clinical signs

Of five groups of chickens infected with avian reoviruses, eight of sixteen chickens of the group inoculated with strain SK98a died within 1 week PI (Table 9). The livers of these chickens were moderately swollen and had focal to diffuse areas of pale discoloration. The surviving chickens had swollen joints at 2 and 3 weeks PI (data not shown). No clinical signs were detected in chickens inoculated with the other avian reovirus strains.

Table 10 and Fig. 9 show body weight gains of all surviving chickens in all groups. The mean body weight gains of chickens inoculated with SK125 were significantly lower ($P<0.05$) than those of the control group.

Table 7. Homologous and heterologous neutralisation titers of fourteen avian reovirus isolates

Trypsin assay[b]	Virus	Antiserum							
		FC	SK73a	Type 24	SK98a	wvu2937	SK114a	Type 59	SK125
R	FC	**2.80**[a]	2.20	2.38	2.20	1.60	2.50	2.08	0.00
S	SK73a	2.81	**2.81**	2.81	2.50	0.00	1.90	1.00	0.00
S	Type 24	1.60	1.60	**2.38**	1.78	1.00	2.20	1.18	0.00
R	SK98a	2.20	1.90	2.38	**2.68**	1.60	2.20	2.38	0.00
R	WVU2937	0.00	0.00	2.81	2.38	**2.20**	3.28	1.30	0.00
S	SK114a	0.00	0.00	2.20	0.00	0.00	**3.41**	0.00	0.00
R	Type 59	0.00	1.30	2.68	0.00	0.00	1.60	**2.81**	0.00
S	SK125	2.81	3.41	2.20	2.50	2.08	2.98	1.90	**2.81**
S	SK164	3.11	3.12	2.20	2.08	2.50	2.38	0.00	2.20
R	SK2[illegible]a	2.81	1.60	2.20	2.81	2.81	2.38	1.00	1.30
R	SK[illegible]3a	0.00	0.00	1.30	2.50	2.38	3.41	1.90	0.00
R	SK131	0.00	0.00	2.50	3.28	2.50	2.50	1.78	0.00
R	SK138a	0.00	0.00	2.20	0.00	2.50	2.81	1.90	0.00
S	SK58a	0.00	0.00	1.30	2.50	0.00	2.50	1.30	0.00

[a] Values represent $\log_{10}$ of geometric mean titer.
[b] Result of trypsin sensitivity assay (from Table 6); R denotes trypsin resistant, and S denotes trypsin sensitive virus strain.

Table 8. Serological relationships between avian reovirus isolates calculated from the neutralisation titers

Trypsin assay[a]	Virus	FC	SK73a	Type 24	SK98a	WVU2937	SK114a	Type 59	SK125
R	FC	100[b]	50	25	29	0	0	0	0
S	SK73a		100	41	29	0	0	2	0
S	Type 24			100	41	35	0	0	0
R	SK 98a				100	35	0	0	0
R	WVU2937					100	20	1	0
S	SK114a						100	0	0
R	Type 59							100	0
S	SK125								100
Sero-type[a]		A	A	A/B	A/B	B	B/C	D	E

[a] Result of trypsin sensitivity assay (from Table 6); R denotes trypsin resistant, and S denotes trypsin sensitive virus strain.
[b] The antigenic relationships calculated as r values (Archetti and Horsfall, 1950), and expressed as a percentage.
[c] A, A/B, B, B/C = 1 serotype; D = 1 serotype; E = 1 serotype.

TABLE 9. Percent cummulative mortality of chicks by 1 week PI

Virus strain	Trypsin assay[a]	Approx. virus dose inoculation	No. chicks used	% cumulative mortality
SK103a	R	5.65	16	0
SK138a	R	5.90	16	0
SK98a	R	7.25	16	50
SK73a	S	6.25	16	0
SK125	S	6.40	16	0
Control	-	-	14	0

[a] Result of trypsin sensitivity assay (from Table 6); R denotes trypsin resistant, and S denotes trypsin sensitive virus strain.

3.6.2 Gross and histopathologic changes

Nine of 16 chickens (56%) inoculated with reovirus strain SK98a had conspicuous areas of hepatic necrosis. The inflammatory response in chickens dying within the first few days of inoculation was essentially non-existent, while those dying later had considerable infiltration of mononuclear cells and occasional heterophils (Fig. 10). The only chicken of this group that survived more than 2 weeks also had areas of hepatic necrosis. None of the controls (Fig. 11), and of the chickens inoculated with other avian reovirus strains had evidence of necrosis or inflammation in the liver. Microscopic changes in tendons and synovial membranes were present in chickens inoculated with avian reovirus strains SK98a, SK138a and SK103a (Table 11). Affected tissues had a diffuse infiltration of lymphocytes and macrophages, and moderate hyperplasia of synoviocytes in both joints and tendon sheaths (Fig. 12, 13, 14, 15, 16 and 17). These lesions were notably more severe in chickens infected with strain SK98a as compared with those infected with strains SK138a and SK103a. In addition, the diaphysis of 5 out of 8 (62%) one-week old chickens inoculated with reovirus strain SK98a had pieces of retained cartilage cone (Fig. 18 and 19). This change in the cartilage cone was not seen in any of the control chickens (Fig. 20) and was seen only in one chicken in each of the groups inoculated with reovirus strain SK125 and SK103a.

Table 10. Mean body weight gains of chicken inoculated with avian reovirus

Avian reovirus	Trypsin assay[a]	No. chicks used	Mean body weight gains ± SD[b] 7 D	14 D	21 D	42 D
SK73a	S	16	69.5±3.6	137.4±10.9	206.2±15.8	508.9±49.1
Control	-	14	75.8±7.7	145.0±16.3	199.6±22.5	517.0±31.5
SK125	S	16	63.2[c]±6.2	113.7± 9.1	193.4±10.8	496.5±42.1
SK98a	R	16	ND[d]	104.1±26.6	158.6±32.5	ND
Control	-	15	74.8±6.5	123.8± 8.4	196.9±35.1	486.9±55.4
SK103a	R	16	69.5±3.6	136.7±17.0	243.1±28.9	551.8±26.2
SK138a	R	16	71.0±6.6	123.2± 9.4	203.0±22.3	531.1±52.7
Control	-	14	67.8±5.0	118.2±13.8	167.7 ± 4.4	501.1±85.1

[a] Result of trypsin sensitivity assay (from Table 6);
R denotes trypsin resistant, and S denotes trypsin sensitive virus strain.
[b] Standard deviation.
[c] Significantly different from the control ($P<0.05$).
[d] Not done because of high mortality by 1 week PI.

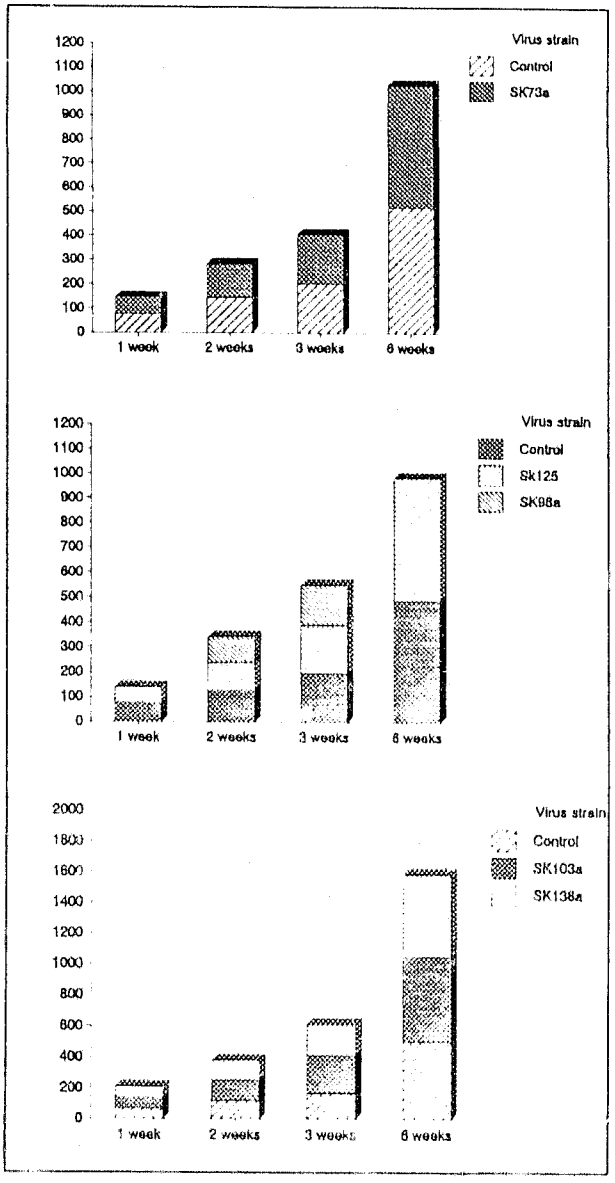

Figure 9. Mean body weight gains of chickens inoculated with avian reovirus

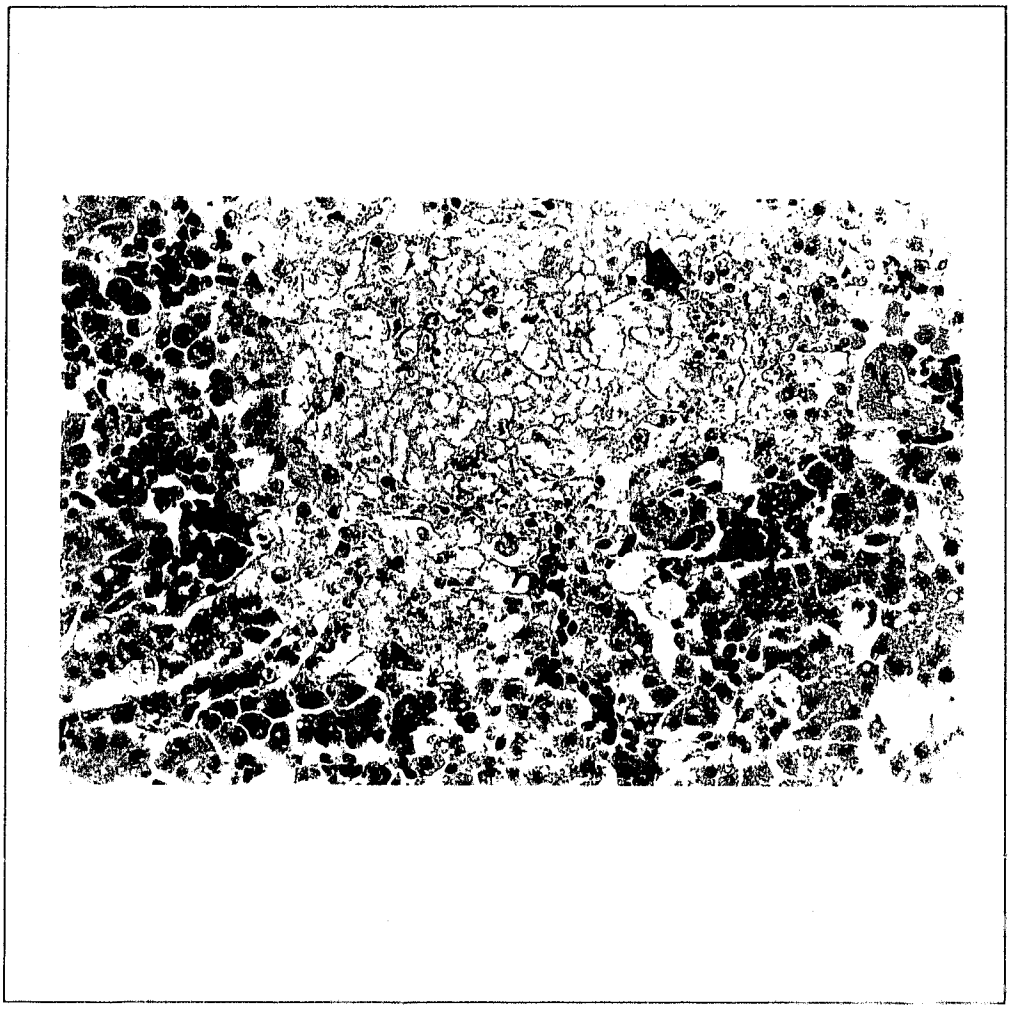

Figure 10. Liver from a chicken dying acutely following inoculation with strain SK98a. X 410.

Large area of coagulative necrosis (arrow)

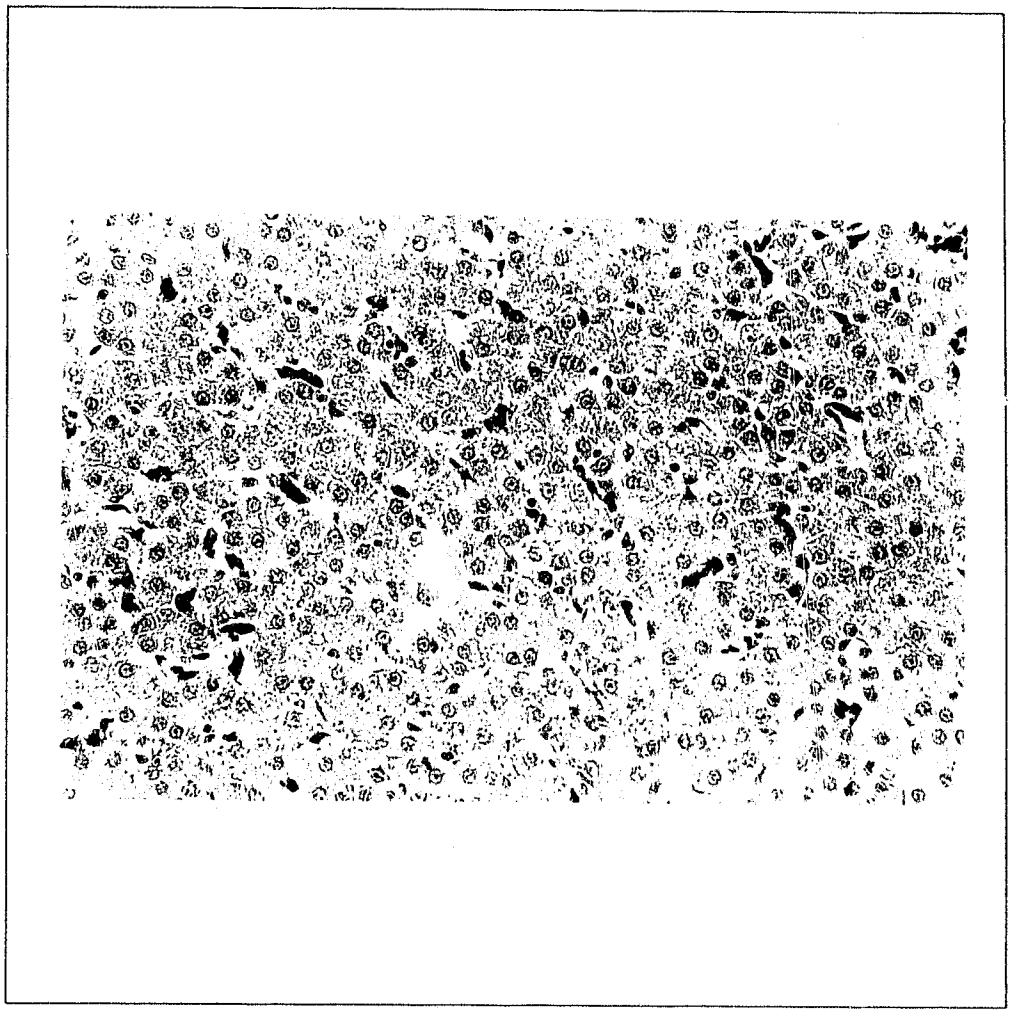

Figure 11. Liver from a control chicken 7 days old. X 310.

Normal architecture of hepatocytes and sinusoids.

Avian reovirus strain SK73a did not elicit any visible change in joints or tendon sheaths of inoculated chickens.

3.6.3 Virus localization in tissues

Table 12 shows virus persistence in the various tissues following inoculation with the 5 avian reovirus strains. The virus isolation rate from cloacal swabs and liver samples were poor for all virus groups except SK98a at 7 days PI. Virus was recovered from heart and hock joint tissues of chickens inoculated with SK103a for up to 21 day PI. Throughout the duration of this experiment, virus was only recovered once from chickens inoculated with strain SK125, from heart samples of 2/4 chickens at 7 days PI (Table 12).

Table 11. Percentage of chickens with tenosynovitis following inoculation with selected strains of avian reovirus

Virus strain	Trypsin assay[a]	Days after inoculation			
		7	14	21	42
Control	-	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
SK73a	S	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
SK125	S	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
SK103a	R	0/4 (0%)	0/4 (0%)	0/4 (0%)	1/4 (25%)
SK138a	R	0/4 (0%)	0/4 (0%)	1/4 (25%)	2/4 (50%)
SK98a	R	0/8 (0%)	4/4 (100%)	4/4 (100%)	ND[b]

[a] Result of trypsin sensitivity assay (from Table 6); R denotes trypsin resistant, and S denotes trypsin sensitive virus strain.
[b] Not done because of high mortality by 1 week PI.

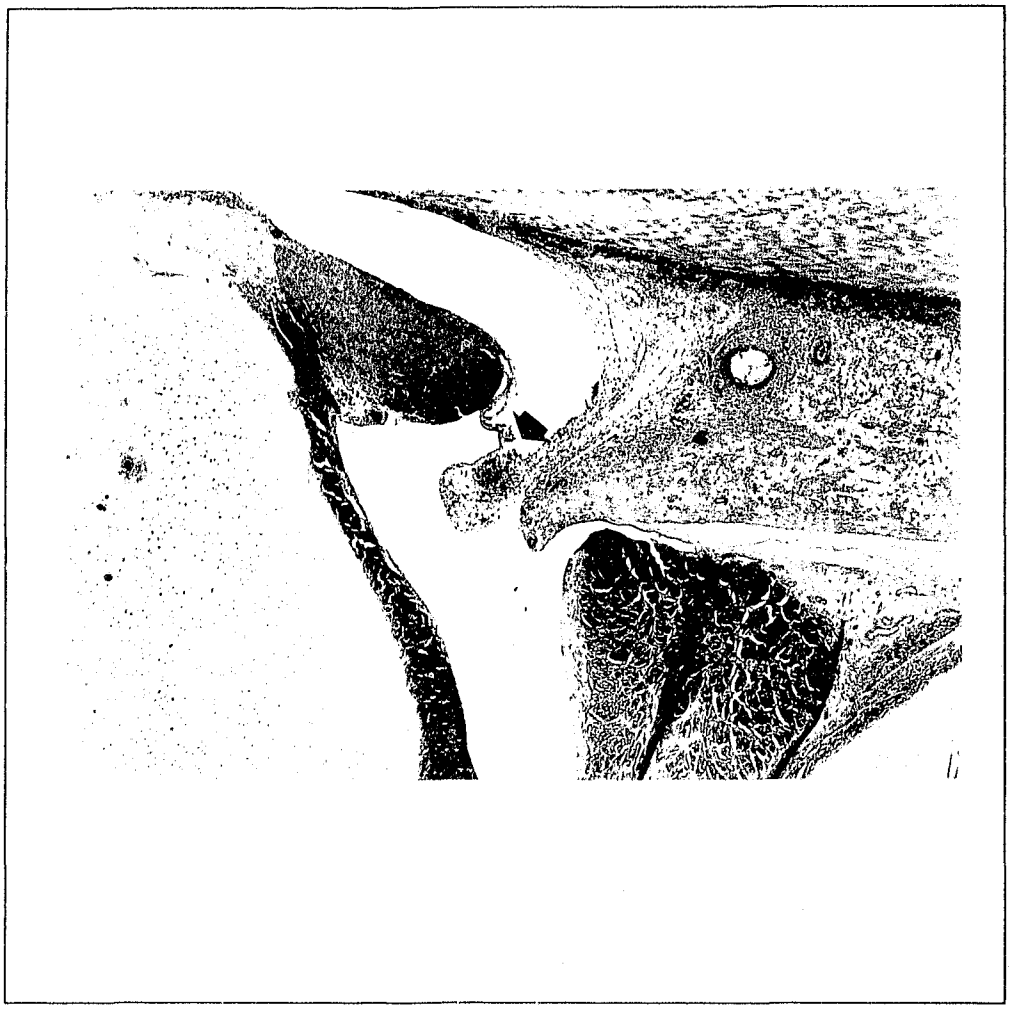

Figure 12. Hock joint of a chicken 21 days after inoculation with SK98a. X 50.

Marked thickening of syrovial membrane due to extensive diffuse infiltration of lympocytes and macrophages in the synovial membrane (arrows)

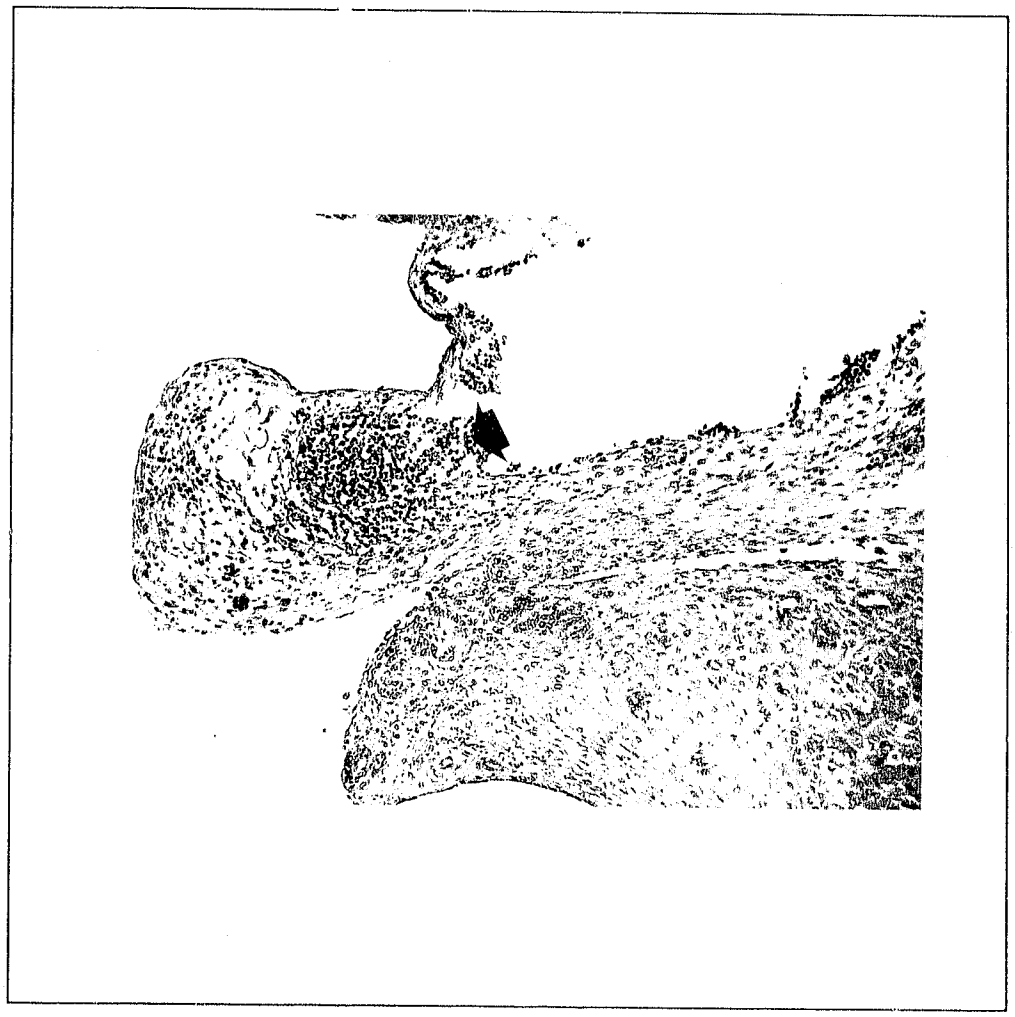

Figure 13. Close up view of a synovial membrane from a hock joint of a chicken 21 days after inoculation with SK98a. X 120.

Extensive infiltration of lymphocytes and macrophages in the synovial membrane (arrow)

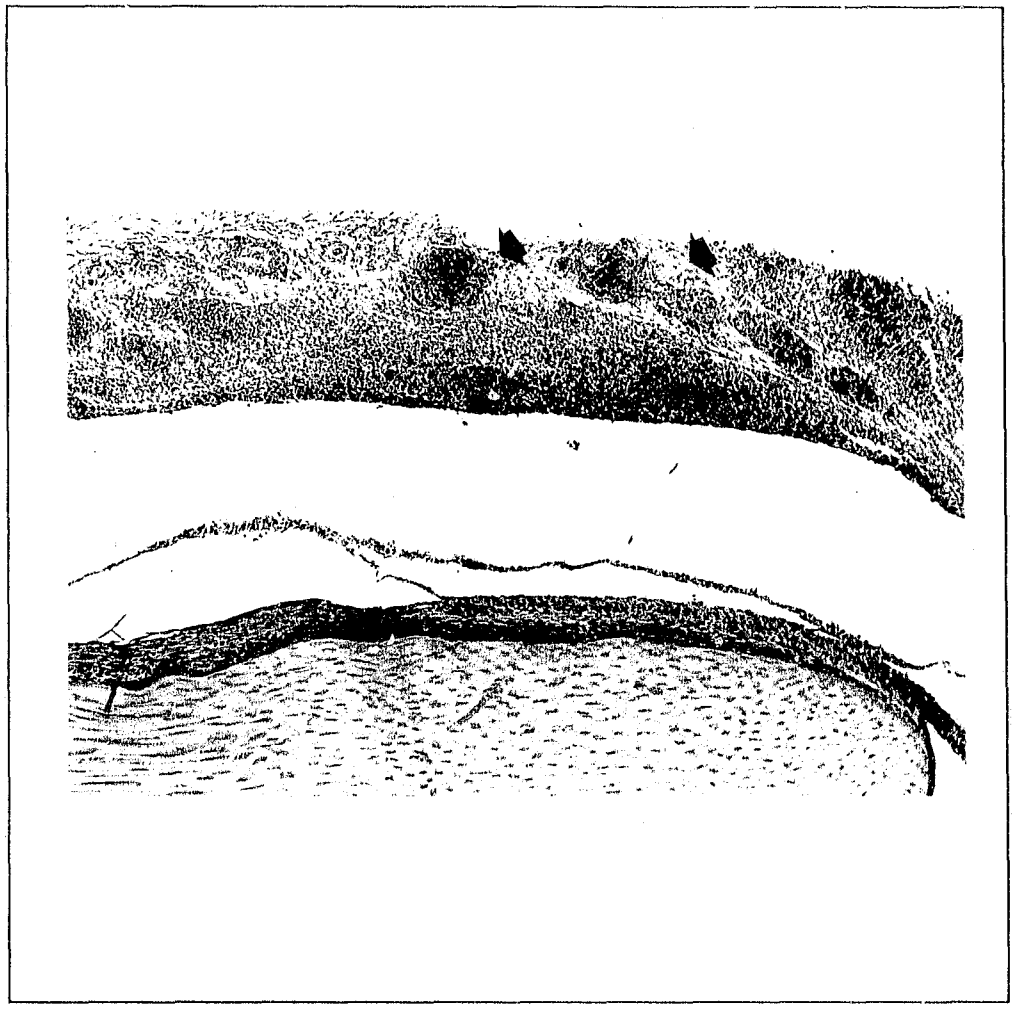

Figure 14. Tendon sheath of a chicken killed 21 days after inoculation with strain SK98a. X 125.

Thickening of synovial membrane due to massive infiltration of mononuclear cells with formation of perivascular lymphoid nodules (arrows).

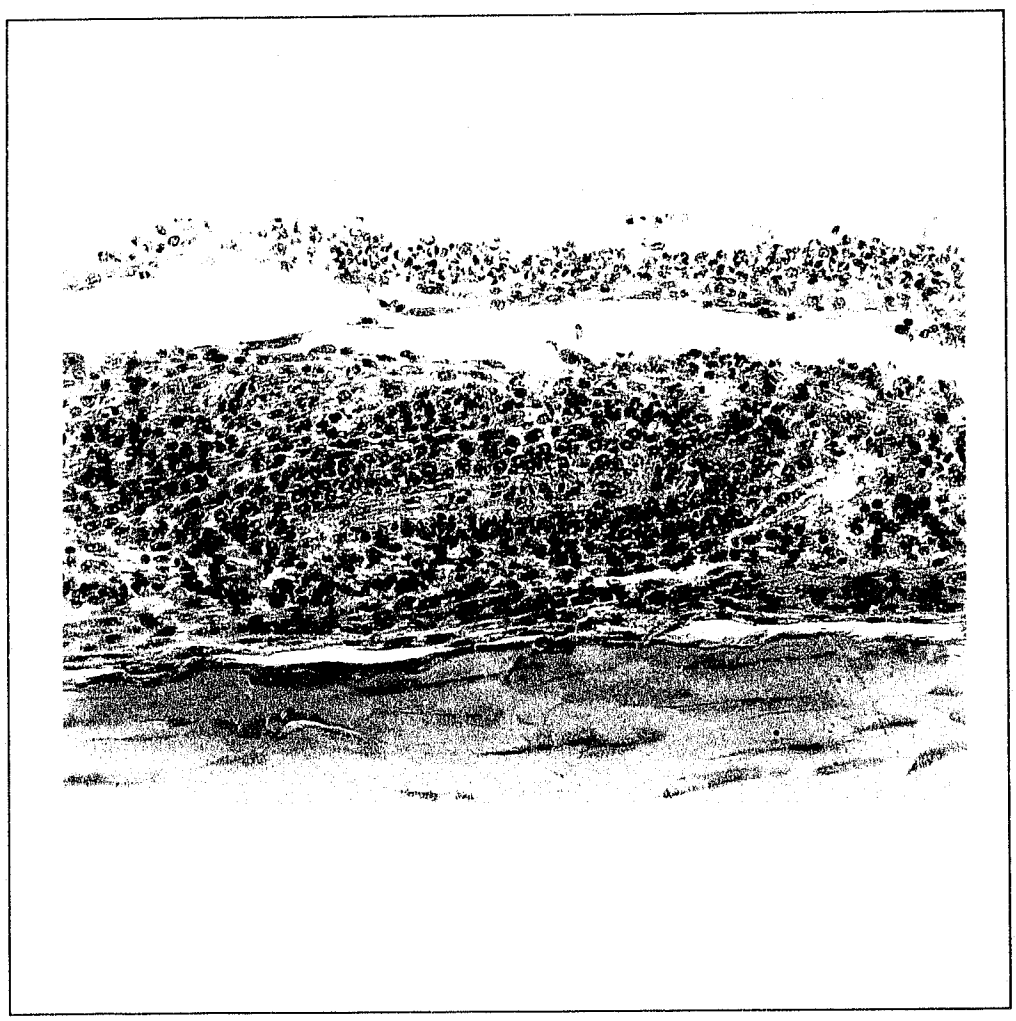

Figure 15. Tendon sheath of a chicken killed 21 days. after inoculation with strain SK98a. X 310.

Synovial membrane showing extensive infiltration of lymphocytes and plasma cells.

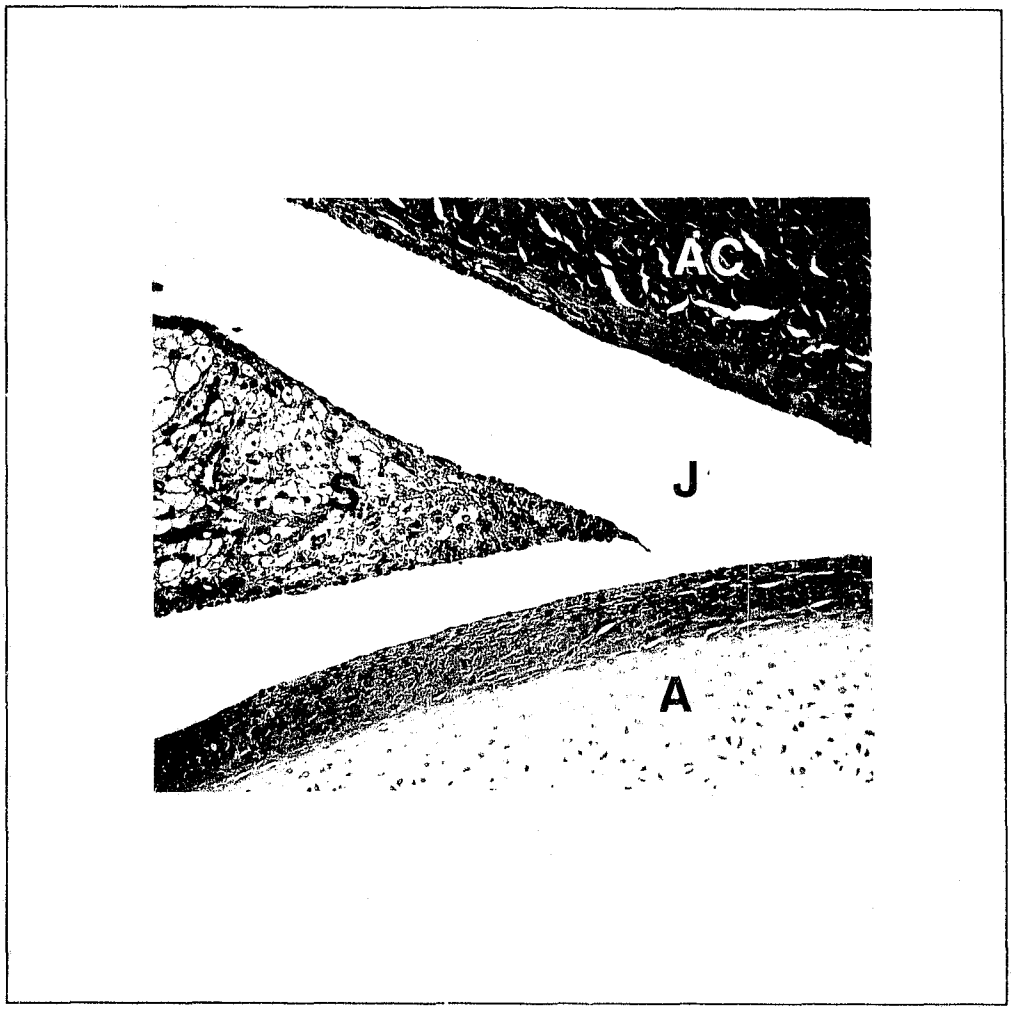

Figure 16. Hock joints from a control chicken 21 days old. X 12.

AC=articular capsule; J=joint cavity; A=articular cartilage; S=synovial membrane.

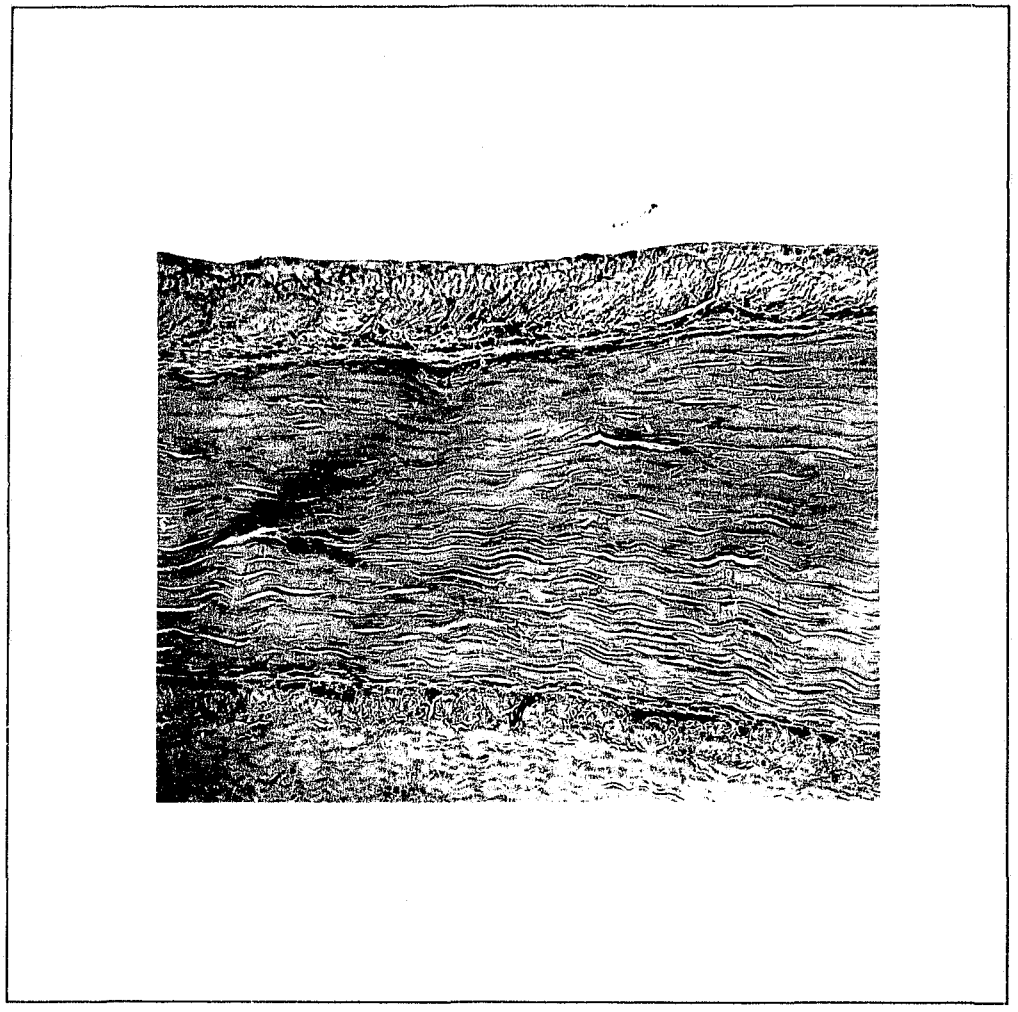

Figure 17. Tendon sheath from a control chicken 14 days old. X 120.

Note ordinarly arrangement of fibres and lack of cellular infiltrates.

Figure 18. Long bone from a chicken 7 days old inoculated with reovirus strain SK98a. X30.

Large pieces of retained cartilage cone in the diaphysis (arrow).

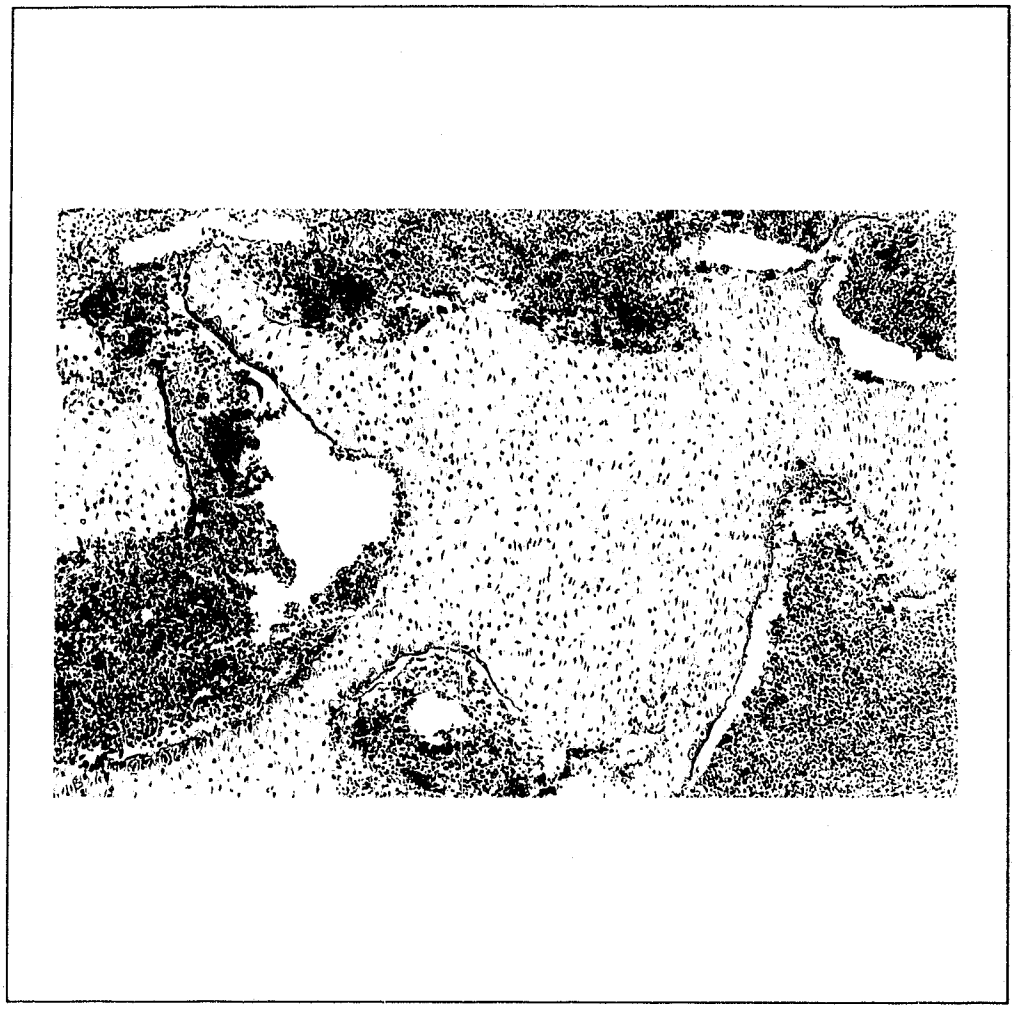

Figure 19. Close up view of a long bone from a chicken 7 days old inoculated with reovirus strain SK98a. X 125.

Large pieces of retained cartilage cone in the diaphysis. Note chondrocytes containing single nucleus.

Figure 20. Long bone from a 7 days old control chicken. X 30.

Note normal bone trabeculae in diaphysis and absence of retained cartilage cone.

Table 12. Virus isolation from tissue samples of chickens inoculated with avian reoviruses

Trypsin assay[a]	Virus strain	7 days PI				14 days PI				21 days PI				42 days PI			
		CS[b]	Liv	Hrt	HkJt	CS	Liv	Hrt	HkJt	CS	Liv	Hrt	HkJt	CS	Liv	Hrt	HkJt
R	SK103a	0[c] 100	0	75		0	0	0	100	0	0	0	75	0	0	0	75
R	SK138a	0	0	75						0	0	75	100	0	0	0	100
R	SK98a	50[d]	100	100	100	0	0	75	75	0	0	100	100	ND[e]	ND	ND	ND
S	SK73a	0	0	100	0	0	100	25	0	0	0	25	75	25	0	0	0
S	SK125	0	0	50	0	0	0	0	0	0	0	0	0	0	0	0	0
-	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Result of trypsin sensitivity assay (from Table 6); R denotes trypsin resistant, and S denotes trypsin sensitive virus strain.
[b] CS = cloacal swab, Liv = liver, Hrt = heart, HkJt = hock joint tissue samples.
[c] The virus isolations were expressed in percentage.
[d] Includes chickens dead by 7 days PI.
[e] ND denotes not done.

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

All 14 avian reoviruses isolated in CELi cells induced serially sustained CPE during successive passages in Vero cells indicating that they were adapted to grow in the Vero cell line. Hussian et. al. (1981) previously reported successful adaptation of three Australian isolates examined, although Wilcox et. al. (1985) could only adapt one strain, designated RAM-1, of six other Australian avian reovirus strains to Vero cells. The RAM-1 strain had been passaged 4 to 6 times more in chicken cells than the unadaptable strains. In the present study, prior to the single passage in CELi cell cultures, most isolates had only been propagated in chicken embryonated eggs 1 to 2 times, suggesting that cell passage history of the reovirus strain is probably not a major factor in adaptation to Vero cells. It is probable that variation either in culture conditions or between batches of Vero cell line, and individual virus strain variation may be responsible for the different success rates of adaptation of avian reoviruses to Vero cells. It has been recently demonstrated that avian reovirus strain S1133 can replicate in Mouse L cells at pH 6.4 and pH 7.2 but not pH 8.2, suggesting that a basic pH in the culture medium is responsible for the inhibition in viral replication (Mallo et. al., 1991a). These authors also showed that the presence of Actinomycin D during infection increased viral replication in L cells by

eliminating host mRNA competition for the translation machinery (Mallo et. al., 1991b). This suggests that virus replication in L cells is also dependent on the multiplicity of virus infection. The same may be true for avian reovirus replication in Vero cells.

The growth curves of 5 avian reovirus strains were determined to further characterize the nature of avian reovirus replication in Vero cells. For FC, SK125, SK73a, and SK103 strains, the growth curves of the cell-associated virus resembled that of total virus which indicated that these virus strains were highly cell-associated, and this may also be true for all avian reoviruses adapted to replicate in Vero cells.

The traditional method of harvesting stock cultures of reoviruses involves freezing and thawing of infected monolayers three times, and clarification of the supernatant by low speed centrifugation. However, this method would result in poor virus recovery if the virus were predominantly cell-associated, as is the case with the Vero-propagated avian reoviruses. It was observed that strain SK125 rapidly produced strong CPE in Vero cells, but stock cultures had low viral titers upon titration. This could be because SK125 makes a lot of fusion protein (Wilcox and Compans, 1982) resulting in strong CPE, but only immature virus or defective interfering particles are produced which are not detectable by titration. However, based on the viral titers in Fig.2, this virus also appeared to be more cell-associated in Vero cells than the

other 4 strains. The SK125 strain was therefore chosen for analysis of different procedures for releasing of cell-associated virus propagated in Vero cells. In the present study, treatment of infected cell monolayers with sterile ddH_2O was the most effective for releasing cell-associated virus (Table 5). In fact this procedure was significantly better than the traditional method of repeated freezing and thawing to lyse cells (Wilcox et. al., 1985). Distilled water is routinely used to lyse virus-infected cells when preparing specimens for electron microscopy, and is probably a more effective procedure for harvesting virus propagated in Vero cells. It was considered that trypsin treatment had a deleterious effect on SK125 because it resulted in a viral titer which was significantly less than that obtained with the standard 3 freeze-thaw cycles. This finding was surprising since all avian reoviruses had previously been reported to be resistant to trypsin (Kawamura et.al., 1965; Petek et.al., 1967; Jones et.al., 1975).

A trypsin sensitivity assay was therefore set up to verify the effect of trypsin on avian reoviruses. The results obtained indicate a wide variation in the degree of sensitivity of avian reoviruses to trypsin. Six of 14 strains examined (42%) were shown to be sensitive to trypsin. Although the present study used 0.83% trypsin concentration for 45 min, a trypsin sensitive avian reovirus, strain TR1, was previously shown to be affected by exposure to as little as 0.01% trypsin

for 30 min (Alfaleq and Jones, 1991).

Since trypsin is one of the digestive enzymes, the infectivity, virulence, and persistence of the trypsin sensitive avian reovirus strains in the intestinal tract following exposure by the oral route may be significantly affected. Indeed it has been shown that the turkey reovirus strain TR1 was less pathogenic for day-old chicks when given orally than when given by footpad inoculation (Afaleq and Jones, 1991). This isolate also showed limited faecal shedding following oral administration, and it was suggested that the respiratory route may be more important in dissemination of such trypsin sensitive avian reoviruses.

It was hypothesized that the trypsin sensitivity may serve as a simple diagnostic test for differentiating between pathogenic and non-pathogenic strains of avian reoviruses. Previous attempts by several workers had failed to find a correlation between genotypes and serotypes of avian reoviruses with the disease conditions they cause in chickens (Gouvea and Schnitzer, 1982). It was therefore decided to determine the relationship between trypsin sensitivity of avian reoviruses and their genotypes, serotypes, and pathotypes. The migration patterns of dsRNA genome segments of avian reovirus have been shown to be heterogenous (Gouvea and Schnitzer, 1982; Clark et. al., 1990). Comparison of the 10 segments among 32 viruses showed no single band of S, M, or L was different for any serotype examined, and no specific

association between migration pattern and disease presentation could be detected (Gouvea and Schnitzer, 1982). In present study, the comparison of those showed there was a correlation between a single band of M2 and trypsin sensitivity assay. When avian reoviruses comprising of serially passaged S1133 strains were grouped according to virulence, the greatest changes were seen in the migration patterns of genome segments S1, M2, and M3, suggesting that these may be associated with the virulence of a given avian reovirus strain (Huang et. al., 1987). In the present study, two main genotypes were recognized on the basis of the migration patterns of the M2 gene (Fig. 8). In mammalian reoviruses the M2 gene is responsible for the differences in response to trypsin treatment. Of the six avian reoviruses in this study whose M2 gene migrated more slowly than the eight remaining viruses, five viruses (80.33%) were sensitive to trypsin, suggesting that the migration pattern of the M2 genomic segment of avian reoviruses is also correlated with sensitivity to trypsin. The M2 gene of trypsin sensitive avian reoviruses migrated more slowly i.e, was larger than that of the trypsin resistant avian reoviruses.

Four genotypes were recognized on the basis of the migration patterns of the S1 genomic segment. The S1 gene encodes the virus attachment protein which is also the antigen for inducing neutralizing antibody production (Tyler and Fields, 1990). However, the four genotypes in this study could

not be assigned to any of the serotypes determined.

Unlike mammalian reoviruses, avian reoviruses show heterogeneity in their neutralizing antigens. More than 77 avian reovirus isolates have been grouped into at least 11 serotypes (Robertson and Wilcox, 1986). In present study, the homologous titers of eight avian reovirus isolates ranged from 2.20 $\log_{10}$ to 3.41 $\log_{10}$ (Table 7). The isolates with a neutralization of less than 1.7 $\log_{10}$ were considered to be of a different serotype to the homologous isolate to which the antiserum was produced (Robertson and Wilcox, 1984). Avian reoviruses antigenically related to strains FC formed the largest serotype. Strain Type 24 and WVU2937 previously shown to belong to separate serotypes (Sahu and Olson, 1975) had considerable cross-reactions with the FC serotype and were grouped as subtypes of this serotype. The predominant FC serotype contained both trypsin sensitive and trypsin resistant strains.

These results indicate that there is no correlation between the serological groups of the avian reoviruses studied and their sensitivity to trypsin treatment. This is not surprising since the protein encoded by the S1 genomic segment of avian reoviruses, which functions as the antigen for inducing neutralizing antibody production, is different from the protein encoded by M2 genome segment, which confers resistance of the outer capsid of virion particles to protease digestion in vivo (Tyler and Fields, 1990).

Hepatic and articular lesions in chickens inoculated with strain SK98a were consistent with those previously reported in natural and experimental infections with avian reoviruses (van der Heide, 1977). High mortality in the first few days post-inoculation with strain SK98a was closely associated with the severity of necrosis in the liver. Myocarditis often reported in reovirus infection was not observed in any group (van der Heide, 1977). Unlike the control and other inoculated groups, chickens infected with strain SK98a had rather large pieces of retained cartilage cone. Although remnants of these cones could occasionally be seen in the metaphysis of chicks younger than two weeks (Riddell, 1981), it appears, based on the findings of the present study, that infection with strain SK98a interfered with the normal removal process of these embryonic chondrocytes.

The present study compared the pathogenicity of 3 trypsin resistant avian reoviruses and 2 trypsin sensitive avian reoviruses. Of the 3 trypsin resistant strains examined only strain SK98a produced mortality in day-old chickens. The difference between the 3 viruses may be due to virus strain variation since a standard virus dose of 5 $\log_{10}$ $TCID_{50}$ was used for all virus groups. Indeed the incidence of tenosynovitis lesions in surviving chickens among the 3 virus groups appeared to be virus strain-dependent, the highest being in the SK98a group and the lowest in the SK103a group. The 2 trypsin sensitive strains SK125 and SK73a produced

neither mortality nor tenosynovitis. When the five virus groups were compared for virus persistence in tissues, again the trypsin resistant virus groups showed a prolonged persistence of virus in heart and hock joints of 21 days and 42 days, respectively. The trypsin sensitive virus groups had shorter durations and fewer numbers of chickens with virus in their tissues (Table 12). These findings are generally in agreement with those of Alfaleq and Jones (1991). However, no difference was found in virus shedding via the cloaca, and virus persistence in the liver, between trypsin resistant and trypsin sensitive virus groups. In fact very poor virus recoveries were obtained from cloacal swabs as a whole. This may be due to the fact that the Vero cells used for virus isolation in the present study are not as sensitive as the commonly used chicken embryo liver cell cultures (Guneratne and Jones, 1982; Nwajei et. al., 1988).

In conclusion, 14 avian reoviruses isolated in CELi cells induced serially sustained CPE during propagation in Vero cells. The growth curves of 5 of the viruses examined showed them to be highly cell-associated, and this may be true for all avian reoviruses adapted to replicate in Vero cells. Of eight procedures tested with SK125 strain, treatment of virus-infected cultures with ddH_2O was the most effective, and treatment with trypsin was the least effective for recovering cell-associated virus. It is envisaged that sterile ddH_2O as demonstrated for SK125, may prove useful for release of cell-

associated virus particularly where large amounts of virus are required for experimental purposes. This study further indicates that trypsin sensitivity among avian reoviruses is heterogenous and not serotype specific, is correlated with the migration patterns of the M2 genomic segment in PAGE, and is related to viral persistence in tissues and its ability to produce tenosynovitis in infected chickens. The biological heterogeneity of avian reoviruses in the virus neutralizing antigens, the migration patterns of genomic segments, and in the pathological conditions these viruses produce complicates etiological diagnosis of avian reovirus infections in poultry. In the present study mortality, hepatic necrosis, and tenosynovitis were in experimentally infected chickens. Since no other pathological manifestations of avian reovirus infection were reproduced, it could not be confirmed if trypsin sensitivity per se would serve as a rapid test for identification of pathogenic avian reoviruses. Additional experiments are needed in day old chickens inoculated via respiratory or intravenous routes, where the viruses are not affected by intestinal trypsin. Alternatively, the experiment in the present study can be repeated with additional viruses.

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