AVIAN PNEUMOVIRUS INFECTION IN CHICKENS AND TURKEYS: STUDIES ON SOME ASPECTS OF IMMUNITY AND PATHOGENESIS

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Dedicated to my parents and in gratitude for
the sacrifices made by my wife Gursharan and
my children Jasreet and Anahat

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Preface

The experimental work described in this thesis was conducted in the Department of Veterinary Pathology, Jordan Building, Leahurst, University of Liverpool, UK between 1995 and 1998, during the tenure of a scholarship awarded to the author by Association of Commonwealth Universities.

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Abstract

This thesis describes experimental work to investigate some aspects of immunity to and pathogenicity of avian pneumovirus (APV) infection in chickens and turkeys.

Local antibody (IgA & IgG) production was demonstrated in the harderian gland and trachea of 4-week old chickens infected with two strains of APV using a method of *in vitro* synthesis of antibody in tissue explants. Local and systemic class-specific antibody responses following primary and secondary APV infection in chicks and poults were also characterised by enzyme-linked immunosorbent assays. A boost in secondary IgA and IgG antibody response in lachrymal fluid and IgG in serum of chicks or poults was found to be associated with resistance to APV infection. The role of cell-mediated immunity to APV infection in chickens and turkeys was evaluated *in vivo* by use of a T-cell suppressant drug cyclosporin A (CSA). Prolongation of respiratory signs, persistence of tracheal lesions and delay in APV clearance from tissues of CSA-treated poults indicated that T-cells play a significant role in the recovery of birds from APV infection. This finding of a role of T-cells in APV infection was exploited to investigate persistence of APV in poults and chicks by inducing CSA based T-cell suppression. No re-excretion of APV could be demonstrated in CSA-treated birds.

The pathogenicity of APV for the chicken reproductive tract was studied *in vitro* in oviduct organ culture (OOC) prepared from young chicks with oestrogen-induced precocious oviducts and *in vivo* in such young chicks and adult female with normal oviducts. While *in vitro* experiments on OOC prepared both from precocious oviducts of young chicks and normal oviducts from adults revealed intrinsic susceptibility of the chicken oviduct to APV infection, results of *in vivo* experiments failed to show APV replication in the chicken ovary and oviduct.

Experimental dual infections of APV were studied with (i) infectious bronchitis virus (IBV) *in vitro* using chick embryo tracheal organ cultures and *in vivo* in 1-week old chicks, and (ii) *Mycoplasma synoviae* (Ms) *in vivo* in one day-old poults. Results of both the *in vitro* and *in vivo* experiments on dual infection with APV and IBV primarily suggested predominance of IBV replication over APV and a significant interference by IBV in the development of antibody response to APV but not vice versa. Following dual infection of poults with APV and Ms, there was no increase in severity of clinical disease or gross and microscopic lesions, and no increased persistence of either APV or Ms. The poults did not seroconvert to Ms and Ms inoculation did not affect the development of antibodies to APV.

List of presentations

The following presentations have resulted from the work described in this thesis:

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Acronyms and abbreviations used

APV	avian pneumovirus
AS	attenuated strain
bp	base pairs
BSA	bovine serum albumin
^{o}C	degree celsius
CBC	carbonate-bicarbonate buffer
CD	clusters of differentiation
CD_{50}	50% ciliostatic dose
cDNA	complementary deoxyribonucleic acid
Ci	curie
cm	centimetres
CMI	cell-mediated immunity
CO_2	carbon dioxide
Con A	concanavalin A
CP	cyclophosphamide
CPE	cytopathic effect
cpm	counts per minute
CSA	cyclosporin A
DNA	deoxyribonucleic acid
ECE	embryonated chicken eggs
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
h	hours
HEPES	2-hydroxy-ethyl-piperazine-N'2-ethane sulphonic acid
HG	harderian gland
IB	infectious bronchitis
IBV	infectious bronchitis virus
IF	immunofluorescence

Ig	immunoglobulin
IIF	indirect immunofluorescence
IL-2	interleukin-2
IP	immunoperoxidase
IU	international units
IV	intravenous
kDa	kilodalton
kg	kilogram
lb	pounds
μ	micron
M	molar concentration
MA	maternal antibody
Mab	monoclonal antibody
MB	mycoplasma broth
MCS	mean clinical score
MEM	minimum essential medium
μg	microgram
mg	milligram
min	minutes
μl	microlitre
ml	millilitre
mm	millimetres
mM	milli-Molar concentration
mRNA	messenger ribonucleic acid
Ms	*Mycoplasma synoviae*
n	number of samples
N	normal concentration
NDV	Newcastle disease virus
nm	nanometre
OD	optical density
O/N	oculonasal
OOC	oviduct organ culture
OPD	o-phenylenediamine

p	probability that null hypothesis is valid
PBS	phosphate buffered saline
pc	post-challenge
PCR	polymerase chain reaction
pi	post infection or post inoculation
ppi	post primary infection
psi	post secondary infection
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RSA	rapid serum agglutination test
RSV	respiratory syncytial virus
sd	standard deviation
SHS	swollen head syndrome
SI	stimulation index
SNT	serum neutralisation test
SPF	specific pathogen-free
sq in	square inch
$TCID_{50}$	50% tissue culture infective dose
TOC	tracheal organ culture
Tris	(hydroxy methyl) aminomethane
TRT	turkey rhinotracheitis
TRTV	turkey rhinotracheitis virus
Tween	polyoxyethylene sorbitan monolaurate
UK	United Kingdom
USA	United States of America
VN	virus neutralisation
VS	virulent strain
WLH	white leghorn (chickens)
w/v	weight/volume

CHAPTER 1

INTRODUCTION AND AIMS

A respiratory disease of young poults termed turkey rhinotracheitis (TRT) was first identified in 1978 in South Africa (Buys et al., 1989a) and subsequently reported in Europe and in certain other parts of the world. Several groups of researchers in the UK and one in France isolated a virus and reproduced the disease (McDougall & Cook, 1986; Giraud et al., 1986b; Jones et al., 1986; Wilding et al., 1986; Wyeth et al., 1986). Later, based on biochemical analysis of viral protein and mRNA, and molecular studies on its genome revealed the causative agent to be a pneumovirus (Cavanagh & Barrett, 1988; Yu et al., 1991). The virus is referred to as turkey rhinotracheitis virus or avian pneumovirus (APV) and it belongs to the genus Pneumovirus of family Paramyxoviridae. Other members of genus Pneumovirus include human, bovine and ovine respiratory syncytial virus, and pneumonia virus of mice (Ling et al., 1995). Further studies using monoclonal antibodies (Collins et al., 1993; Cook et al., 1993b) and nucleic acid sequencing (Juhasz & Easton, 1994) revealed that various APV strains belong to two antigenic subtypes A and B.

TRT is a major cause of both respiratory infection in young turkeys and reduced egg production in turkey breeders. The economic importance of the disease is due to poor feed conversion and weight gain, decline in egg production and quality, and sometimes severe mortalities associated with secondary infections. APV also causes a mild respiratory infection in chickens. The relative economic impact of disease in chickens has not yet been clearly understood. Although, in the literature swollen head syndrome (SHS), a pathological condition which affects chickens of all types but mainly broilers and broiler parents, has been strongly associated with APV, its role in the development of SHS has not been clearly demonstrated. Field evidence suggests that the APV can cause reduced egg production in chickens as it does in turkeys, but little is known whether it is capable of infecting the reproductive tract of chickens.

TRT and pneumovirus infection in chickens have been controlled by use of vaccines with variable degree of success in the field. The precise mechanism of protection afforded by vaccines is not clearly understood. At present, cross-protection between the two existing antigenically distinct subtypes A and B has been documented (Cook et al., 1995; Naylor et al., 1997a). However, there are some unpublished reports of emergence of a new antigenically distinct strain of APV referred to as 'Colorado strain' in the United states, the country which was thought to be free of APV infection until recently. Certain aspects of the epidemiology, such as maintenance of reservoir of

infection in nature and the role of APV in pathogenicity with regard to certain other infectious poultry pathogens are yet to be explored.

The aims of the work described in this thesis were to study some aspects of the immune responses to and pathogenesis of APV in turkeys and chickens. The major areas which have been studied are as follows.

1. Demonstration of local antibody production in chickens infected with a virulent or an attenuated strain of APV, and characterisation of local and systemic class-specific antibody responses following primary and secondary APV infection in chicks and poults.

2. The role of T-cells in primary and secondary APV infection in turkeys and only primary infection in chickens was investigated using a T-cell suppressant drug cyclosporin A (CSA).

3. A preliminary investigation was undertaken to explore the possibility of long-term persistence of APV in poults and chicks using CSA immunosuppression.

4. The pathogenicity of APV for the chicken reproductive tract was evaluated in *in vitro* and *in vivo* studies, using young chicks with precocious oviducts and mature female chickens with normal oviducts.

5. Dual infection of tracheal organ cultures (*in vitro*) and chicks (*in vivo*) with APV and infectious bronchitis virus were carried out to investigate the interaction between these two agents.

6. The effect of dual infection of turkey poults with APV and *Mycoplasma synoviae* was also studied.

CHAPTER 2

REVIEW OF THE LITERATURE ON AVIAN PNEUMOVIRUS INFECTIONS

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CHAPTER 2

REVIEW OF THE LITERATURE ON AVIAN PNEUMOVIRUS INFECTIONS

2.1. AVIAN PNEUMOVIRUS

2.1.1. Taxonomy

The structural features, biological properties and morphogenesis of virus particles, together with the number and mobility of the viral polypeptides and mRNA species on gels (Wyeth et al., 1986; Cavanagh & Barrett, 1988; Collins & Gough, 1988; Ling & Pringle, 1988; O' Loan et al., 1992) provided some evidence for the classification of the cause of turkey rhinotracteitis (TRT) as a member of the genus Pneumovirus in the family Paramxyoviridae. Subsequent molecular analyses confirmed the close genetic homology of avian pneumovirus (APV) with human and bovine respiratory syncytial viruses (RSV), the type member of the genus Pneumovirus of the sub-family Pneumovirinae, and pneumonia virus of mice, the other known mammalian pneumovirus. However, the genetic organisation of APV was found to be different from mammalian pneumoviruses (Yu et al., 1991; Ling et al., 1992; Yu et al., 1992a; Yu et al., 1992b).

2.1.2. Structure of the virus

In negatively stained preparations under the electron microscope, APV appear as enveloped and highly pleomorphic, sometimes spherical but often bizarre shapes and possessing long filaments (see Naylor & Jones, 1993). Virus particles measure from 50 to over 500 nm with a mean diameter between 100 and 200 nm. Filamentous forms can be 1000 to 2000 nm long. Virus particles are covered with a layer of closely-spaced surface projections with a length of 13 to 15 nm. A helical nucleocapsid is present with a diameter of 14 nm and the pitch of the helix is 6-7 nm (McDougall & Cook, 1986; Wyeth et al., 1986; Giraud et al., 1986b; Collins & Gough, 1988; Gough & Collins, 1989; O'Loan et al., 1992). The smaller diameter of nucleocapsid of APV distinguishes it from other genera Paramxyovirus and Morbillivirus of family Paramxyoviridae, which have a diameter of about 18 nm, together with a smaller pitch (Collins & Gough, 1988) and groups it with the genus Pneumovirus.

In ultrathin sections, mainly filamentous forms with a diameter of 12-13 nm are seen budding from the plasma membrane of infected cells which was considered to correspond to the virus nucleocapsid (O'Loan et al., 1992).

2.1.3. Physico-chemical properties of the virus

APV has a buoyant density of 1.21g/ ml in sucrose gradients (Collins & Gough, 1988). It is sensitive to lipid solvents, stable between pH 3.0 to 9.0, and is inactivated at 56 °C after 30 minutes (Collins et al., 1986; Giraud et al., 1986b). The lack of haemagglutination property is a feature characteristic to pneumoviruses (Wyeth et al., 1986; Buys et al., 1989a).

2.1.4. Viral nucleic acids & polypeptides

Viral nucleic acids

APV contains non-segmented single stranded, negative sense RNA (Yu et al., 1991). In infected cells, 10 mRNA species, ranging in molecular ratio from 0.22 to 2.0 x 10^6 are generated. The viral mRNA profile of APV closely resembles that of RSV (Huang & Wertz, 1983; Cavanagh & Barrett, 1988). The amino acid sequences of these mRNAs have been established by cDNA cloning (Yu et al., 1991; Yu et al., 1992 a; Yu et al., 1992b; Ling et al., 1992; Juhasz & Easton, 1994; Ling et al., 1995; Randhawa et al., 1996a; Randhawa et al., 1996b; Li et al., 1996; Naylor et al., 1998). The order of the known genes in the APV genome (3'N-P-M-F-M2-SH-G-Polymerase 5') is somewhat different from that of the mammalian pneumovirus (3'N-P-M-SH-G-F-M2-Polymerase 5'). However APV conforms to the pattern seen in all members of the family Paramyxoviridae, with the N gene being the most promoter-proximal of the three major virus structural protein genes (Li et al., 1996).

Viral polypeptides

In APV, approximately 8 viral polypeptides with molecular weights of between 14 and 200 kDa have been identified (Cavanagh & Barrett, 1988; Collins et al., 1986; Collins & Gough., 1988; Ling & Pringle, 1988). Cavanagh and Barrett (1988) summarised the 3 studies by comparing the relative molecular masses of viral polypeptides of APV with those of other pneumoviruses. They concluded identities of viral polypeptides to be L: large protein (200kDa), G: large glycopolypeptide (82-84 kDa), F: fusion protein (68kDa), the fusion protein cleavage products F_1 (53-54 kDa) and F_2 (14-15kDa), N: nucleocapsid (38-43KDa), P: phosphorylated (35-40 kDa), M: matrix (30-35kDa) and unidentified (19-22kDa). Two of these, G and F were found to be glycosylated. In addition to these, a 19 kDa virus-specific polypeptide referred to as a non-structural protein was detected by Ling and Pringle (1988).

Other polypeptides were also detected but these were considered to be precursors or modifications of those listed above. Three polypeptides discriminate the pneumoviruses from morbilliviruses and paramyxoviruses, the case also seen in APV. The proteins N and P are smaller than those of the other 2 genera. The small polypeptide 22kDa termed

M_2 (Wunner & Pringle, 1976) is not found in the other two genera. The amino acid sequences of the P, M, F, M2 and N proteins of APV have approximately 30 to 41 % identity with those of mammalian pneumoviruses (Li et al., 1996).

Two subtypes (A & B) of APV have been defined on the basis of a very low level (38 %) of amino acid identity between their G glycoprotein (Juhasz & Easton, 1994). This resembles the situation with two subgroups of human RSV, having 53 % identity in their G protein (Johnson et al., 1987).

2.1.5. Antigenicity

Early comparisons of different strains of APV by double immuno-diffusion, indirect immunofluorescence (IIF), serum neutralisation test (SNT) and enzyme-linked immunosorbent assays (ELISA) using polyclonal sera, revealed that they were antigenically similar (Baxter-Jones et al., 1987; Gough & Collins, 1989). Analyses using monoclonal antibodies (Mab) indicated that APV isolates formed at least two antigenic subtypes (Collins et al., 1993; Cook et al., 1993b), a finding later confirmed by nucleic acid sequencing (Juhasz & Easton, 1994). Cook et al. (1993b) using Mabs showed that all British isolates collected between 1985 and 1990 were very closely related antigenically among themselves as well as with a strain isolated in South Africa in 1978, but somewhat different from European isolates made in France, Spain, Italy, Hungary and Netherlands. Collins et al. (1993) came to similar conclusions, except that the 1986 French isolate 1556 (Giraud et al., 1986b) was considered to be very similar to the pre-1990 British isolates. The division of APV strains into subtypes A and B on the basis of G glycoprotein differences of Juhasz and Easton (1994) is now well recognised.

2.2. AVIAN PNEUMOVIRUS INFECTIONS

2.2.1. Natural hosts

The most important host for APV is the turkey where the virus causes a severe respiratory disease designated TRT and disorders of reproductive tract in breeders resulting in reduced egg production and poor egg quality. It also causes respiratory infection in chickens (sometimes associated with swollen head syndrome), pheasants and guinea fowl (Picault et al., 1987; Jones et al., 1987; Gough et al., 1988). APV antibodies have been demonstrated in the sera of ostriches (Cadman et al., 1994).

2.2.2. Transmission

When TRT was first encountered in the UK, it spread rapidly among turkey flocks in England and Wales within 6 months. This lead to the assumption that transmission must be airborne (Anon, 1985). Whether the airborne transmission is important has never been established.

Horizontal transmission was demonstrated by contamination of specific pathogen-free (SPF) turkey contacts from inoculated turkey poults kept in separate cage (Giraud et al., 1986b). Cook et al. (1991) reported that experimentally, virus did not transfer from infected to uninfected poults placed in the same room even though the air flow was favourable, but virus could be transmitted from inoculated poults to APV-free poults placed in direct contact, but only during the first 9 days after inoculation. With mammalian pneumoviruses, contact also has been shown to be important for horizontal transmission (Hall et al., 1981; Ploeger et al., 1986).

The possibility of a role for migratory wild birds and feed lorries in APV transmission has been also suggested (see Naylor & Jones, 1993). The role of carriers or latently or persistently infected birds in transmission has not been investigated, though in our present state of knowledge, it seems to be less likely, as infected turkeys shed virus for relatively short period of time, usually from 3 to 9 or 10 days after infection (Cook et al., 1991). Preliminary work to investigate the possibility of persistence of APV in turkeys or chickens is described in the Chapter 6.

Although virus is known to infect the epithelium of the oviduct of laying turkeys and causes drop in egg production (Jones et al., 1988; Cook et al., 1996), it has never been demonstrated that vertical transmission can occur.

2.2.3. Geographical distribution

APV is distributed in many parts of the world where turkeys are kept commercially and in several countries with few or no turkeys. The disease entity called TRT was first reported in South Africa (Buys & Du Preez, 1980; Buys et al., 1989a) and subsequently in UK (Anon, 1985), France (Giraud et al., 1986a), Spain (Anon, 1985), Italy (Anon, 1985), Israel (Weisman et al., 1988), Germany (Hafez & Woernle, 1989), Hungary (Lantos, 1990) and certain other parts of Europe. APV antibodies have been demonstrated in the sera of ostriches in Zimbabwe (Cadman et al., 1994).

In addition to Africa and Europe, pneumovirus isolation or seroconversion to it has been reported in Asia (Lu et al., 1994), and South and Central America (Jones, 1996). In some countries, notably USA (Jones, 1996), Canada (Heckert & Myers, 1993) and

Australia (Bell & Alexander, 1990), there has been no evidence of pneumovirus infection. However, at the time of writing there are unpublished reports of a pneumovirus-like agent so-called "Colorado strain' affecting turkeys in the midwest of the USA (R.C. Jones, personal communication).

Swollen head syndrome (SHS) in chickens has been reported in several countries, and although APV has been strongly implicated as one of its causes, the exact aetiology and role of APV in its pathogenesis have not been clearly understood. SHS was first reported in broiler chickens in South Africa (Morley & Thomson, 1984) and considered to be possibly caused by a coronavirus and *Escherichia coli.* (*E. Coli*). Since then, SHS has been reported in UK (O'Brien, 1985), France (Picault et al., 1987), Israel (Perelman et al., 1988), Yemen (Sarakbi, 1989), Germany (Hafez & Lohran, 1990), Japan (Uramoto et al., 1990), Taiwan (Lu et al., 1994) and the USA (Droual & Woolcock, 1994; Goodwin & Waltman, 1994). Antibodies against APV were present in many cases of SHS mentioned above. APV has been isolated from affected chicken flocks (Picault et al., 1987; Buys et al., 1989b; Gough et al., 1994; Lu et al., 1994; Maharaj et al., 1994; Tanaka et al., 1995).

SHS in the USA has been shown not to be associated with APV (Droual & Woolcock, 1994; Goodwin & Waltman, 1994). Infectious bronchitis virus (IBV) and *E.coli* (Shirai et al., 1993; Droual & Woolcock, 1994) or multiple viruses, bacteria and *Cryptosporidium bailleyi* (Goodwin & Waltman, 1994) were implicated as aetiological agents.

2.2.4. Clinical signs

The severity of clinical signs in turkeys largely depends upon certain environmental and management factors, and invasion by secondary agents (see Naylor & Jones, 1993). The characteristic respiratory signs of TRT include initially a clear nasal discharge, which becomes turbid and frothy, but then clearing. Infraorbital sinuses show swelling during this period together with sneezing, coughing, eye discharge, head shaking and depression, and within 10 to 12 days, birds may recover completely (Lister & Alexander, 1986; McDougall & Cook, 1986; Wyeth et al., 1986; Giraud et al., 1986b; Jones et al., 1986; Buys et al., 1989a; Stuart, 1989). There is poor feed conversion in growing birds. The morbidity rate can reach up to 100% (Anon, 1985; Stuart, 1989), but mortality is highly variable and seen in complicated cases mainly associated with poor management conditions and invasion by secondary agents. In some cases, mortality has been reported to be as high as 30% (Anon, 1985; Buys et al., 1989a) or even over 50% (Stuart, 1989).

In laying turkeys, the virus is also known to cause mild respiratory infection but the most important effect of APV in breeders is drop in egg production (Anon, 1985; Stuart, 1989). This can drop to 40% of expected levels and in the worst cases to as low as 2% (see Naylor & Jones, 1993). Recovery takes approximately three weeks and many of the eggs laid during this period are white and have misshapen thin shells and the number of settable eggs is severely reduced (Anon, 1985; Stuart, 1989).

SHS is a pathological condition which affects chicken of all types, but mainly broilers and broiler parents. Clinical signs of SHS have been described by Morley and Thomson (1984), O'Brien (1985) and Pattison et al. (1989). The broilers may be affected from 4 to 6 weeks of age while broiler breeders flocks are affected from peak of lay, at about 30 weeks, until 52 weeks of age. In most cases it lasts 2-3 weeks. Percentage mortalities and morbidities are low, usually in the region of 1% (Morley & Thomson, 1984; Pattison et al., 1989).

In broilers, clinical signs consists of head swelling which gives the face a puffy appearance caused by subcutaneous oedema around the eyes which extends over the head, and down into the intermandibular tissue and wattles. Respiratory signs include coughing and sneezing and many affected birds have a severe tracheitis, ear and eye discharge, red conjunctivitis, incordination, torticollis, foul-smelling green diarrhoea and often die from secondary septicaemia caused by *E.coli* (Morley & Thomson, 1984; Pattison et al., 1989). Shirai et al. (1993) reported SHS also in layer chickens and clinical aspects included black scars on the combs, and preceding that, subcutaneous oedema of the head.

Experimental infection of young chickens with APV has been shown to result either in mild or no respiratory signs (Jones et al., 1987; Buys et al., 1989a,b; Cook et al., 1993a). Cook et al. (1993a) in an experimental study, found that a turkey and a chicken strain were both capable of causing signs of infection in both turkeys and chickens, although the signs tended to be more severe in the species from which APV was isolated initially.

Little is known about the effects of APV on the reproductive tract of chickens. There is continued speculation in the field that the virus can cause reduced egg production and perhaps poor shell quality, as it does in turkeys.

2.2.5. Influencing factors

Several factors may influence disease following APV infection.

Age

All ages of turkeys are susceptible to TRT (Anon, 1985) but the clinical disease is more severe in young poults (Alexander et al., 1986; Stuart, 1989). Williams et al. (1991b) found that it was necessary to increase the dose of challenge virus with age in order to induce similar clinical disease.

There is no field evidence regarding age of susceptibility leading to clinical disease of chickens but experimental studies have shown mild respiratory signs in chicks from one day to 8 weeks old (Jones et al., 1987; Cook et al., 1993a). SHS has been mainly found to occur in broilers at 4 to 6 weeks of age and broiler breeders at 30 weeks of age (see Naylor & Jones, 1993).

Breed

There is no information published regarding difference in breed susceptibility to APV. However, Cook et al. (1993a) studied susceptibility of seven inbred lines of chickens and did not find any marked differences in incidence of clinical infection or antibody response between these inbred lines.

Intercurrent infections

TRT may not occur as a single entity in the field. In general, the disease has been seen to be more severe in the field than in experimental conditions (Stuart, 1989), where it was thought that certain bacteria as secondary agents may be involved. *E.coli*, *Bordetella* and *Pasteurella* spp are found to be frequently associated with TRT infection, resulting in septicaemia lesions thus contributing to the enhanced mortality (see Naylor & Jones, 1993). Secondary agents have also been shown to increase severity in experimental conditions. *Bordetella avium*, *Pasteurella*-like organisms (Cook et al., 1991), *Mycoplasma gallisepticum* (Naylor et al., 1992) and *Mycoplasma imitans* (Ganapathy et al., 1998) have been shown to exacerbate and prolong the clinical disease. Chapter 9 describes the effect of dual infection of APV and *Mycoplasma synoviae* in turkey poults.

E.coli infection has been commonly reported following natural outbreaks of TRT (Anon, 1985; Stuart, 1989) but no experimental work involving dual infection of poults with APV and *E.coli* has been published.

In naturally infected turkey flocks, Hafez (1990) reported that the seroconversion to APV was accompanied by a significant increase in the number of positive sera to the

avian adenovirus celovirus. Whether celovirus is another important secondary agent has not been established.

Goodwin and Waltman (1994) found SHS accompanied by infection with multiple viruses and bacteria, possibly the viruses being the primary causal agent or trigger leading to invasion of secondaries. Among viruses, the coronavirus (IBV) (Morley & Thomson, 1984; Shirai et al., 1993; Droual & Woolcock, 1994; Goodwin & Waltman, 1994) and pneumovirus (Picault et al., 1987; Buys et al., 1989b; Gough et al., 1994; Lu et al., 1994; Maharaj et al., 1994; Tanaka et al., 1995) are most frequently encountered. The involvement of *E.coli* in SHS is almost invariable, although other bacteria like *Staphylococcus spp.* and *Pasteurella spp.* and *Cryptosporidium baileyi* have also been found (Morley & Thomson, 1984; Pattison et al., 1989; Shirai et al., 1993; Droual & Woolcock, 1994; Goodwin & Waltman, 1994; Lu et al., 1994; Nakamura et al., 1997).

Attempts to reproduce SHS have been made by experimental dual infections of chickens with APV and *E.coli* but resulted in variable outcome. While Majo et al. (1997) reported that dual infection of chickens by eyedrop or intranasal routes of inoculation resulted in increase in severity of clinical signs, and macroscopic and microscopic lesions, Nakamura et al. (1998) could not demonstrate any clinical signs or significant lesions either following single or dual infection of chickens with APV and/or *E.coli* by intranasal route. However, instead, the latter workers demonstrated typical SHS lesions in groups of chickens following inoculation of four different strains of *E.coli* (not APV) into the submucosal tissue of the nasal membrane or subcutaneous tissue of eyelids.

Capua et al. (1994) recorded a severe natural outbreak of a IBV variant strain in chickens, which was also accompanied by antibodies to APV. Likewise, Gough et al. (1994) isolated a pneumovirus from SHS-affected broiler chickens which had seroconverted to a IBV variant strain. However, no experimental work on the interaction of these two infectious agents has been published [Chapter 8 reflects the outcome of dual infection of tracheal organ cultures and chicks with APV and IBV].

Management conditions

The severity of the clinical disease in turkeys is considerably affected by management practices such as poor ventilation and hygiene, high stocking densities, and cold and damp weather (Anon, 1985; Stuart, 1989). Mixed aged sites are also thought to influence the disease severity by allowing continued re-exposure of the susceptible stock.

2.3. PATHOGENESIS

2.3.1. Replication and persistence in the tissues

Following experimental infection of turkeys by eyedrop or intranasal inoculation, APV replicates in epithelial cells of respiratory tissues, namely nasal turbinates, trachea and lungs (Jones et al., 1988; Buys et al., 1989a; O'Loan & Allan, 1990; Cook et al., 1991; Cook et al., 1993a; Majo et al., 1995). Studies using immunofluorescence (IF) and immunoperoxidase (IP) have shown the detection of the viral antigens in turbinates from days 2 to 6 after experimental infection and in tracheas from days 1 to 7 post infection (pi) (Jones et al., 1988; Majo et al., 1995). Majo et al. (1995) were able to demonstrate the viral antigens in ciliated brush border of surface epithelial cells of the bronchus on days 4 and 5 pi.

Virus can be readily recovered from turbinates and trachea between 2 and 5 days after experimental infection of turkeys and peak titres are observed between day 3 to 5 pi (Jones et al., 1988; Cook et al., 1991; Cook et al., 1993a). Virus has been also isolated from lungs as early as day 5 pi and air-sacs from days 5 to 11 pi, however small amounts of virus in respiratory tissues may continue to be detected up to day 14 pi (Cook et al., 1991). Using the polymerase chain reaction (PCR), Jing et al. (1993) demonstrated viral RNA in dry tracheal swabs from turkey poults for days 17 to 19 pi, nearly 2 weeks after peak virus titres were recorded.

Virus replication in epithelium of the reproductive tract of turkey hens has been shown experimentally (Jones et al., 1988; O'Loan & Allan, 1990). Using IF staining, Jones et al. (1988) detected the viral antigens in epithelium of uterus on day 7 pi and in all regions of the oviduct on day 9 pi. Virus could only be isolated from middle magnum and vagina on day 9 pi. Using IP staining, O'Loan and Allan (1990) were also able to demonstrate viral antigens in surface epithelial cells of the uterus day 9 pi.

In chickens, virus has been isolated from nasal secretions, 6 days after experimental infection of day-old SPF chicks with a turkey strain (Jones et al., 1987) and from tracheal swabs on day 6 pi with a chicken strain (Jones et al., 1991). However, a detailed study by Cook et al. (1993a), revealed some differences in biological properties between a turkey and a chicken strain, following experimental infection of the chicken. High titres of virus were recovered from the nose on days 3 and 5 pi and from trachea on day 3 pi with a chicken isolate. However, only small amounts of virus were recovered from nose and trachea of chickens inoculated with a turkey strain. In experimentally infected chickens, the virus replication in epithelial cells of the turbinates and trachea have been demonstrated up to day 5 pi by IF and IP techniques (Jones et

al., 1987; Majo et al., 1995) and in turbinates on days 3 and 5 pi by electron microscopy (Majo et al., 1996).

There is no documented evidence of APV replication in the chicken reproductive tract. Chapter 7 describes an attempt to investigate replication of APV in the chicken oviduct.

2.3.2. Gross lesions

In natural field outbreaks of TRT, where secondary bacteria become involved, typical post mortem lesions such as pericarditis, perihepatitis, air-sacculitis, pneumonia and adhesions between the pericardial sac and the epicardium have been described (Anon, 1985; Stuart, 1989).

Following experimental infection of turkeys with APV alone, Jones et al. (1988) found greyish, watery to mucoid, exudate in turbinates and excess mucus in the trachea. Following mixed infection of turkeys with APV and *Bordetella avium* or *Pasteurella*-like organisms, thickened air-sacs were found between days 7 and 14 pi (Cook et al., 1991). Similarly, dual infection of turkey poults with APV and *Mycoplasma gallisepticum*, revealed an increase in gross abnormalities namely nasal, sinus and tracheal exudate, congested lungs and cloudy air sacs (Naylor et al., 1992).

In mature female turkeys experimentally infected with APV, a variety of reproductive tract abnormalities was found including egg peritonitis, folded shell membrane in the oviduct, mishapen eggs, ovary and oviduct regression, and scattering of white masses of inspissated albumen with free abdominal yolk material deposition (Jones et al., 1988).

2.3.3. Histopathology

Following experimental APV infection of turkeys, histopathological changes in the trachea (Jones et al., 1986; Picault et al., 1987; Majo et al., 1995), turbinates (Picault et al., 1987; Majo et al., 1995) and lungs (Majo et al., 1995) have been described. Microscopic changes occur in the mucosa of the nares and trachea between days 2 and 10 pi. The first abnormality includes focal loss of the surface cilial layer at day 2 pi, along with increase in glandular activity. By day 4 or 5 pi, loss of cilia becomes more extensive with focal discontinuity of the epithelial layer and within the epithelium there is vacuolation and occasional cell debris. Sub-epithelial hyperaemia is also present with intraepithelial heterophilic and lymphocytic infiltration. Copious inflammatory exudate in the lumen is observed. These degenerative and inflammatory changes become more marked between days 5 and 10 pi; at the later stage, subepithelial lymphocyte accumulation becomes conspicuous and regenerative changes begin. The acidophilic

cytoplasmic inclusions in epithelial cells of turbinates and trachea observed by French workers (Picault et al., 1987) were not seen by others.

Majo et al. (1995) observed certain changes in mucosa and submucosa of the bronchi subsequent to experimental infection of turkeys. These consisted of inflammatory exudate in the bronchial lumen, an increase in glandular activity, hyperplasia of the bronchial epithelium and presence of an abundant mononuclear inflammatory infiltrate in the submucosa.

Following experimental infection of three-week-old commercial broiler chickens with APV, a small number of birds developed either focal heterophil and leucocyte infiltration of congested nasal epithelium or focal karyorrhexis of tracheal epithelium on day 4 pi (Jones et al., 1987). Majo et al. (1995) described microscopic lesions in the turbinates of APV infected broiler chickens to be similar to that for turkeys but in the trachea the changes were of a very mild order.

2.3.4. Ultrastructural changes

There are no published reports on ultrastructural changes associated with experimental APV infection in turkeys. However, Majo et al. (1996) studied the ultrastructural changes associated with APV in turbinates of chickens following an experimental infection. The ciliated and non-ciliated epithelial cells of the turbinates showed numerous intracytoplasmic nucleocapsid aggregates of APV as well as budding virus particles at days 3 and 5 pi. This was accompanied by different ultrastructural abnormalities including cytoplasmic blebs, clumping and loss of cilia in the apical cell membrane of many infected cells, and desquamation of epithelial cells. Regenerative changes were observed by day 7 pi.

2.4. IMMUNE RESPONSE TO APV

2.4.1. Humoral immune responses

Serological methods comprising ELISA (Grant et al., 1987; Chettle & Wyeth, 1988; Cook et al., 1988; O'Loan et al., 1989), SNT (Cook et al., 1988; Baxter-Jones et al., 1989; O'Loan et al., 1989) and IIF (Baxter-Jones et al., 1986; Baxter-Jones et al., 1989; O'Loan et al., 1989) have been used to detect humoral antibody responses of turkeys or chickens to APV. Several workers have studied the kinetics of antibody in turkeys after experimental inoculation or natural infection with APV. Baxter-Jones et al. (1989) could detect virus neutralising (VN) antibodies with peak titres as early as 5 days after appearance of clinical signs following natural infection of APV in turkeys.

The VN and IF antibody titres were found to be decreasing by day 13, whereas ELISA antibodies peaked on day 13, and were in decline by day 34.

Following experimental infection of turkeys with APV, ELISA antibody titres reached significant levels at day 14 pi and continued to rise until day 29 pi after which they declined between days 30 to 35 pi (Naylor et al., 1992). In an earlier study (Jones et al., 1988), experimental infection of mature turkey hens resulted in ELISA and VN antibodies, both reaching high titre by day 12 pi and these were maintained at a high levels throughout the period of observation of 89 days.

Inoculation of attenuated APV strains in poults also result in seroconversion and protect the birds against virulent challenge (Cook et al., 1989b; Williams et al., 1991b). However, in certain instances, correlation between presence of antibody and protection of the respiratory tract has been found to be poor. In an experimental study, vaccinated poults, chemically bursectomised using cyclophosphamide and unable to seroconvert, were still found to be protected against challenge with virulent virus (Jones et al., 1992).

In a long-term study, turkey hens given live TRT vaccine at 12 days of age were protected against challenge at 22 weeks, even though they had no significant levels of ELISA antibody at the time of challenge (Williams et al., 1991b). The serological response to vaccination in poults with maternal antibodies (MA) to APV, sometimes has been found to be poor (Cook et al., 1989b; Williams et al., 1991b), but this did not affect protection against challenge. Thus the evidence cited above indicates that humoral antibody at least detected by ELISA may not be a good indicator of protection against APV challenge. However, it is probable that in laying turkey hens, circulating antibody is important in protecting the reproductive tract from viraemic virus which might affect egg production (Jones, 1996).

The fusion F glycoprotein of APV induces antibodies detectable by ELISA and SNT, and has been shown to induce partial protection in turkey poults when administered using a fowl pox vector in two doses (Yu et al., 1994). No work has been published on antibody responses to other specific viral proteins *in vivo*, however the neutralising capacity of Mab that recognises G glycoprotein has been demonstrated *in vitro* (Cook et al., 1993b). Nothing has been published on immunoglobulin class-specific responses to APV. Some work related to this topic is described in the Chapter 4.

2.4.2. Maternal antibodies

The presence of MA do not interfere with live TRT vaccination of turkey poults at a young age (Cook et al., 1989b). Furthermore, Naylor et al. (1997b) have recently shown that MAs in turkey poults are not involved in protection against virulent APV challenge.

2.4.3. Local immune responses

The local immune responses to APV have not been studied. Since APV replicates primarily in the upper respiratory tract of turkeys and chickens, it appears that local immunity could be important in protection against APV. A study on the local antibody responses in APV-infected poults and chicks is described in the Chapter 4.

2.4.4. Cellular immune response

The role of cell mediated immunity in protection against APV has not been studied. As discussed earlier, the humoral antibody against APV in certain instances, do not appear to completely correlate with protection against virulent challenge and MA has been shown not to play a part either. The role of T-cells in APV infections is investigated in turkeys and chickens using the T-cell suppressor drug cyclosporin A (Chapter 5).

2.5. DIAGNOSIS

2.5.1. Clinical signs

The clinical signs of TRT in the turkey and APV infection in the chicken are not pathognomonic, hence diagnosis is based on demonstration of the virus or of rising APV antibody titres. However, the severity of clinical signs has been used for assessing protection afforded by vaccines following challenge under experimental conditions (Cook et al., 1989a; Jones et al., 1992).

2.5.2. Isolation and demonstration of the virus

Since APV primarily replicates in the upper respiratory tract, turbinates and trachea or swabs from these sites are the preferred samples for APV isolation, and they should be taken in early stages of clinical disease. For both species, the virus typically sheds most strongly 3 to 5 days following experimental infection (McDougall & Cook, 1986, Wilding et al., 1986; Jones et al., 1988; Buys et al., 1989a; Cook et al., 1991; Cook et al., 1993a). The system of choice for isolation of APV is tracheal organ culture (TOC) prepared from embryonic chickens or turkeys (McDougall & Cook, 1986, Wyeth et al., 1986; Jones et al., 1986; Buys et al., 1989a). Ciliostasis usually follows within one week of inoculation but further passages may be necessary. Embryonated turkey or

chicken eggs are not commonly used for primary isolation of this virus, although embryo deaths with stunting and liver lesions after several passages using yolk sac route of inoculation have been described (Giraud et al., 1986b; Wyeth et al., 1986).

The virus has been adapted to grow on the chick embryo chorio-allantoic membrane (Buys et al., 1989a) and in chick embryo fibroblasts (Grant et al., 1987), chick embryo liver cells (Williams et al., 1991a) and vero cells (Buys et al., 1989a; Williams et al., 1991a), but none has been used for primary isolation.

The demonstration of the virus can be achieved by techniques such as IF staining of tracheal smears or sections (Baxter-Jones et al., 1986; Jones et al., 1986), IP staining of tissue sections (O'Loan & Allan, 1990; Majo et al., 1995), IF on unfixed infected TOC rings (Naylor, 1993), IF on cell-cultures (O'Loan et al., 1990), virus neutralisation test using reference antisera (Cook et al., 1993b) and electron microscopy of concentrated organ cultures or cell culture fluids (McDougall & Cook, 1986; Wyeth et al., 1986; Giraud et al., 1986b; Buys et al., 1989a).

A PCR based method has been described for identification of APV in tracheal or oropharyngeal swabs from turkeys (Jing et al., 1993). It was shown that following experimental infection of turkeys, viral RNA could be detected for about 2 weeks longer than virus could be isolated in TOCs. Mase et al. (1996) also reported another sensitive and specific PCR method for detection of APV in the chicken turbinates and trachea from the field cases of SHS. The advantage of the PCR is that it is quicker and detects virus in small quantities, but the PCR technique identifies only the viral RNA and not the infectious virus which may be needed for further studies. However, by using primers to make PCR subtype-specific, it can be employed for strain characterisation and is a valuable tool for epidemiological surveys (Jing et al., 1993; Naylor et al., 1997a).

2.5.3. Serology

Procedures described for serodiagnosis of APV include IIF (Baxter-Jones et al., 1989; O'Loan et al., 1990), ELISA (Grant et al., 1987; Chettle & Wyeth, 1988; Cook et al., 1988; O'Loan et al., 1989; Etteradossi et al., 1992; Naylor et al., 1992) and SNT (Cook et al., 1988; Baxter-Jones et al., 1989; O'Loan et al., 1989; Collins et al., 1993).

Baxter-Jones et al. (1989) comparing these three tests, concluded that all were capable of detecting APV antibodies within 5 days of the appearance of clinical respiratory disease in naturally infected commercial turkeys. However, they found ELISA and

SNT to be more reliable and sensitive than the less practicable IIF and suggested the use of ELISA for routine diagnosis for being less laborious, more sensitive and with ease of automation.

In contrast, O'Loan et al. (1990) found the IIF test to be the most broadly based, presenting the widest range of antigens for antibody detection, since some sera from TRT infected birds, which were negative by ELISA proved to be positive by IIF. Cook et al. (1988) demonstrated a good correlation between ELISA and SNT in detecting APV antibodies in commercial chicks. However, they found some samples which were negative in SNT were positive in ELISA, reflecting that the ELISA can detect a wide range of antibodies which combine with the viral antigens, whereas the SNT detects only those antibodies which bind to particular antigenic determinants involved in virus neutralisation.

Various ELISA systems have also been compared with respect to their efficiency in serological testing in turkeys challenged with different APV strains, with or without previous administration of live TRT vaccine (Etteradossi et al., 1992, Etteradossi et al., 1995; Toquin et al., 1996). These workers reported some discrepancies in results among different ELISAs leading to the conclusion that the seroconversion to either live vaccination or infection is significantly better demonstrated using ELISA with homologous antigen i.e. antigen of same APV subtype. Etteradossi et al. (1995) suggested that the use of an inadequate antigen for serological testing may account for an apparent lack of immunogenicity of the vaccine or can hinder the early diagnoses of APV infection. Therefore, a careful choice of the antigen and testing of appropriate control sera appear to be major requisites for reliable APV-ELISA monitoring following infection or vaccination (Etteradossi et al., 1995).

Mekkes and De Wit (1998) compared three commercial ELISAs kits (Pathasure, Svanovir and Flockscreen) in their ability to detect APV vaccine-induced antibodies in chickens. Pathasure ELISA failed to detect antibodies after live vaccination with either French or UK vaccines, but detected antibodies following a second inoculation with inactivated vaccines. The two other ELISAs responded to both live vaccinations equally well and both detected a rise in antibody level 8 days after the second inoculation with inactivated vaccine(s). However, all the three ELISAs showed similar sensitivity in detecting APV antibodies in field sera from turkey flocks in The Netherlands.

2.6. CONTROL

There is no specific treatment for APV infections. Administration of antimicrobials can be useful in controlling secondary bacterial infections (Stuart, 1989). Improvement of management practices including ventilation, stocking densities, litter conditions and general hygiene, helps in reducing losses (Stuart, 1989). However, the main approach for controlling pneumovirus infections has been through the use of live attenuated vaccines in young stock for both turkeys and chickens (Cook et al., 1989b; Buys et al., 1989a; Williams et al., 1991b; Cook et al., 1995) and combinations of live attenuated and inactivated vaccines in turkey layers or breeders before the onset of lay (Cook et al., 1996).

Methods of attenuation have included passage of virus on the chorio-allantoic membrane of the fertile chicken eggs (Buys et al., 1989a), alternate passage in TOCs and chick embryos (Cook & Ellis, 1990), and multiple passage in vero-cells (Buys et al., 1989a; Williams et al., 1991a). Vaccines prepared by the above methods have all been reported to confer protection against experimental infection (Buys et al., 1989a; Cook et al., 1989b; Williams et al., 1991b; Cook et al., 1995). In the field, results with vaccines have been variable following aerosol, spray or drinking water administration and one problem associated with live attenuated TRT vaccines is that some strains of pneumovirus have a tendency to revert to virulence (Jones, 1996). Naylor and Jones (1994) using an *in vitro* and *in vivo* screening technique demonstrated a virulent sub population in a prototype live attenuated TRT vaccine which was thought to account for occasional disease in young poults following multiple back passage of the vaccine.

Under experimental conditions, inoculation with live attenuated vaccine virus usually leads to a strong antibody response in the turkeys (Cook et al., 1989b; Williams et al., 1991b), though it has been reported that minimum efficacious doses of vaccine may not lead to seroconversion (Cook et al., 1989b). Reports from the field sometimes also highlight a poor serological response following vaccine application, leading to difficulties in ascertaining immune status of birds (Jones, 1996).

The demonstration of some antigenic differences among various APV strains and subsequent confirmation of the existence of two subtypes (A & B) (Cook et al., 1993b; Collins et al., 1993; Juhasz & Easton, 1994), lead to cross protection studies for drawing better vaccine strategies. Cook et al. (1995) showed that poults or chicks vaccinated with a subtype A vaccinal strain were protected against experimental challenge with strains belonging to both the subtypes A & B. Etteradossi et al. (1995) have reported that poults which were vaccinated with a subtype B vaccine did not show any signs of respiratory disorder when challenged with virulent subtype A or B

viruses. It indicates that the vaccine of one subgroup will induce protection against the other. Similarly, Naylor et al. (1997a) also showed cross-protection among virulent type A & B viruses during experimental reinfection studies suggesting that type A and B viruses do have protection-inducing epitopes in common, although a very small proportion of poults inoculated at two days of age with virulent type A virus were not protected against challenge with type B virus 21 days later.

Studies with the pneumovirus human RSV have shown that the G and F surface glycoproteins play major roles in the induction of protective immunity (Stott et al., 1987; Connors et al., 1991). A very low amino acid identity (38%) between the G protein of the two subtypes of APV suggests that at least some of the protection-inducing epitopes reside in protein (s) other than G (Naylor et al., 1997a). Vaccination of turkey poults with a fowl pox virus recombinant expressing the APV F-glycoprotein resulted in partial protection against homologous challenge (Yu et al., 1994). These results showed that the immune responses to the F protein play a major role in protection against APV and indicated that recombinant viruses expressing APV F protein have potential as recombinant vaccines against APV infections.

For the immediate future, it seems likely that conventional APV vaccines will continue to be used until recombinant ones with better protective ability are developed that prove to be at least as cost-effective as current ones.

CHAPTER 3

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CHAPTER 3

GENERAL MATERIALS AND METHODS

This section describes the general materials and methods used routinely while undertaking this work. Details of specialised procedures used are described in relevant chapters. The composition of buffers, solutions and media are presented in the Appendix.

3.1. GLASSWARE

All items of glassware were collected and cleaned in the laboratory according to the following procedures before use.

3.1.1. Contaminated glassware

All glassware that had been used with contaminated material was autoclaved at 121°C for 30 minutes (min) at a pressure of 15 lb/sq in, before cleaning and sterilisation. After use, all pasteur pipettes were soaked in sodium hypochlorite (Chloros, Golden Grain Products Ltd., Liverpool, England) and discarded into safety buckets. All used disposable plasticware was put in plastic bags after disinfection by overnight soaking in the disinfectant Virkon (ANTEC International Sudbury, Suffolk, England). These plastic bags were then autoclaved and incinerated.

3.1.2. Cleaning of glassware

All glassware which was new or had been autoclaved was cleaned automatically using a glass washing machine (Miele, Hamburg, Germany). The glassware was washed in the washing machine, with a detergent (Neodisher GK, Chemische Fabrik Dr. Weigert, Hamburg, Germany), following a cycle which gives 2 tap water rinses and 3 ultra pure water rinses and then dries automatically.

3.1.3. Sterilisation of glassware

Bottles were closed with plastic or metallic caps and were sterilised in an autoclave at 121°C for 30 min at a pressure of 15 lb/sq in. Flasks and measuring cylinders were closed with aluminium foil and sterilised in a hot air oven at 160°C for 2 hours (h). Pasteur pipettes were plugged with cotton wool and were sterilised in stainless steel

containers in a hot air oven. Pestles and mortars were wrapped in plastic bags and autoclaved. All autoclaved glassware was dried thoroughly in a drying oven.

3.2. EMBRYONATED EGGS

Embryonated chicken eggs (ECE) were used for preparation of tracheal organ culture (TOC), virus propagation and as a source of day-old specific pathogen-free (SPF) chicks. Embryonated turkeys eggs free of maternal antibodies (MA) to APV were used as a source of day-old poults.

3.2.1. Source of the eggs

The SPF chicken eggs were obtained from a commercial supplier (Wickham Laboratories, Wickham, Hants, UK). The parent flock was free of major infectious disease agents including APV. Turkey eggs free of MA to APV were obtained from Nicholas Turkey Breeding Farms, Kilwinning, Ayr, Scotland.

3.2.2. Incubation

The eggs were usually set for incubation within the first week after being laid. Incubation was carried out in a commercial incubator with automatic hourly turning. Fertile eggs were used at the age required.

3.3. CHICKEN EMBRYO TRACHEAL ORGAN CULTURES

The method for preparation of TOC has been described by Cook et al. (1976). Briefly, the embryos were removed from fertile 19- or 20-day old SPF chicken eggs and the tracheas collected from them after decapitation. Tracheas were trimmed of adventitious tissue and placed in a petri dish containing warm (37°C) TOC medium (see Appendix). They were then cut into 0.6 mm thick rings using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK) and each ring was placed in a sterile tube (Nunc, Life Technologies Ltd, Paisley, Scotland) containing 0.7 ml of medium. Tubes were rotated in a roller drum at 8 revolutions per hour at 37°C. After 24 h, the rings were checked for ciliary activity and used as required.

3.4. VERO CELLS

Vero cells orginally obtained from Duphar BV (Weesp), Netherlands, were maintained and stored in this department in liquid nitrogen. When required, the cells were revived in growth medium (see Appendix). Each week, cells were split using trypsin treatment (see Appendix) and diluted four times in growth medium. Cells were incubated at 37°C in a CO_2 incubator. Once confluent sheets had formed, the cells were ready for infection or further splitting.

Only confluent cell sheets were inoculated with APV, because infection of growing cells failed to result in cytopathic effects (CPE). Medium was removed for inoculation then one hour afterwards maintenance medium (see Appendix) was added. Positive infection resulted in a scruffy syncytial-type CPE after four to seven days.

3.5. EXPERIMENTAL BIRDS

Turkeys and chickens were used for the experimental infections. ECE were checked for their fertility on the 18th day of incubation, and turkeys eggs on the 25th day of incubation. They were then transferred into a tray kept at the bottom of the incubator in a static condition, for hatching after 21 days for chickens and 28 days for turkeys. For some experiments, one day-old turkey poults free of MA to APV were obtained from the Nicholas Turkey Breeding Farms.

3.5.1. Housing and management

The poults or chicks were maintained in isolation pens equipped with a supply of filtered air under negative pressure. Foot baths with glutaraldehyde disinfectant (GPC 8, Evans Vanodine International PLC, Lancashire, UK) and protective clothing including overalls, masks, gloves and boots were used at all times when visiting birds. Heat was supplied from 1.5 Kilowatt radiant electric heaters. The birds were placed on wood shaving litter in the isolation pens. Feed and water were provided *ad libitum.*

3.6. ASSESSMENT OF CLINICAL DISEASE

Following inoculation of birds with APV, clinical disease was assessed and scored as per method of Jones et al. (1992). This involved examination of head, paying particular attention to nostrils, eyes and area between overlaying the infraorbital sinuses. The eyes were viewed for excessive aerated liquid and the sinus region was monitored for swelling. Lastly, the nostrils were examined for signs of nasal exudate, but if none was found, the thumb was slid with moderate pressure, from sinus region towards the nostrils, to eject any internal mucous from the nostrils. The signs were scored on 0 to 3 scale. 0: no signs, 1: clear nasal exudate, 2: turbid nasal exudate, 3: frothy eyes and/or swollen infraorbital sinuses in conjunction with nasal exudate.

3.7. VIROLOGICAL METHODS

3.7.1. APV strains

Virulent field virus

APV strain #8544 (Wilding et al., 1986) originally isolated from a field outbreak of TRT, was used for most of the experimental work. This strain has been maintained in

this department for several years and had been passaged 22 times in chick embryo TOC. Before use, it was further passaged twice and titrated in TOC. A virulent APV strain of chicken origin designated, CP-1 was also used in the study. The details of this strain are given in Chapter 7. Both the strains belong to APV subtype A.

Vero-grown vaccine virus

Strain #8544 of APV, previously attenuated and adapted to grow in vero cells (Williams et al., 1991a) was used for this study. This vero-grown vaccine strain was obtained from a commercial supplier (Solvay Animal Health, Southampton, England). It was used for vaccination of either poults or chicks (Chapter 4) or cultivated in vero cells for obtaining purified antigen for enzyme-linked immunosorbent assay (ELISA) (see section 3.8). Before use, it was passaged once and titrated in vero cells.

3.7.2. Processing of samples for virus isolation

In various experiments, virus isolation was attempted either from swabs or macerated tissues. After sampling, the swabs were dipped into 1ml of TOC medium containing x10 the normal amount of antibiotics, shaken vigorously and stored at -70°C until used. Tissues were macerated by grinding in a sterile pestle and mortar, using sterile sand and a small volume of medium. Then more medium was added to the ground tissue to make a final 1:10 (w/v) dilution of the sample. Following centrifugation at 1500 g for 10 min, the supernatants were collected and stored at -70°C until used.

3.7.3. Virus isolation in TOC

Twenty four hours after preparation, each tracheal ring was checked microscopically and only those showing vigorous ciliary beating were used. The medium was removed and rings were inoculated with 0.1 ml of the sample suspension. Virus was allowed to adsorb at 37°C for 1 hour. After adsorption, the inoculum was removed, the rings were washed with medium and then overlaid with 0.7 ml of TOC medium and maintained in a roller drum at 37°C. The tubes were examined daily and the occurrence of complete ciliostasis was taken to be indicative of the presence of virus. All the samples were passaged up to three times at 7 day intervals before being considered negative. Three rings were inoculated for each sample.

3.7.4. Virus titration

For virus titration in TOC, the method described by Cook et al. (1976) was followed. Briefly, replicates of 3 to 5 tubes of TOC were inoculated with ten-fold dilutions of samples mentioned above. The occurrence of complete ciliostasis was taken as the endpoint. The titres were calculated according to the method of Reed and Muench (1938) and expressed as median ciliostatic doses ($\log_{10}$ CD_{50}) per 0.1 ml.

For virus titration of vaccine virus, the method described by Williams et al. (1991b) was followed. Briefly, ninety six well cell culture plates (Costar UK Ltd., High Wycombe, Bucks, England) were seeded with vero cells. Replicates of 5 wells with confluent cell sheets was inoculated with ten-fold dilutions of cell-free suspension of the virus. The occurrence of 80-100% CPE was regarded as the endpoint. The titres were calculated according to the method of Reed and Muench (1938) and expressed as median tissue culture infective doses ($\log_{10}$ $TCID_{50}$) per 0.1 ml.

3.7.5. Identification of virus

To demonstrate APV-specific antigens on unfixed TOC rings or cryostat sections of tissue, an immunofluorescence (IF) technique was used.

IF staining of unfixed TOC rings

Identification of ciliostatic virus in unfixed TOC rings was performed following the method described by Bhattacharjee et al. (1994) with modifications. Briefly, 72 to 96 h after inoculation, TOC rings were placed in a 48-well cell culture plate (Costar UK Ltd.) and washed with phosphate buffered saline (PBS) pH 7.2 (see Appendix) using a plate shaker (Titertek, Irvine, Scotland). Then, each well received 0.15 ml of optimally diluted hyperimmune serum (see section 3.9.1) to strain #8544 and the plate was set on a shaker with gentle shaking for 1 h at room temperature. After washing with PBS, the cultures were treated with 0.15 ml of 3% bovine serum albumin (BSA) (Sigma, Poole, Dorset, England) for 30 min for blockage of non-specific binding sites. After another washing, the cultures were treated with 0.15 ml of optimally diluted commercial affinity-purified fluorescein conjugated goat antibody to turkey IgG or chicken IgG (Kirkegaard & Perry Laboratories, Inc. Gaithersburg, USA) for another hour. Following washing, the rings were mounted on teflon-coated slides (ICN Biomedicals Inc., Costa Mesa, California, USA) with fluoromount-G (Kirkegaard & Perry Laboratories) and examined under a fluorescent microscope (Leica UK Ltd., Milton Keynes, UK) equipped with incident UV/blue illumination. Specific fluorescence in the epithelial cells of TOC was taken as an indicator of the presence of virus-specific antigens.

IF staining of tissue sections

Small pieces of tissues were collected in aluminium foil cups containing an embedding medium, OCT compound (Miles Inc., Elkhart, Indiana, USA) and snap-frozen in liquid nitrogen. Each sample was then collected in a self-sealing polythene bag and stored at -70°C until used for sectioning. Cryostat sections were cut at a thickness of 4 to 6 µm, collected onto poly-L-lysine coated slides (Sigma) and air-dried and fixed in

acetone at room temperature for 10 min. The acetone-fixed sections were rinsed briefly in PBS and then flooded with a pre-determined optimal dilution of hyperimmune serum to strain 8544 and incubated at 37°C for 1 h in a humid chamber. Slides were washed in PBS for 15 min in a bath using a magnetic stirrer. After washing, the slides were incubated with 3% BSA (Sigma) for 30 min. This was followed by washing of slides in two changes of PBS for 15 min each, after which the appropriate secondary antibody was added and incubated similarly. Following washing, the slides were mounted and examined as described above.

TOCs inoculated with samples from uninfected birds or sections of tissues from uninfected birds and the use of sera prepared from control birds in place of primary antibody, were used as controls to assess the specificity of IF staining.

3.8. PURIFICATION OF VIRUS

For ELISA use, the vero-grown vaccine strain derived from APV strain 8544 (Williams et al., 1991a) (see section 3.7.1) was propagated by passage through the vero cell-line as described above. When extensive CPE had developed, the cells were harvested and clarified. Supernatants were collected and centrifuged at 100,000 g for 3 hours over 25% sucrose in GNTE buffer (see Appendix), in a swing-out rotor (AH 629) using an ultracentrifuge (Sorvall Instruments, Dupoint, USA) according to the method described by Cavanagh and Barrett (1988). The pellet was resuspended in a small volume of GNTE buffer in the centrifuge tube and this constituted the purified viral preparation. It was divided into aliquots and stored at - 70°C. The optimum dilution of this virus for use in ELISAs was determined by checker-board titration.

3.9. SEROLOGY

3.9.1. Preparation of hyperimmune serum

Six week-old SPF chickens were infected oculo-nasally with 3.5 log_{10} CD_{50} of APV strain #8544. Three weeks later, an identical booster dose was given and the birds were bled after two-weeks. Sera separated from these birds were heat inactivated (56°C for 30 min) and used for IF staining, serum neutralisation test (SNT) and ELISA.

3.9.2. Serum neutralisation test

These were carried out in chick embryo TOC following the method described by Cook et al. (1988) with some modifications. Briefly, serial two-fold dilutions (from 1:8) of heat-inactivated (56°C for 30 min) hyperimmune serum were made in TOC medium

and each dilution was mixed with an equal volume of virus (strain 8544) suspension containing log_{10} 2.0 CD_{50} in 0.1 ml. The mixture was incubated at room temperature for 30 min. After removing the medium from TOC tubes, 0.2 ml of the virus-serum mixture was added into each tube and incubated at 37°C for one hour. Then, the tubes were overlaid with 0.5 ml of TOC medium and incubated in a roller drum at 37°C. Replicates of 3 to 5 tubes was used for each serial dilution. The test was read between 7 to 10 days after inoculation by examining the cultures for complete ciliostasis. The endpoint titres were calculated according to the method of Reed and Muench (1938).

3.9.3. Enzyme-linked immunosorbent assay

APV antibodies were measured using the ELISA originally described by Chettle and Wyeth (1988) and modified by Naylor et al. (1992). Briefly, 96 well ELISA plates (Falcon 3912, Becton Dickinson, Labware, Oxnard, CA) were coated overnight with 50 µl of purified viral antigen (see above) diluted in carbonate-bicarbonate (CBC) buffer (see Appendix) at 37°C. The plates were coated alternatively with the antigen and CBC buffer. This was followed by washing four times with wash-dilution buffer (see Appendix) using a plate washer (Nunc-Immuno Wash 8, Life Technologies Ltd, Paisley, Scotland). After washing, the plates were air-dried and then the test sera along with positive and negative control sera diluted 100 times in wash-dilution buffer were added. The sera were tested using duplicate wells and reagents were used as 50 µl volumes per well. The plates were incubated for 1 h at 37°C then washed five times and dried as before.

In the next step, affinity purified, peroxidase conjugated goat-antibody to turkey IgG (H+L) (Kirkegaard & Perry Laboratories) was added at a predetermined dilution, and incubated for 1 h at 37°C then washed off as before. Finally, 100 µl of ortho-phenylenediamine (OPD) (Sigma) substrate (see Appendix) was added in each well and plates were left at room temperature. The reaction was stopped 20 min later by addition of 25 µl of 5 N sulphuric acid (see Appendix) per well. Absorbance values were read at 490 nm (test filter 3) with a reference reading at 630 nm (reference filter 5) using microplate ELISA reader (model MR700, Dynatech, Billingshurst, Sussex, England). Using a software designed in BASICS on an Amstrad computer, the results were expressed as log_2 titres with threshold of significance being >6.1 log_2 (Naylor et al., 1992).

CHAPTER 4

LOCAL AND SYSTEMIC CLASS-SPECIFIC ANTIBODY RESPONSES FOLLOWING PRIMARY AND SECONDARY INFECTION OF AVIAN PNEUMOVIRUS IN CHICKENS AND TURKEYS

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CHAPTER 4

LOCAL AND SYSTEMIC CLASS-SPECIFIC ANTIBODY RESPONSES FOLLOWING PRIMARY AND SECONDARY INFECTION OF AVIAN PNEUMOVIRUS IN CHICKENS AND TURKEYS

4.1. INTRODUCTION

Development of humoral antibodies following APV infection in both chickens and turkeys has been well documented (Cook et al., 1993a; see Naylor & Jones, 1993). Enzyme-linked immunosorbent assay (ELISA) (Chettle & Wyeth, 1988; Etteradossi et al., 1992) and serum neutralisation test (SNT) (Cook et al., 1988; O'Loan et al., 1989) are commonly used serological methods for the demonstration of humoral antibody responses to APV.

Under experimental conditions, inoculation of poults with live attenuated vaccine viruses results in serum antibody response and protects the birds against virulent challenge (Cook et al., 1989b; Williams et al., 1991b). However, in certain instances, correlation between the presence of antibodies and protection of the respiratory tract has been found to be poor. In a recent experimental study, the presence of high levels of maternal antibodies (MA) in turkey poults did not prevent the development of clinical disease (Naylor et al., 1997b). Also, MA-positive turkey poults showing a poor serological response to attenuated APV (Cook et al., 1989b; Williams et al., 1991b), were nonetheless protected against virulent challenge. In a long-term study, turkey hens given live APV vaccine at 12 days of age were protected against challenge at 22 weeks even though they had no significant levels of ELISA antibody in the serum at the time of challenge (Williams et al., 1991b).

The importance of locally produced antibodies against other viral respiratory infections of poultry such as infectious bronchitis (Davelaar et al., 1982; Toro & Fernandez, 1994) and Newcastle disease (Russell & Ezeifeka, 1995; Takada & Kida, 1996), and in other pneumoviral infections comprising human and bovine RSV (McIntosh et al., 1979; see Kimman, 1993) has been well documented. To-date, there is no published work on the local antibody production and their importance in APV infections. In this chapter, the systemic and local virus-specific IgA and IgG are compared in an attempt to relate resistance to APV infection with any of these antibodies.

The main objectives of this study were as follows. (1) To demonstrate local antibody (IgA & IgG) production in the harderian gland (HG) and trachea of chickens infected with a virulent or an attenuated strain of APV. (2) To characterise local and systemic

class-specific antibody (IgA & IgG) responses following challenge of chicks and poults primed with a virulent or an attenuated strain of APV.

PART I: LOCAL ANTIBODY PRODUCTION IN THE HARDERIAN GLAND AND TRACHEA OF CHICKENS

4.2. MATERIALS AND METHODS

4.2.1. Chickens

Commercial SPF eggs of WLH chickens were obtained and hatched in this department. Chicks were reared in strict isolation with food and water *ad libitum*.

4.2.2. Viruses

A virulent strain (VS) and an attenuated strain (AS) of APV were used for this study. The VS #8544 (Wilding et al., 1986) described in Chapter 3 was used after titration in tracheal organ cultures (TOC). The AS was essentially a commercial vaccine prepared by passaging strain #8544 in the vero cell line described previously (Williams et al., 1991a). Before use, the commercial vaccine was passaged once and titrated in vero cells (Chapter 3).

4.2.3. Experimental design

Immunoglobulins in lachrymal fluid, tracheal washes and serum

At four weeks of age, eighty-one chickens were randomly divided into 3 groups. One was infected with the VS, the second with the AS and the third kept as an uninfected control group. According to the infected group, each bird was inoculated by eyedrop with 3.5 log_{10} CD_{50}/0.1ml of VS or with 5.5 log_{10} $TCID_{50}$/0.1ml of AS. Ten birds in each group were examined daily for clinical signs. On days 3, 7, 14 and 21 post infection (pi), five chickens from each group were bled and lachrymal fluid was collected (see below). The birds were euthanased for collecting tracheal washes (see below) and selected tissues for assessment of local antibody production. Samples of serum, lachrymal fluid and tracheal washes were tested for virus-specific and class-specific antibodies (IgA and IgG) using ELISA and SNT.

Immunoglobulins in tissues

The assessment of local antibody secretion in tissues was performed as per the method of Zigtermann et al. (1993). On days 3, 7, 14 and 21 pi, five birds from each group were sacrificed as mentioned above. Pieces of HG, trachea and spleen were removed and washed thoroughly in minimum essential medium (MEM) containing 250 mg ml^{-1} of streptomycin and 250 IU ml^{-1} of penicillin. Pieces 3 mm^2 in size were prepared and

three pieces of each tissue were placed in a single tube with 1 ml medium. Immediately, 200 µl of medium was collected from each tube (t = 0). Duplicate sets of tissues were incubated at 37°C and at 4°C. After 18 h (t = 18), the medium was collected and assayed for APV-specific and isotype-specific (IgA and IgG) antibodies using ELISA. The increase in optical density (OD) was calculated by comparison with t = 0 values. A significant reduction in OD at 4°C was deemed to be indicative of local active secretion of antibodies. Levels of significance were calculated using student's t-test ($p<0.05$). Five birds were used each for tracheal and splenic cultures but for HG because of the small size, the samples from 5 birds were divided in 3 by pooling samples of 2 birds each and the fifth bird HG remaining separate.

On days 3, 5 and 7 pi, pieces of HG and trachea were also collected for virus isolations.

4.2.4. Clinical signs

The severity of signs was scored on a 0-3 scale by the method of Jones et al. (1992) described in Chapter 3.

4.2.5. Virus isolations and titrations

Virus isolations from tissues were performed in TOC (Chapter 3) using complete ciliostasis as the criterion for presence of virus. A minimum of three passages was given and the ciliostatic virus was identified by immunofluorescence (IF) staining of unfixed TOC rings (Chapter 3). All the samples positive by virus isolation were titrated in TOC according to the method described in Chapter 3.

APV isolations in samples from birds infected with the AS were also attempted by passaging three times in a vero cell line (Chapter 3).

4.2.6. Collection of lachrymal fluid and tracheal washes

Lachrymal fluid samples were collected as described previously (Toro & Fernandez, 1994). Briefly, a few crystals of sodium chloride were placed in each eye of the bird and the excess lachrymal fluid was collected with a micropipette.

For collecting tracheal washes the birds were euthanased. The trachea was removed and clamped midway between the larynx and the thoracic inlet, and 200 µl of sterile PBS was injected into the lumen. The trachea was gently massaged and the washes aspirated using a needle and 1ml syringe.

4.2.7. Monoclonal antibodies

These were tissue culture fluids specific for chicken IgA (reference no. p9) and IgG (reference no AV-G3), obtained from Dr. T.F. Davison, Institute for Animal Health, Compton, UK.

4.2.8. Enzyme-linked immunosorbent assays

For measuring class- and virus-specific IgA and IgG, the method of Naylor et al. (1992) described in Chapter 3 was modified as follows. Briefly, ELISA plates (Falcon 3912) were coated overnight at 4°C with purified viral antigen diluted 1: 50 in CBC buffer. The wells were coated alternatively with antigen and CBC buffer. The following day, the wells were blocked with PBS containing 1.0% BSA (Sigma). Based on a checkerboard titration, the samples were tested at a single dilution, with tracheal washes at 1: 5, lachrymal fluid at 1: 10 and serum at 1: 100. Each was diluted in PBS containing 0.05 % tween-80 (dilution buffer). Mouse monoclonal antibodies (Mab) against chicken IgA and IgG were used as primary antibodies at a dilution of 1: 10 followed by affinity-purified goat anti-mouse IgG-peroxidase conjugate (Sigma) at a dilution of 1: 500. The enzyme substrate OPD was added and the reaction stopped after 20 min by adding 25 µl of 5 N sulphuric acid. The plates were read at 490 nm (test filter 3) with a reference reading at 630 nm (reference filter) in a microplate ELISA reader (model MR700, Dynatech). Volumes of 50 µl were used for each step, except for the substrate, where 100 µl was used. The plates were washed three times after each step except for the blocking step and washing buffer was the same as the dilution buffer.

For lachrymal fluid, tracheal washes and serum, the baselines were established from mean plus twice standard deviation of the absorbance values of twenty normal samples collected from 2 and 3 week old SPF chickens.

4.2.9. Serum neutralisation test

Like samples of lachrymal fluid, tracheal washes or serum on each sampling occasion from chickens in the same group were pooled and examined for virus neutralising (VN) antibodies to strain #8544 (100 CD_{50}) in TOC as described in Chapter 3.

4.2.10. Statistical analysis

The mean clinical score and number of virus isolations were compared by using chi-square test ($p<0.05$). The results of virus titrations and ELISAs were analysed by student's t-test ($p<0.05$).

4.3. RESULTS

4.3.1. Clinical signs

Following infection of chickens with the VS, clinical signs were observed from days 3 to 8 pi, comprising clear to turbid nasal exudate (data not shown). No clinical signs were observed in birds inoculated with the AS or in uninfected controls.

4.3.2. Virus isolation and titration

APV was isolated in TOC on days 3 and 5 pi from both the HG and trachea of chickens infected with VS (not shown). No virus was detected from like tissues of birds infected with the AS or uninfected controls, either in TOC or vero cells.

The results of virus titres are shown in Fig. 4.1. In chickens infected with the VS, titres were similar in both the HG and trachea with peak between day 3 and 5 pi, and by day 7, virus was not detectable.

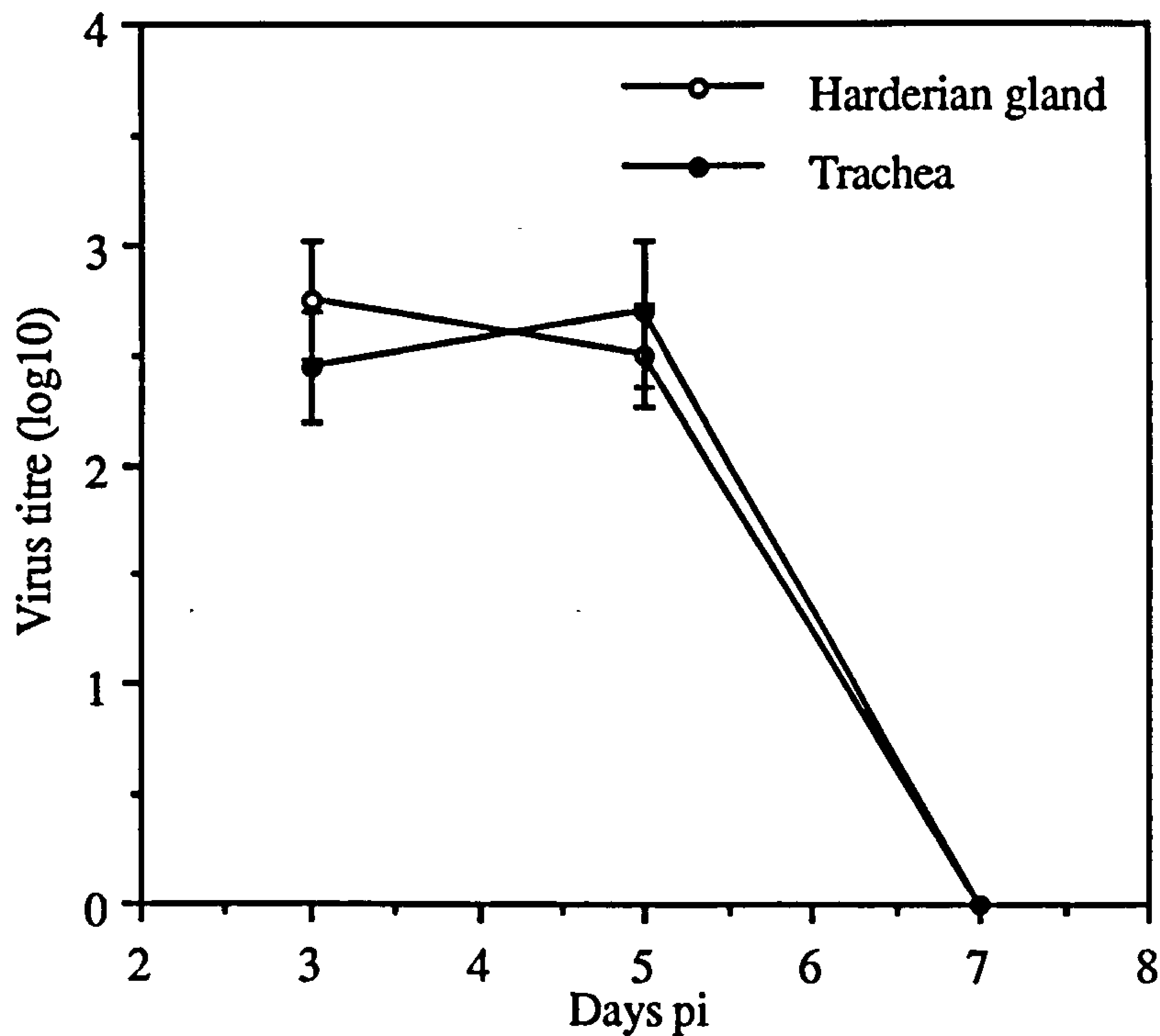

Fig. 4.1. Virus titres (mean ± sd) in tissues from 4-week old chickens following infection with the VS. Titres expressed as CD_{50} log_{10}/g tissue.

4.3.3. IgA antibody in lachrymal fluid, tracheal washes and serum

Fig. 4.2 shows antiviral IgA profiles in lachrymal fluid from APV-infected birds. A statistically significant elevation in lachrymal IgA was detected between days 3 and 21 following infection of chickens with either VS or AS. However, the OD values for chickens infected with AS on day 7 pi were below the baseline (0.05) and on all sampling occasions, the values were lower compared to the VS. The IgA response was highest on day 21 pi in both groups. The OD values for lachrymal fluid from controls birds (C) remained below the baseline.

The absorbance readings for IgA antibody in tracheal washes (Fig. 4.3) and serum (Fig. 4.4) from infected and control birds remained below the baseline levels (0.04).

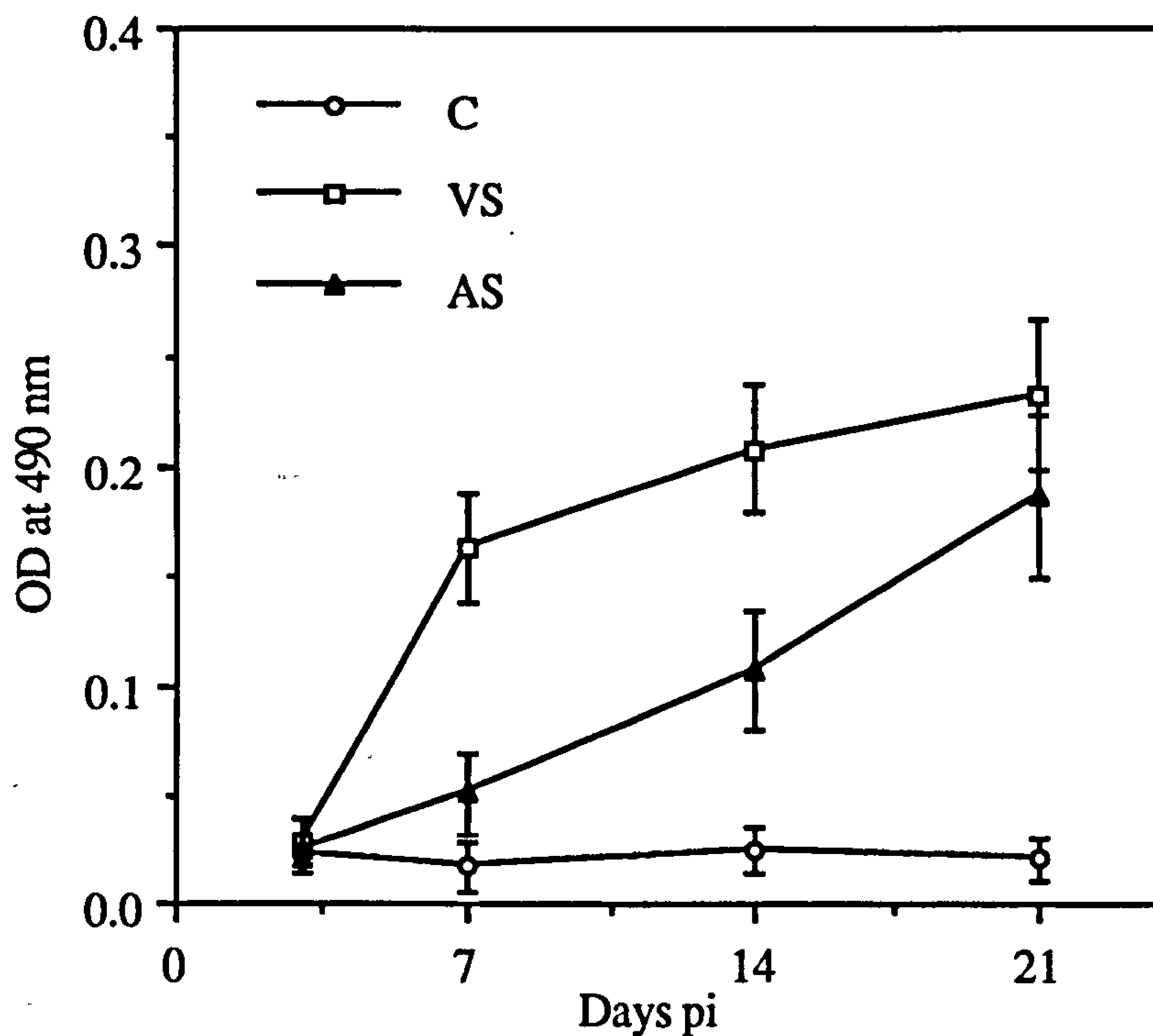

Fig. 4.2. Virus-specific IgA antibody (mean ± sd) in lachrymal fluid from 4-week old chickens following infection with either VS or AS.

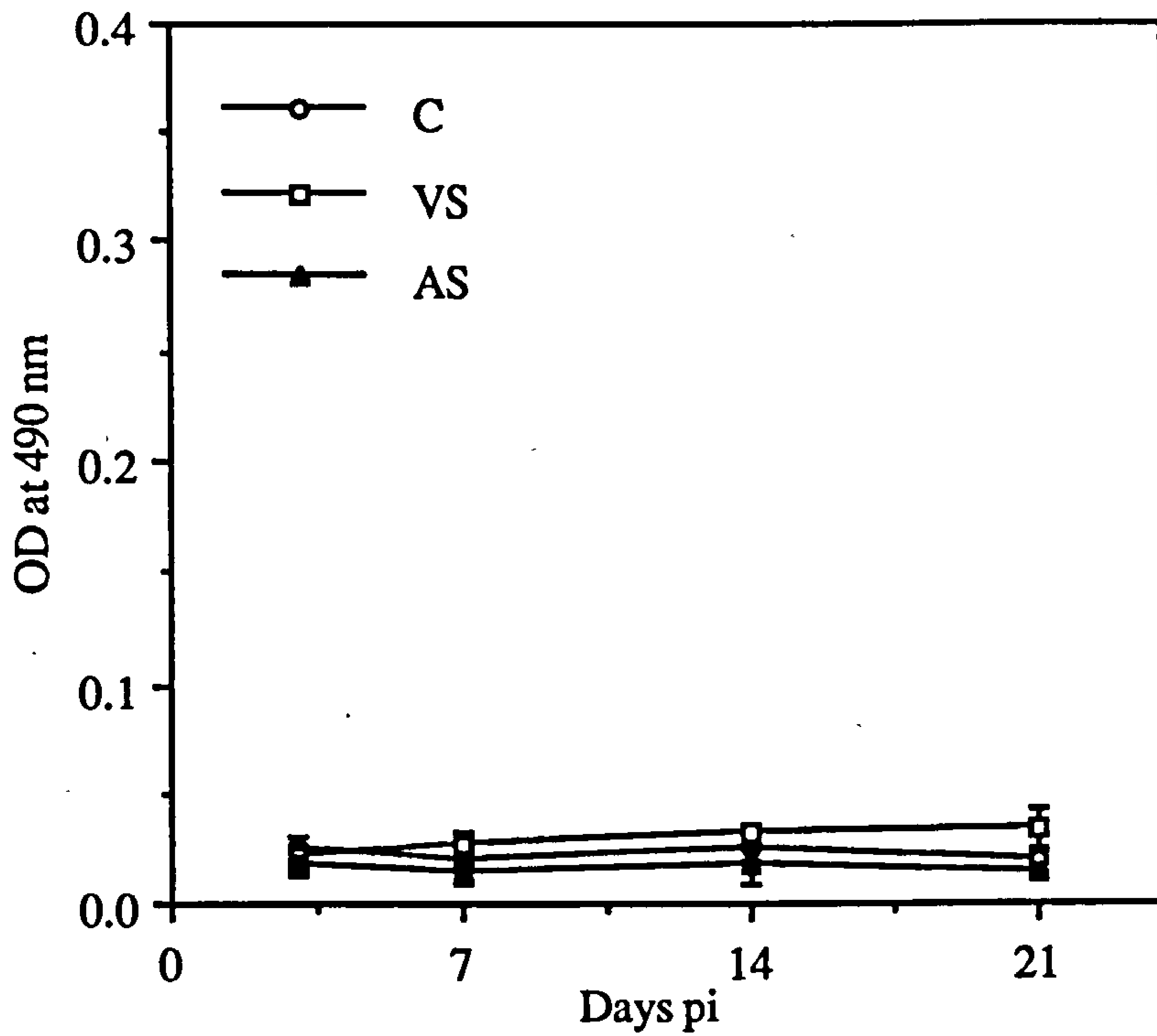

Fig. 4.3. Virus-specific IgA antibody (mean ± sd) in tracheal washes from 4-week old chickens following infection with either VS or AS.

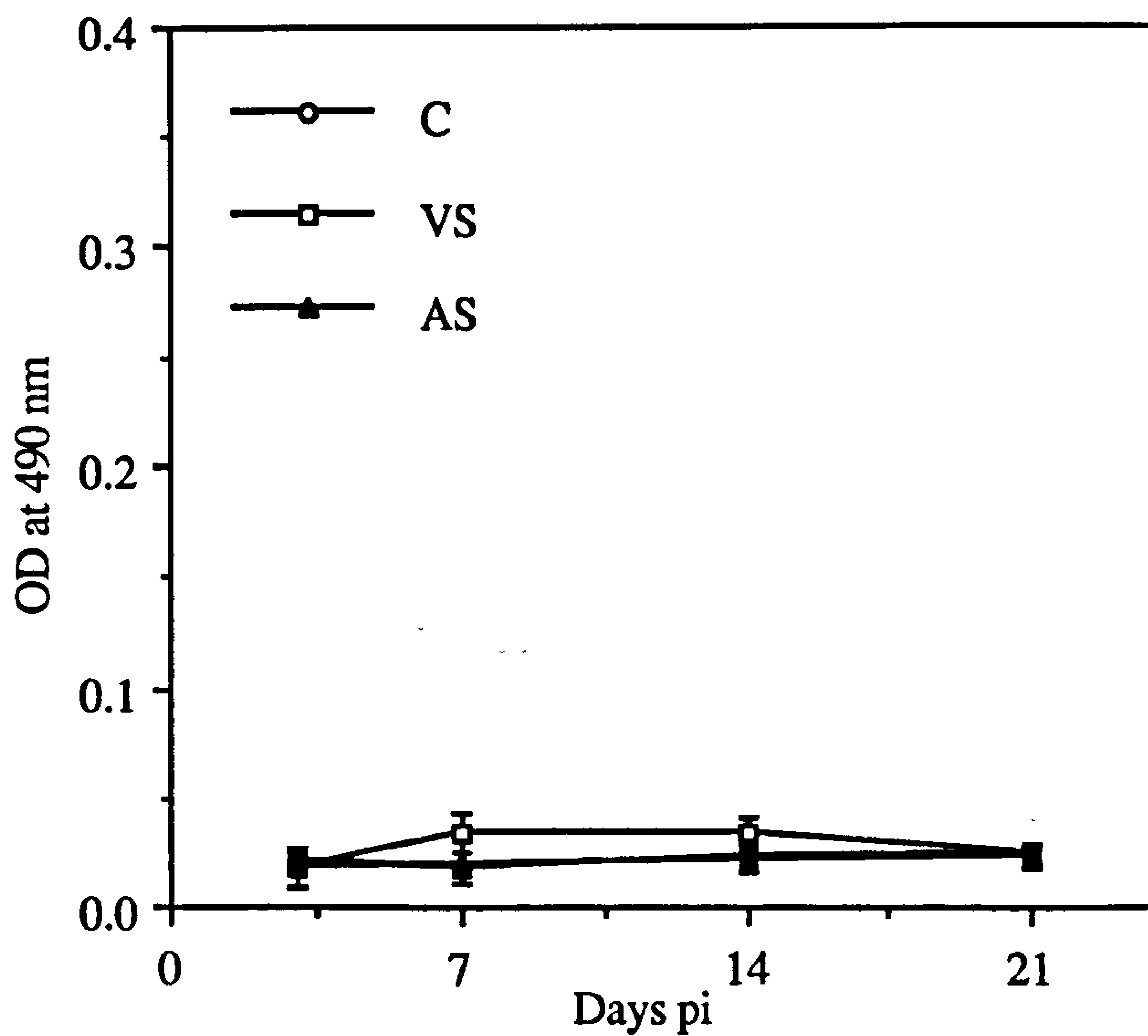

Fig. 4.4. Virus-specific IgA antibody (mean ± sd) in serum from 4-week old chickens following infection with either VS or AS.

4.3.4. *In vitro* IgA antibody production

Figs. 4.5 to 4.7 depict local IgA production in tissues from APV-infected chickens. There was no evidence of IgA production in any tissue on day 3 pi (data not shown). On day 7 pi (Fig. 4.5), there was a significant reduction of IgA antibody at 4°C in the HG of birds infected with the VS only, indicating local production of this antibody. On day 14 pi (Fig. 4.6), there was evidence of substantial amounts of IgA produced by the HG from both groups and small amounts in the trachea of the group infected with VS, even though there was no evidence of significant levels of this antibody in the tracheal washes at this time (see Fig. 4.3). By day 21 pi (Fig. 4.7), the reduction of IgA antibody at 4°C was only significant in the HG from the virulent group. Although at this time IgA in the tracheas in virulent group and HG in attenuated group decreased at 4°C, the reduction was not significant. IgA production in the spleen was not evident from either of infected group at any time. There were no differences in OD values at 4°C and 37°C in any tissues from uninfected controls.

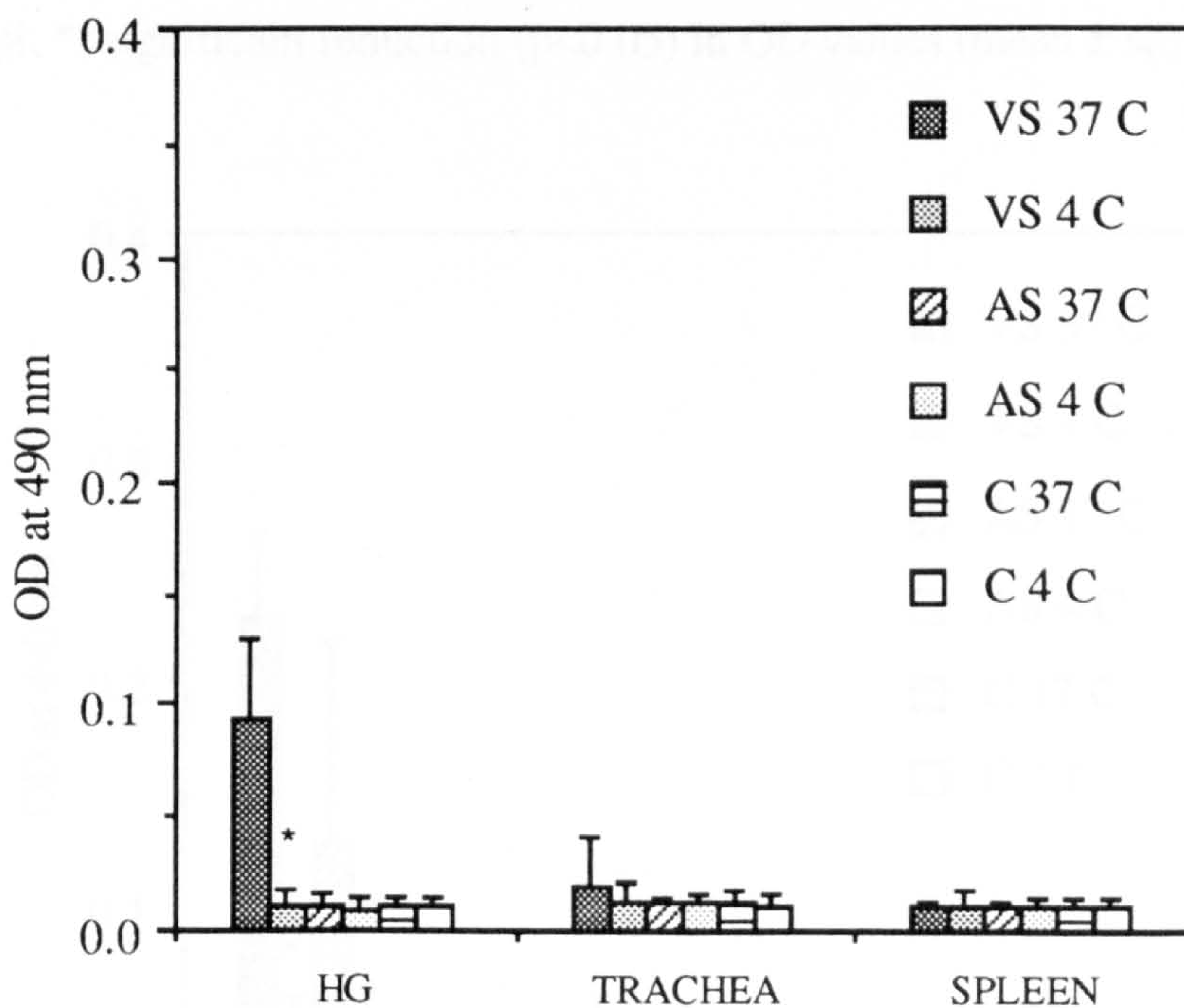

Fig. 4.5. Local IgA production in supernatants of tissue explants from chickens on day 7 pi. *: significant reduction ($p<0.05$) in OD values (mean ± sd) at 4°C compared to value at 37°C within group, indicative of local IgA production.

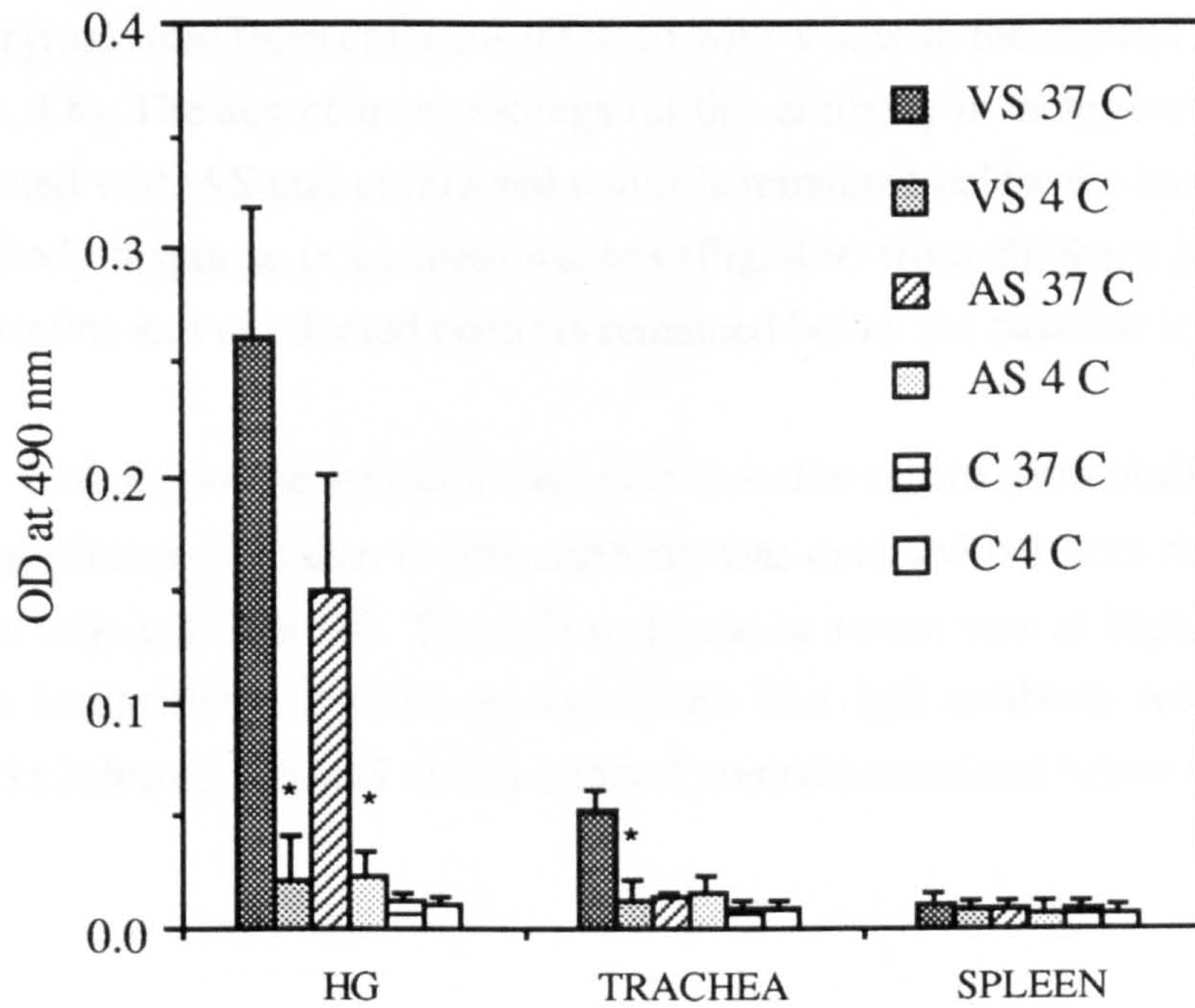

Fig. 4.6. Local IgA production in supernatants of tissue explants from chickens on day 14 pi. *: significant reduction ($p<0.05$) in OD values (mean ± sd) at 4°C within group.

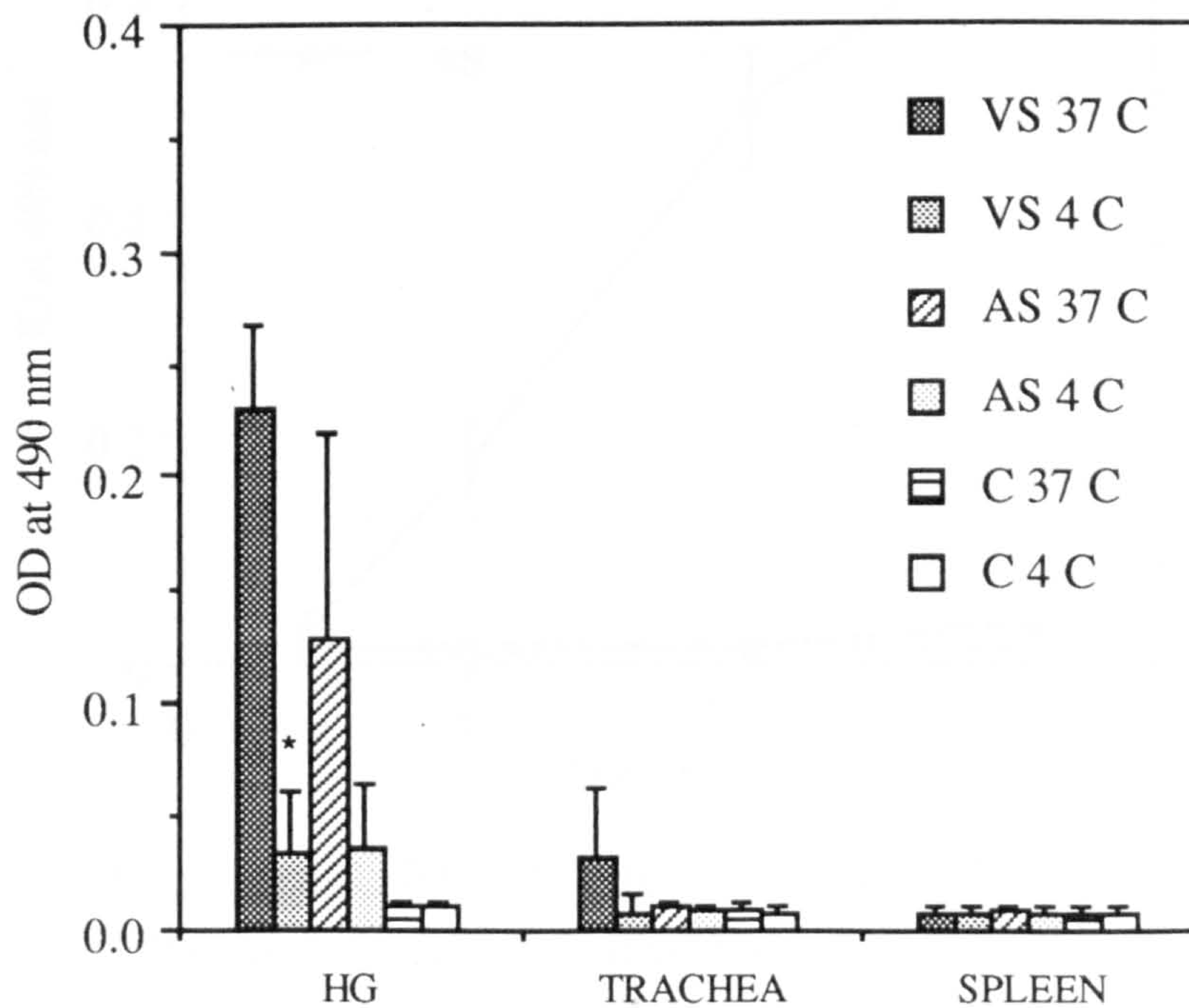

Fig. 4.7. Local IgA production in supernatants of tissue explants from chickens on day 21 pi. *: significant reduction ($p<0.05$) in OD values (mean ± sd) at 4°C within group.

4.3.5. IgG antibody in lachrymal fluid, tracheal washes and serum

A significant rise in IgG antibody was detected between days 3 and 21 pi in the lachrymal fluid from chickens infected with VS, with the highest response on day 21 pi (Fig. 4.8). The absorbance readings for this antibody in lachrymal fluid from the chicks infected with AS and uninfected controls remained below the baseline (0.05). The IgG antibody response in tracheal washes (Fig. 4.9) from chickens infected with either of the strains and uninfected controls remained below the baseline level (0.04).

Fig. 4.10 shows the IgG antibody profile in the serum from birds after APV infection. A significant elevation in this antibody was detected between days 3 and 21 pi in the birds infected with VS. The IgG response in serum was at highest level on day 14 pi with insignificant decline on day 21 pi. The IgG antibody response in serum from chicks infected with AS and uninfected controls remained below the baseline (0.05).

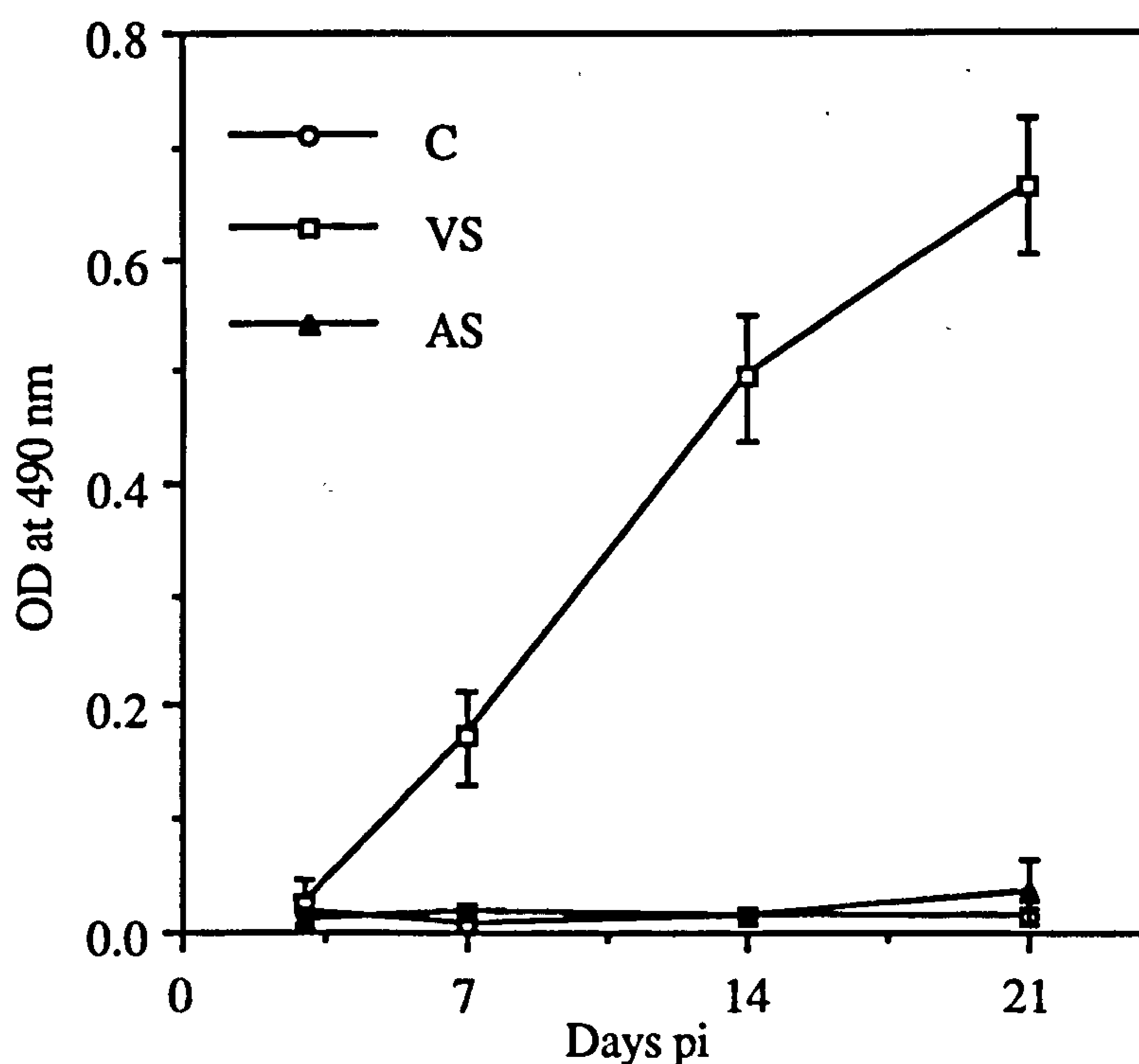

Fig. 4.8. Virus-specific IgG antibody (mean ± sd) in lachrymal fluid from 4-week old chickens following infection with either VS or AS.

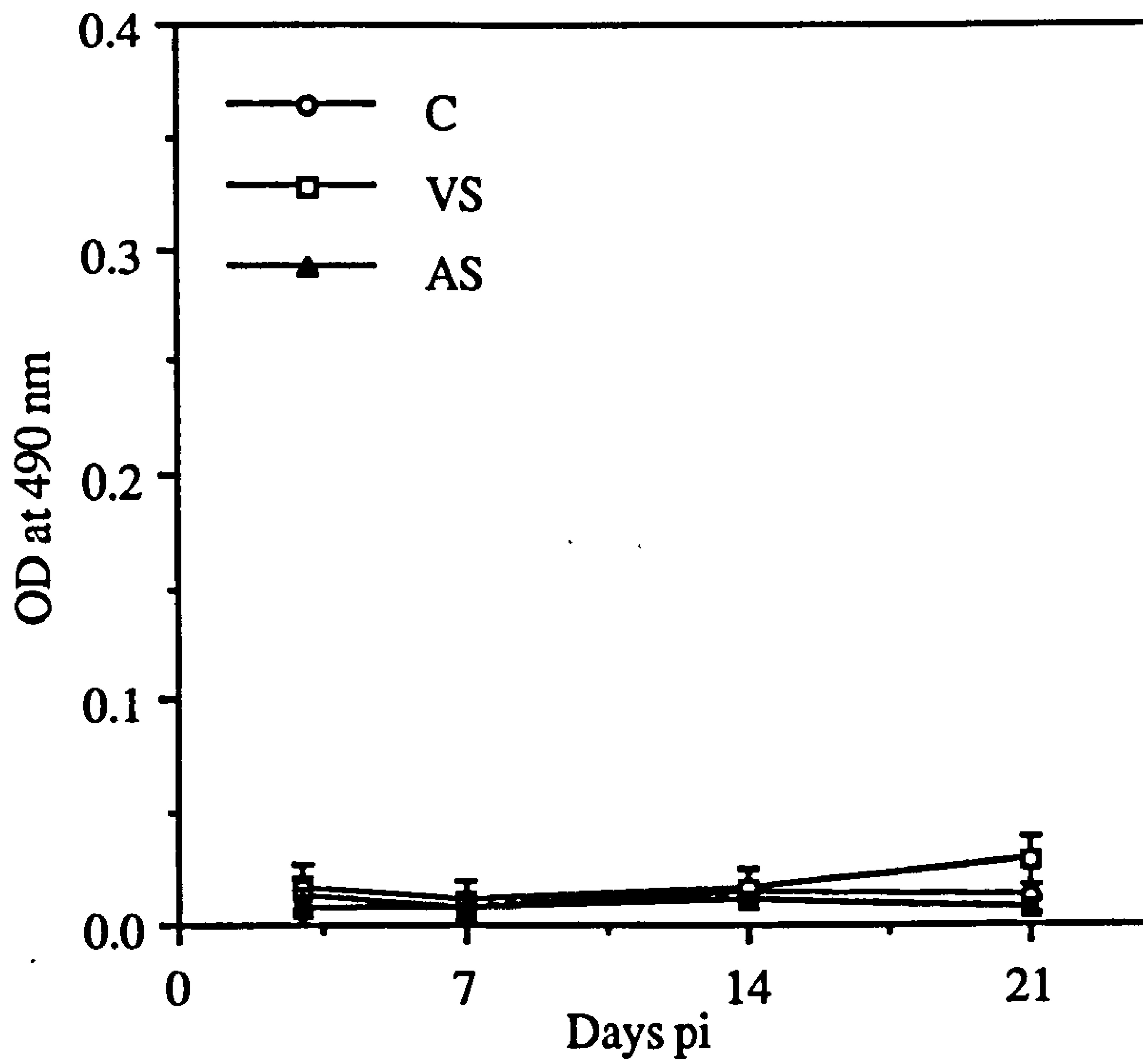

Fig. 4.9. Virus-specific IgG antibody (mean ± sd) in tracheal washes from 4-week old chickens following infection with either VS or AS.

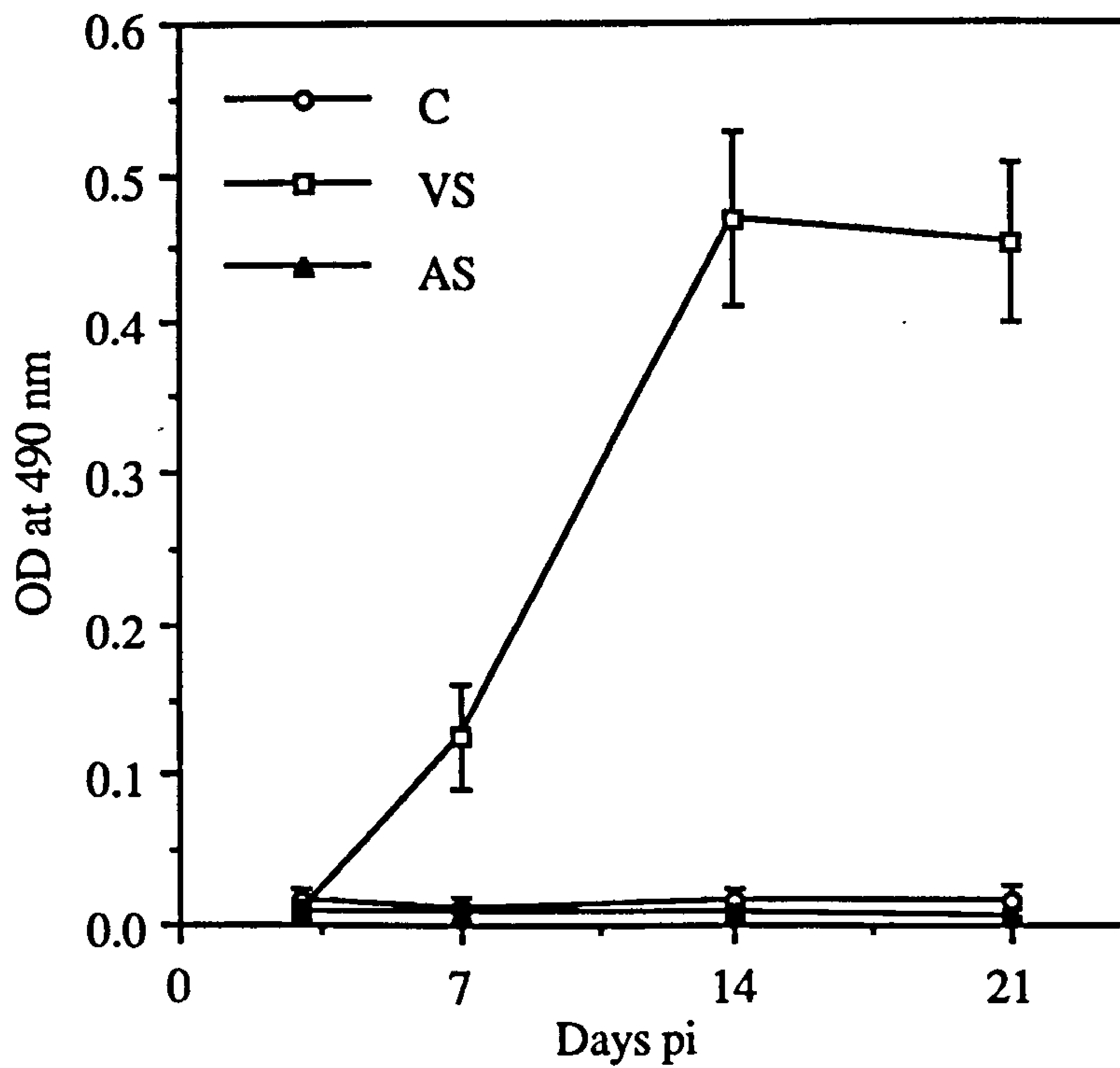

Fig. 4.10. Virus-specific IgG antibody (mean ± sd) in serum from 4-week old chickens following infection with either VS or AS.

4.3.6. *In vitro* IgG antibody production

Figs. 4.11 to 4.13 show *in vitro* IgG antibody production in tissues of APV-infected chickens. No local IgG synthesis was detected from any of the tissues on day 3 pi (data not shown). On day 7 pi (Fig. 4.11), a small but significant reduction of this antibody was detected at 4°C in the HG of chicks infected with the VS only. On day 14 pi (Fig. 4.12), significant reduction in IgG antibody at 4°C in the HG and trachea from the virulent group was also detectable. On day 21 pi (Fig. 4.13), local production of IgG antibody was still seen in the HG from the virulent group. Insignificant reductions in OD at 4°C in the trachea on day 21 pi and in the spleen on days 14 and 21 pi from the virulent group were also detected, but there was no evidence of local IgG synthesis in any of the tissues from chickens infected with the AS. There was no difference in OD values at 4°C and 37°C in any tissue from uninfected controls.

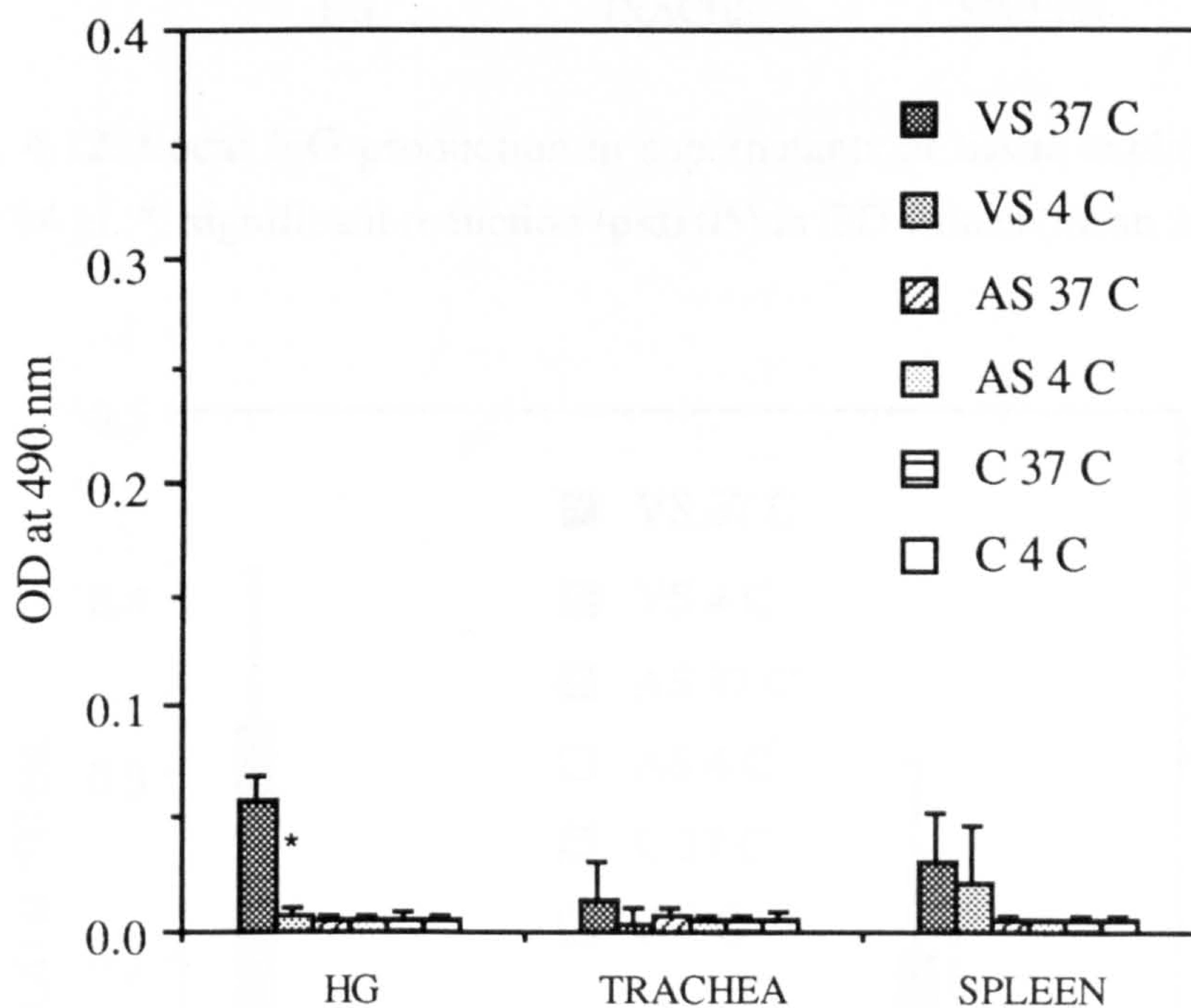

Fig. 4.11. Local IgG production in supernatants of tissue explants from chickens on day 7 pi. *: significant reduction ($p<0.05$) in OD values (mean ± sd) at 4°C compared to value at 37°C within group, indicative of local IgG production.

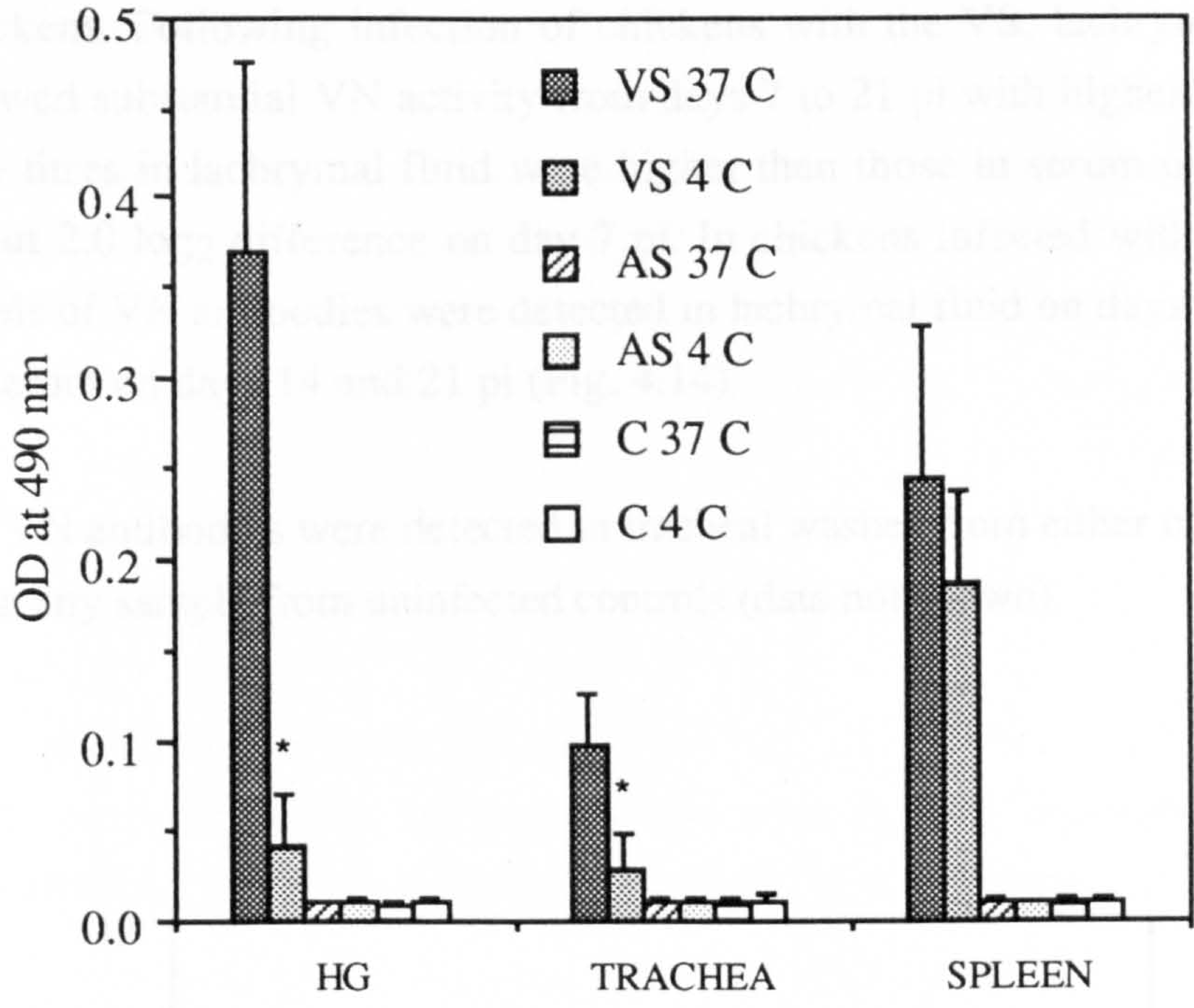

Fig. 4.12. Local IgG production in supernatants of tissue explants from chickens on day 14 pi. *: significant reduction ($p<0.05$) in OD values (mean ± sd) at 4°C.

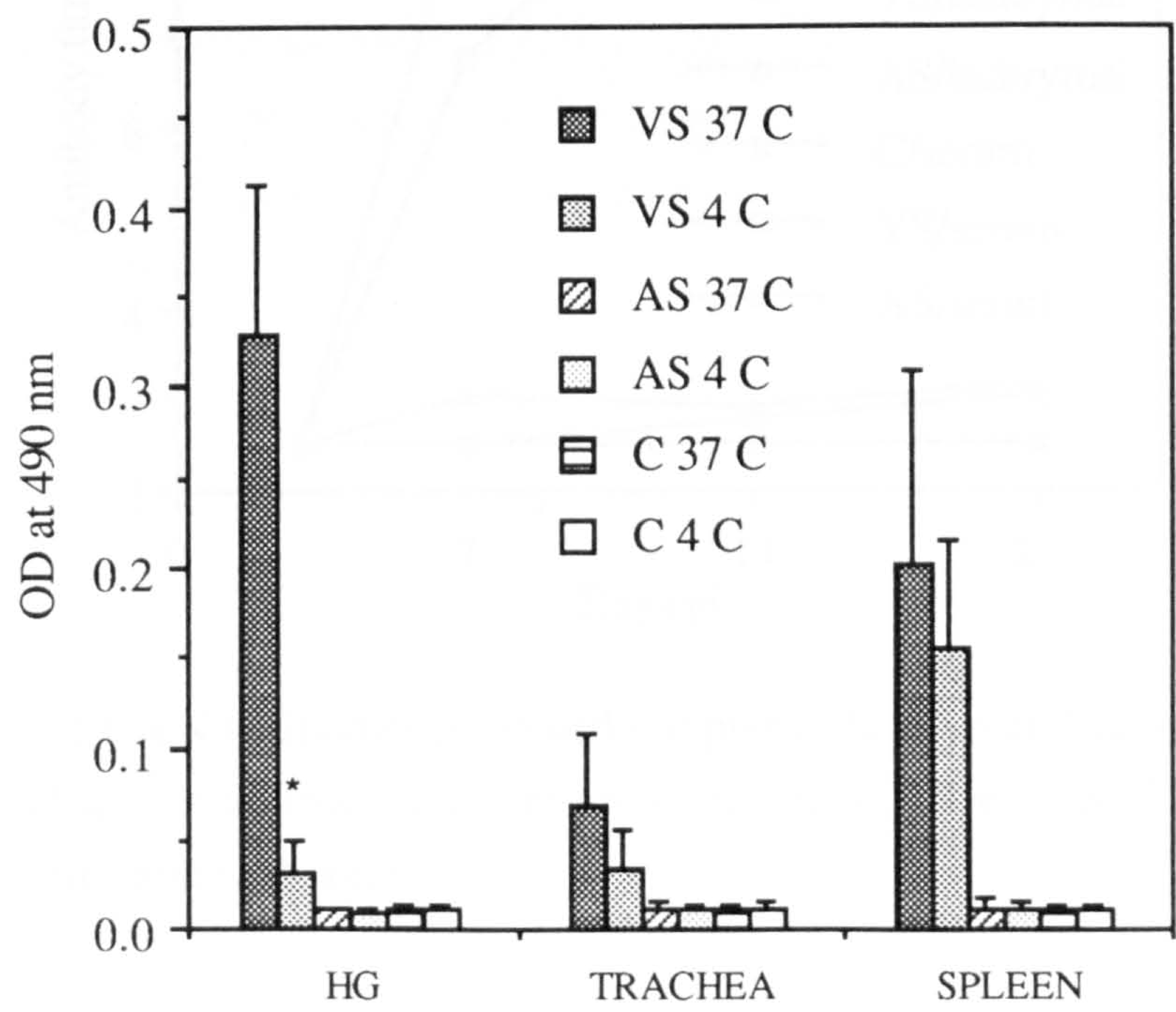

Fig. 4.13. Local IgG production in supernatants of tissue explants from chickens on day 21 pi. *: significant reduction ($p<0.05$) in OD values (mean ± sd) at 4°C.

4.3.7. Virus neutralising antibodies

Fig. 4.14 show VN antibody titres in lachrymal fluid and serum from APV-infected chickens. Following infection of chickens with the VS, lachrymal fluid and serum showed substantial VN activity from days 7 to 21 pi with highest titres on day 21 pi. The titres in lachrymal fluid were higher than those in serum on all occasions with about 2.0 $\log_2$ difference on day 7 pi. In chickens infected with the AS, very small levels of VN antibodies were detected in lachrymal fluid on days 7, 14 and 21 pi, and in serum on days 14 and 21 pi (Fig. 4.14).

No VN antibodies were detected in tracheal washes from either of the infected groups or in any sample from uninfected controls (data not shown).

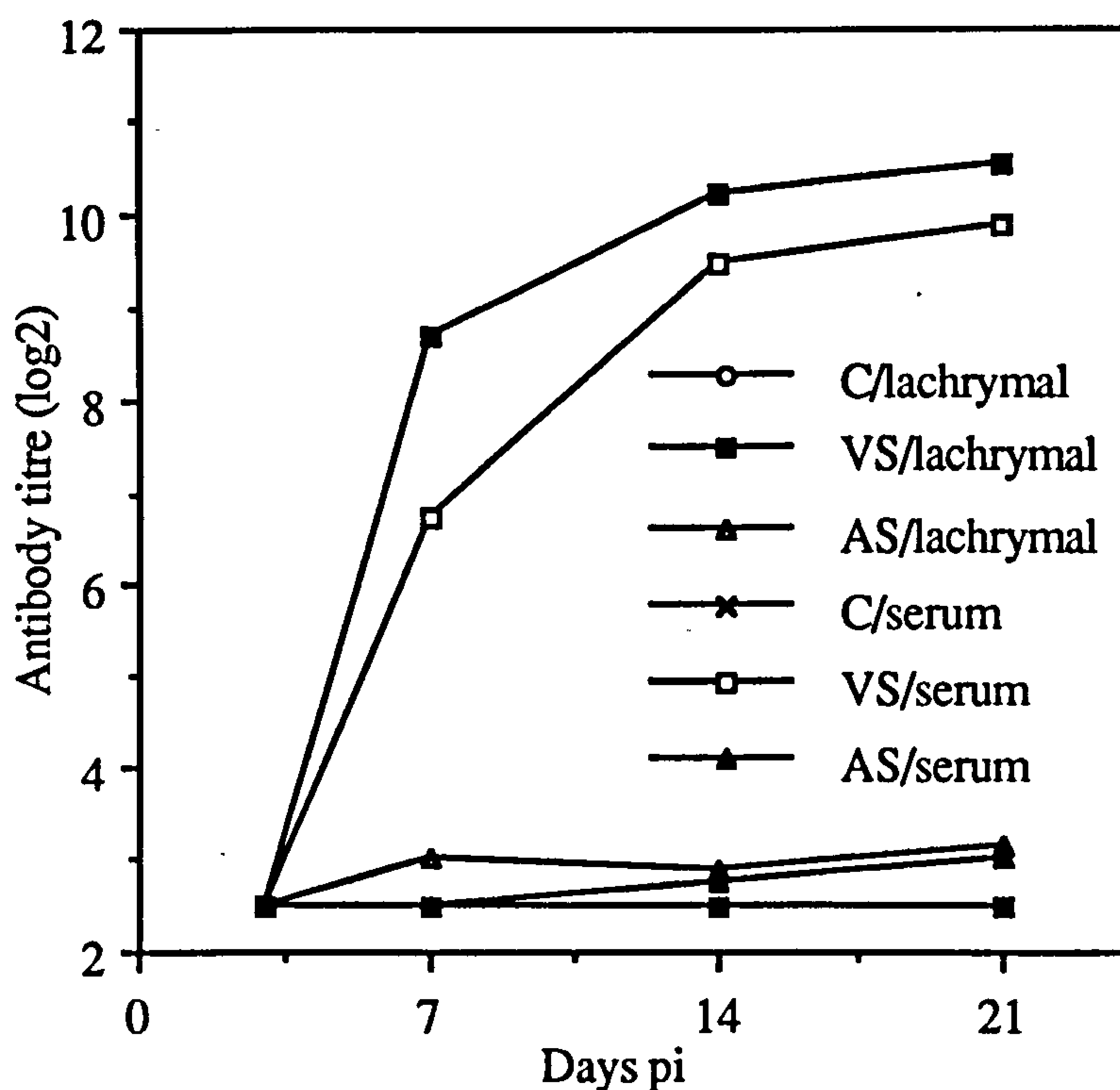

Fig. 4.14. VN antibodies in pooled samples of lachrymal fluid or serum from 4 week-old chickens following infection with either VS or AS. The lowest detectable concentration <2.5 $\log_2$.

PART II: LOCAL AND SYSTEMIC ANTIBODY RESPONSES FOLLOWING CHALLENGE OF CHICKS AND POULTS PRIMED WITH A VIRULENT OR AN ATTENUATED STRAIN

The following two experiments describe local and systemic class-specific antibody responses after challenge of chicks (Experiment 1) and poults (Experiment 2) primed with either a VS or an AS. Attempts were also made to relate the local or systemic antibodies with resistance against APV infection.

Experiment 1. Chicks

4.4. MATERIALS AND METHODS

The source of chicks and virus strains used for this experiment was the same as described for Materials and Methods of Part I of this chapter.

4.4.1. Experimental design

A comparison of virulent and attenuated APVs was made in relation to immunoglobulin (Ig) profile and protection against challenge. Chicks were primed with either the VS or the AS at 1 week of age and 3 weeks later, they were challenged with the virulent APV. The Ig profiles in lachrymal fluid, tracheal washes and serum were monitored by APV-specific and class-specific (IgA & IgG) ELISA and SNT. The protection against challenge was assessed by scoring clinical signs and attempting challenge virus re-isolation. All procedures are described in Materials and Methods of Part I of this chapter. The data was analysed as described in section 4.2.10.

Primary inoculation

One hundred and sixty four birds were divided into three groups (i) infected with VS, (ii) infected with AS and (iii) uninfected controls (Table 4.1). Infections were done at one week of age and according to the group, each bird was given either 3.5 $\log_{10}$ CD_{50} of the VS or 3.5 $\log_{10}$ $TCID_{50}$ of the AS in 0.1 ml volumes by eyedrop. Ten birds in each group were monitored daily for clinical signs. On days 3, 7, 14, 21, 28 and 35 pi, four birds from each group were bled and lachrymal fluids were collected. The birds were subsequently euthanased for collecting tracheal washes. Pieces of HG, nasal turbinates and trachea were collected for virus isolation on days 3, 5 and 7 pi.

Challenge

Three weeks following primary inoculation, birds in each group (Table 4.1) were identified and for challenge were housed in a single pen. The unchallenged ones were kept separately. Each bird was challenged with 2.75 $\log_{10}$ CD_{50}/0.1ml of virulent APV by eyedrop. Ten to fifteen birds in each challenged group were examined daily for the presence of clinical signs and severity of signs scored. Serum and lachrymal fluid were collected from five birds in each challenged group on days 3, 7 and 14 post challenge (pc). The birds were subsequently euthanased and sampled for tracheal washes. For virus isolation, pieces of HG, turbinates and tracheas from 5 birds in each group were collected on days 3, 5, & 7 pc.

Table 4.1. Experimental design

Group	Primary inoculation	Challenge
C	–	–
AS/–	+	–
VS/–	+	–
–/ch	–	+
AS/ch	+	+
VS/ch	+	+

4.5.1. Clinical signs

Primary inoculation of chicks with the VS resulted in clinical signs in the form of clear to turbid nasal exudate between days 3 to 9 pi, but no signs were observed in chicks inoculated with AS or unprimed controls (data not shown). Following challenge 21 days later, no signs were recorded in the chicks primed with VS (VS/ch), but those primed with the AS (AS/ch) showed signs comprising nasal exudate which appeared on gentle beak squeezing. The mean clinical scores of these chicks (Fig. 4.15) were insignificantly lower compared to unprimed but challenged controls (–/ch) on days 3 to 6 pc during the onset of signs but there was no difference in the mean scores on days 7 and 8 pc.

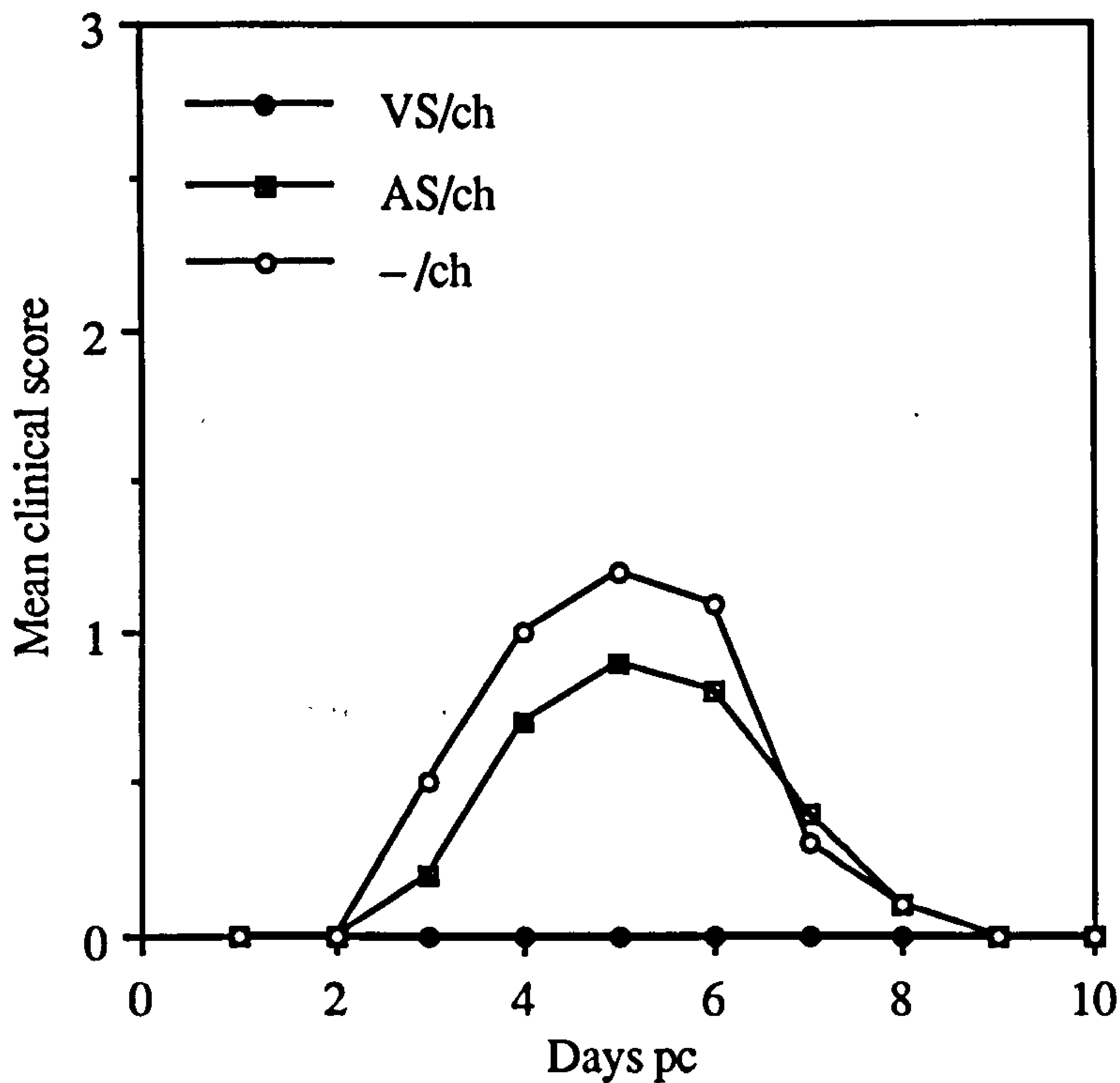

Fig. 4.15. Mean clinical scores following challenge of chicks primed with VS or AS.

4.5.2. Virus isolations

Following priming of chicks with the VS, virus was recovered on days 3 and 5 pi from HG, turbinates and trachea but not from the tissues of those primed with the AS or unprimed controls (not shown). Following challenge, no virus was isolated from any of the tissues in the group primed with the VS, indicative of complete protection, but APV was isolated on days 3 and 5 pc from the HG, turbinates and trachea of those primed with AS, like the unprimed challenge controls (Table 4.2). There was no statistical difference in total number of virus isolations between these two groups.

Table 4.2. Virus isolations from tissues of chicks following challenge

		Group		
Days pc	Tissues	–/ch	VS/ch	AS/ch
3	HG	5*	0	5
	Turbinates	5	0	4
	Trachea	4	0	3
5	HG	4	0	4
	Turbinates	4	0	4
	Trachea	4	0	3
7	HG/turbinates/trachea	0	0	0
Total		26^a	0^b	23^a

Values with different superscripts indicates a statistical significance ($p<0.05$).

*: No. positive of 5 samples tested each time.

4.5.3. Virus titration

Figs. 4.16 to 4.18 depict virus titres following challenge of chicks primed with the AS. The mean titres in the HG (Fig. 4.16), turbinates (Fig. 4.17) and tracheas (Fig. 4.18) of chicks primed with the AS were lower than in the unprimed challenge controls suggestive of partial protection. The differences were greater on day 3 pc as compared to day 5 pc.

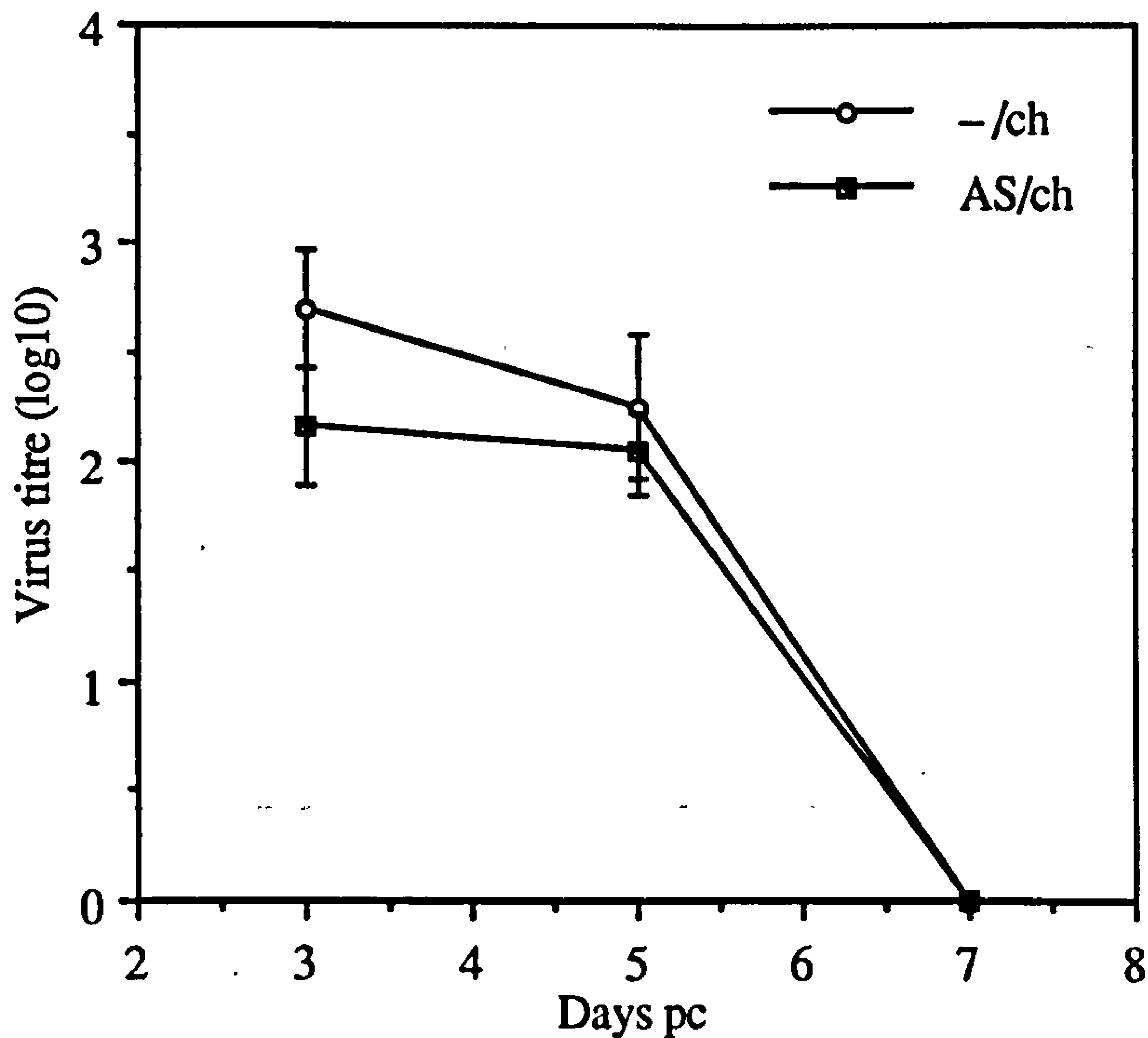

Fig. 4.16. Virus titres (mean ± sd) in harderian gland of chicks following challenge. Titres expressed as CD_{50} log_{10}/g tissue.

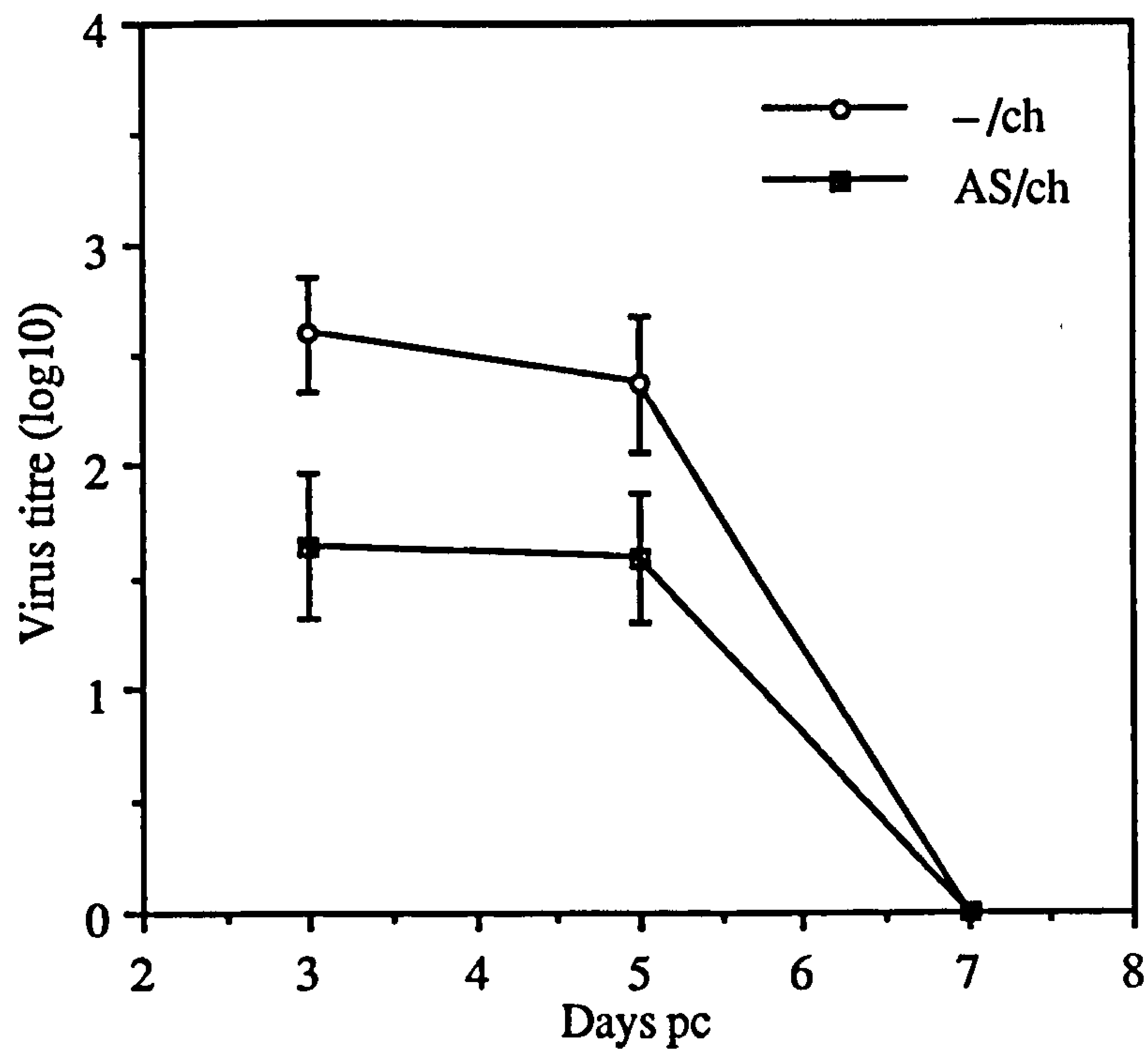

Fig. 4.17. Virus titres (mean ± sd) in turbinates of chicks following challenge. Titres expressed as CD_{50} log_{10}/g tissue.

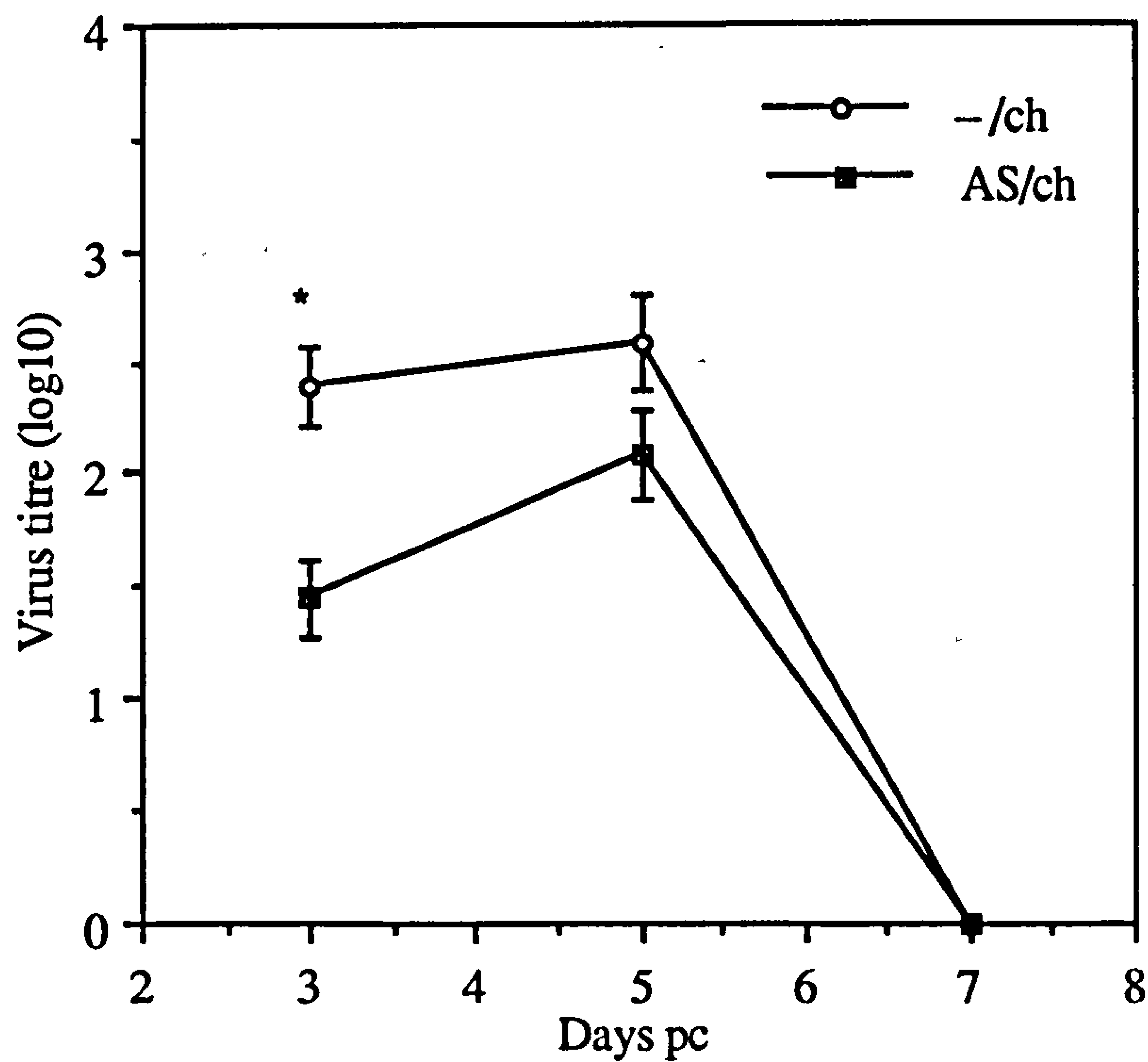

Fig. 4.18. Virus titres (mean ± sd) in tracheas of chicks following challenge.
*: significant differences ($p<0.05$) between two groups. Titres expressed as CD_{50} log_{10}/g tissue.

4.5.4. IgA antibody in lachrymal fluid, tracheal washes and serum

Fig. 4.19 shows IgA antibody in lachrymal fluid from chicks following priming and challenge at day 21 pi. After priming with the VS, there was a gradual and significant rise in IgA between days 3 and 28 pi, then slight decline on day 35 pi. The OD values on days 3 and 7 pi were below the baseline (0.05). Following challenge, there was a significant elevation in secondary IgA response on days 3 and 7 pc. In chicks primed with the AS, lachrymal IgA did not reach the level of significance and subsequent to challenge, there was no difference in absorbance readings compared to unprimed but challenged controls.

IgA was not demonstrable in tracheal washes (Fig. 4.20) or serum (Fig. 4.21) of chicks primed with either strain. After challenge, OD values were still below the baselines (0.04) and an insignificant elevation was noted in tracheal washes (Fig. 4.20) or serum (Fig. 4.21) in the group primed with VS only.

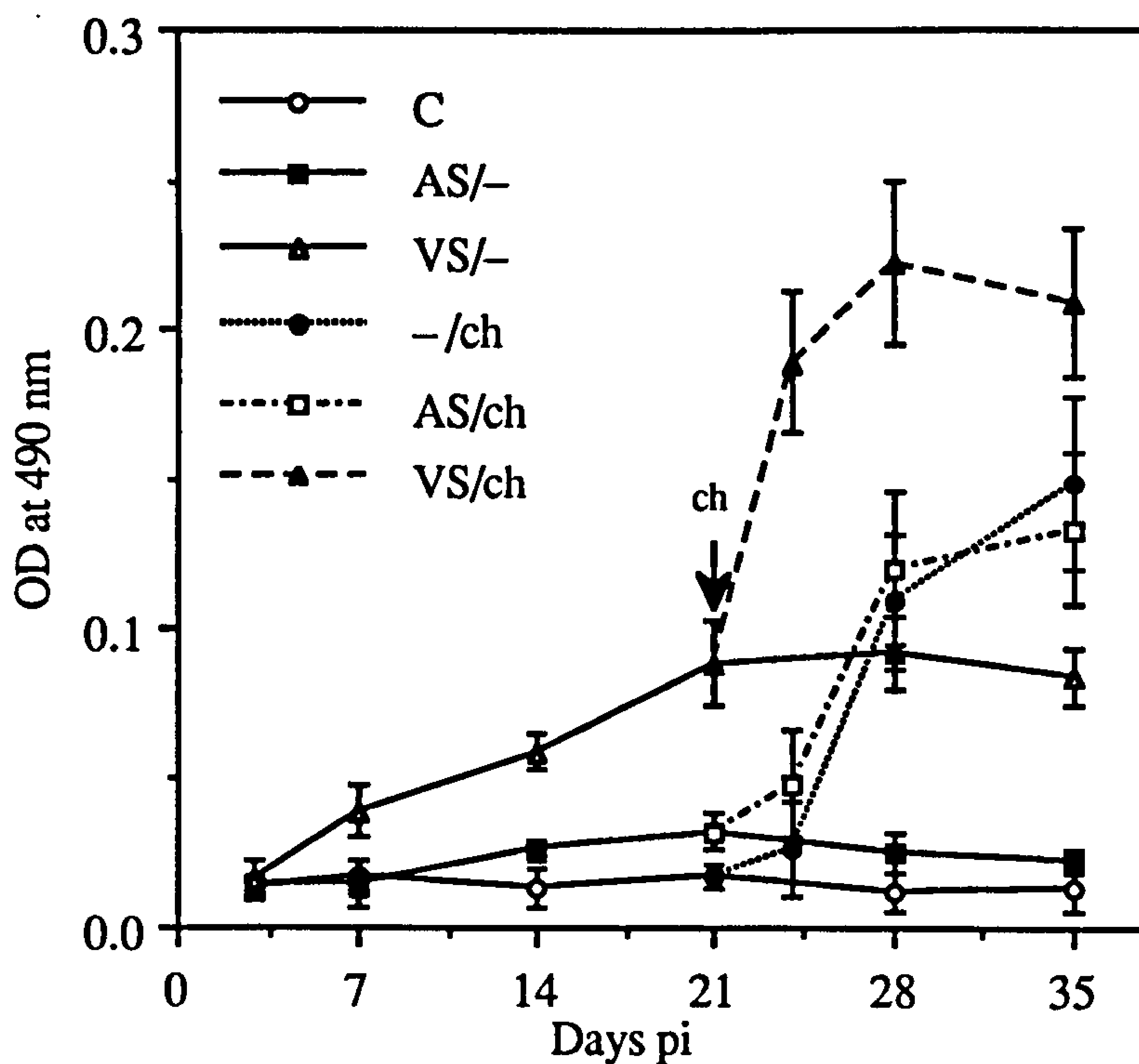

Fig. 4.19. Virus-specific IgA antibody (mean ± sd) in lachrymal fluid of chicks following priming and challenge.

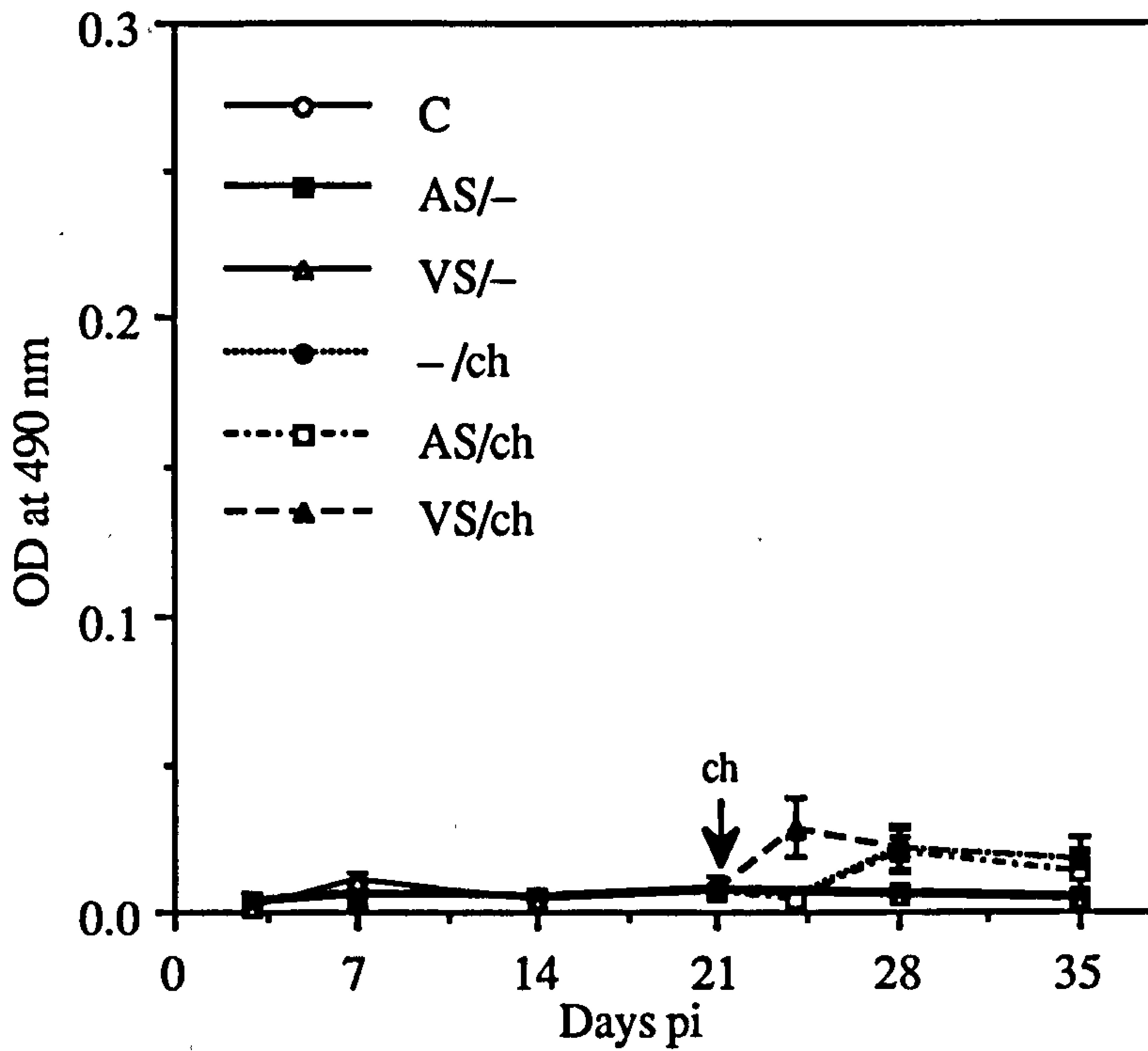

Fig. 4.20. Virus-specific IgA antibody (mean ± sd) in tracheal washes of chicks following priming and challenge.

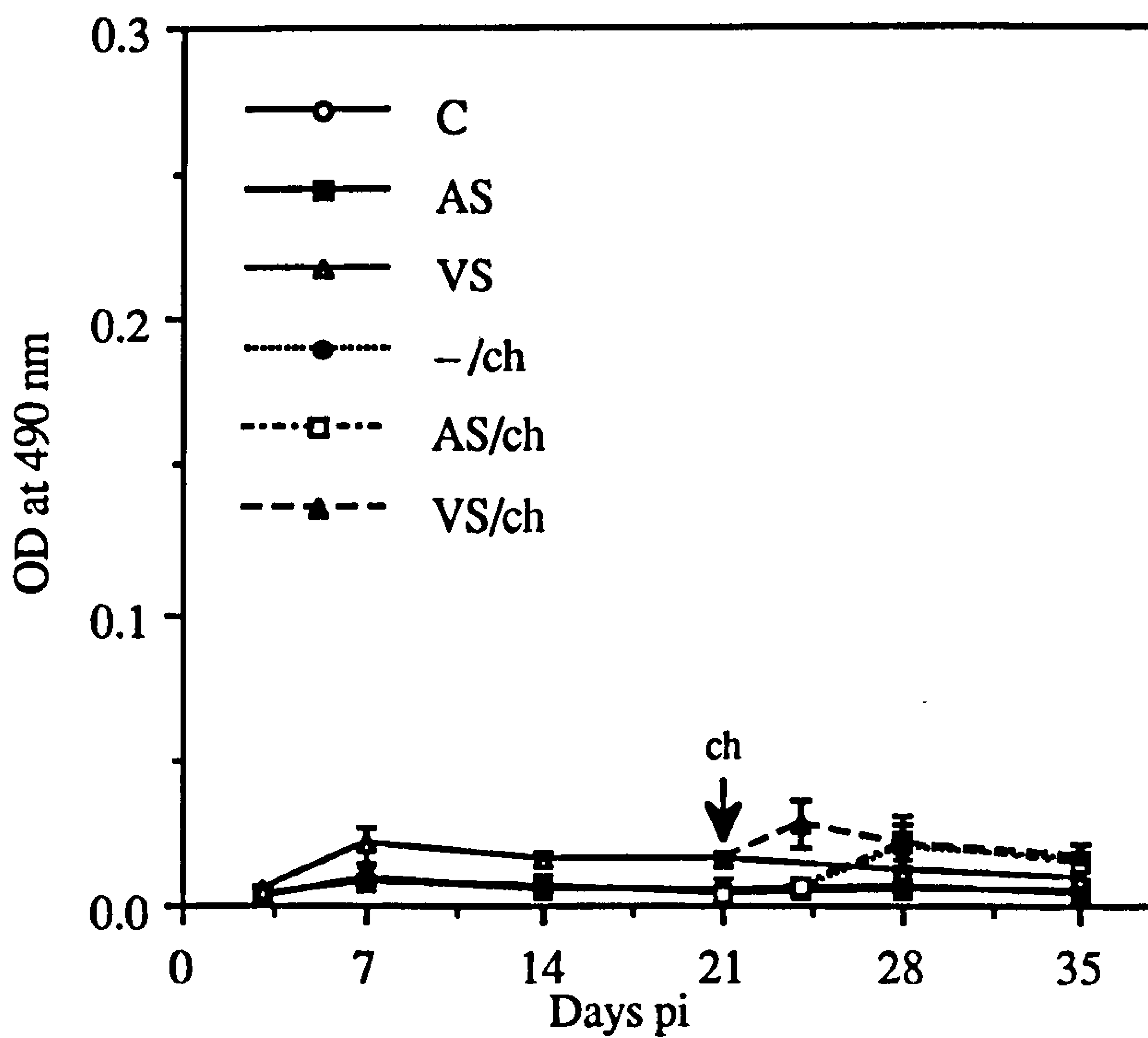

Fig. 4.21. Virus-specific IgA antibody (mean ± sd) in serum of chicks following priming and challenge.

4.5.5. IgG antibody in lachrymal fluid, tracheal washes and serum

A gradual and significant rise in lachrymal IgG was detected between days 3 and 21 pi of chicks primed with the VS (Fig. 4.22). This was followed by an insignificant decline in IgG levels maintained above the baseline (0.05) at least until day 35 pi. Following challenge, a rapid and significant rise in secondary IgG was evident on days 3 and 7 pc. There was no detectable lachrymal IgG in chicks primed with the AS and subsequent to challenge, there was no difference in IgG levels compared to unprimed but challenged controls (Fig. 4.22).

In tracheal washes, no IgG response was detectable (baseline = 0.04) in chicks primed with either strain (Fig. 4.23). Following challenge, there was no significant difference in IgG levels compared to unprimed challenge controls.

In the group primed with VS, serum IgG (baseline = 0.05) rose gradually and significantly between days 3 and 21 pi and the levels were maintained until day 28 pi followed by a slight decline on day 35 pi (Fig. 4.24). Following challenge, a rapid and significant elevation in IgG was demonstrable on days 3 and 7 pc. No serum IgG antibody could be detected in chicks primed with the AS and following challenge, IgG levels were similar to those for the unprimed challenge group (Fig. 4.24).

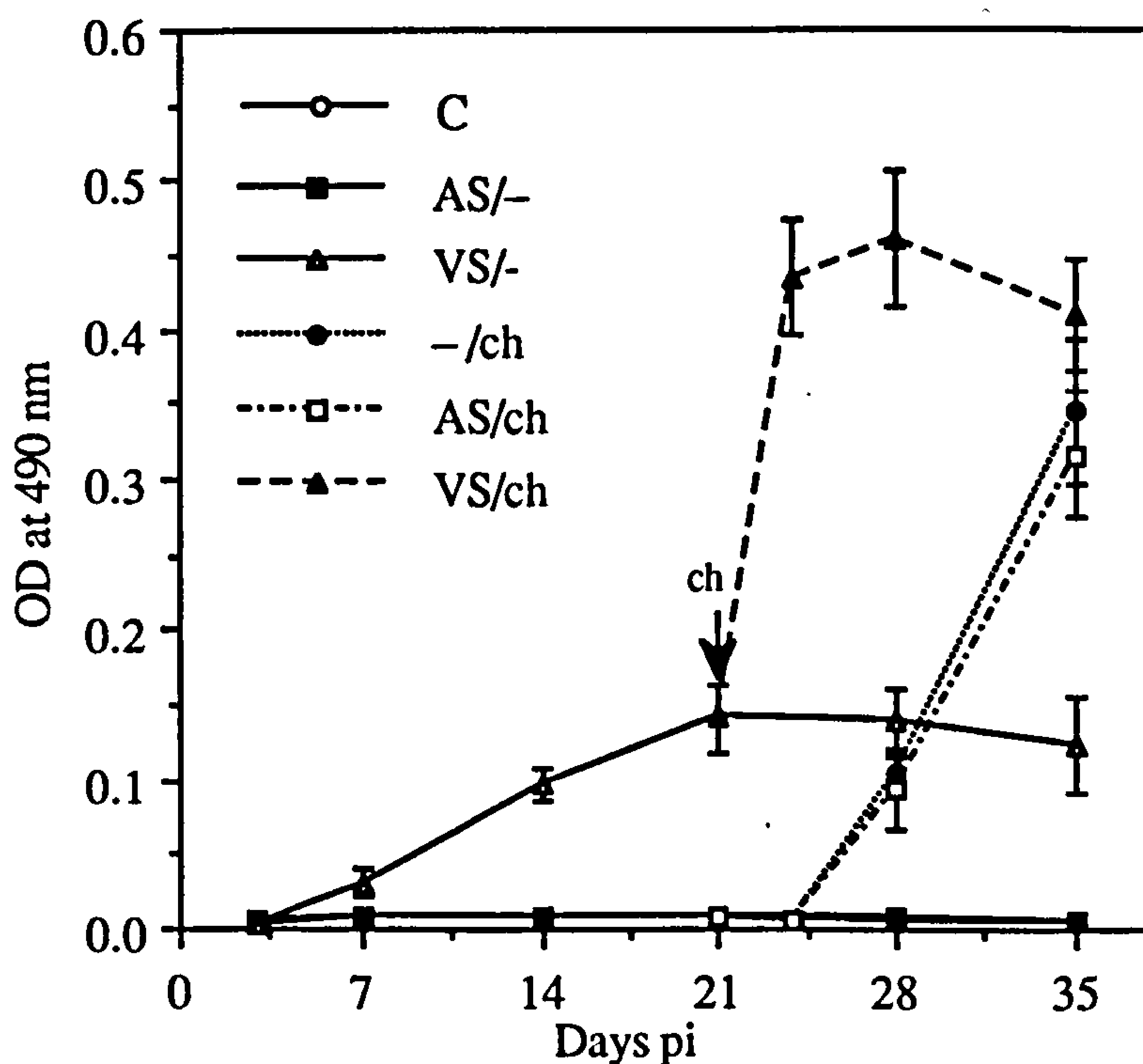

Fig. 4.22. Virus-specific IgG antibody (mean ± sd) in lachrymal fluid of chicks following priming and challenge.

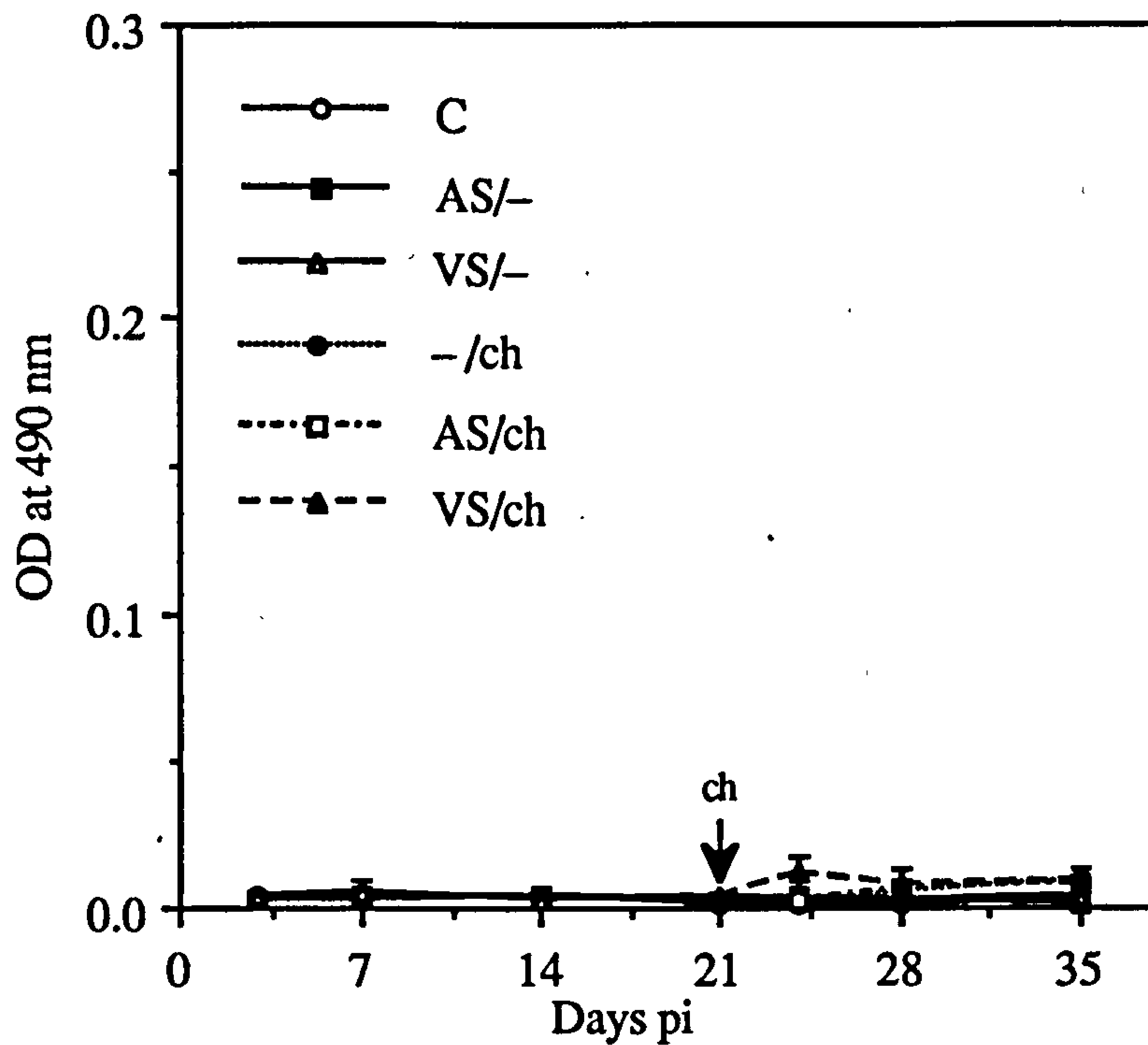

Fig. 4.23. Virus-specific IgG antibody (mean ± sd) in tracheal washes of chicks following priming and challenge.

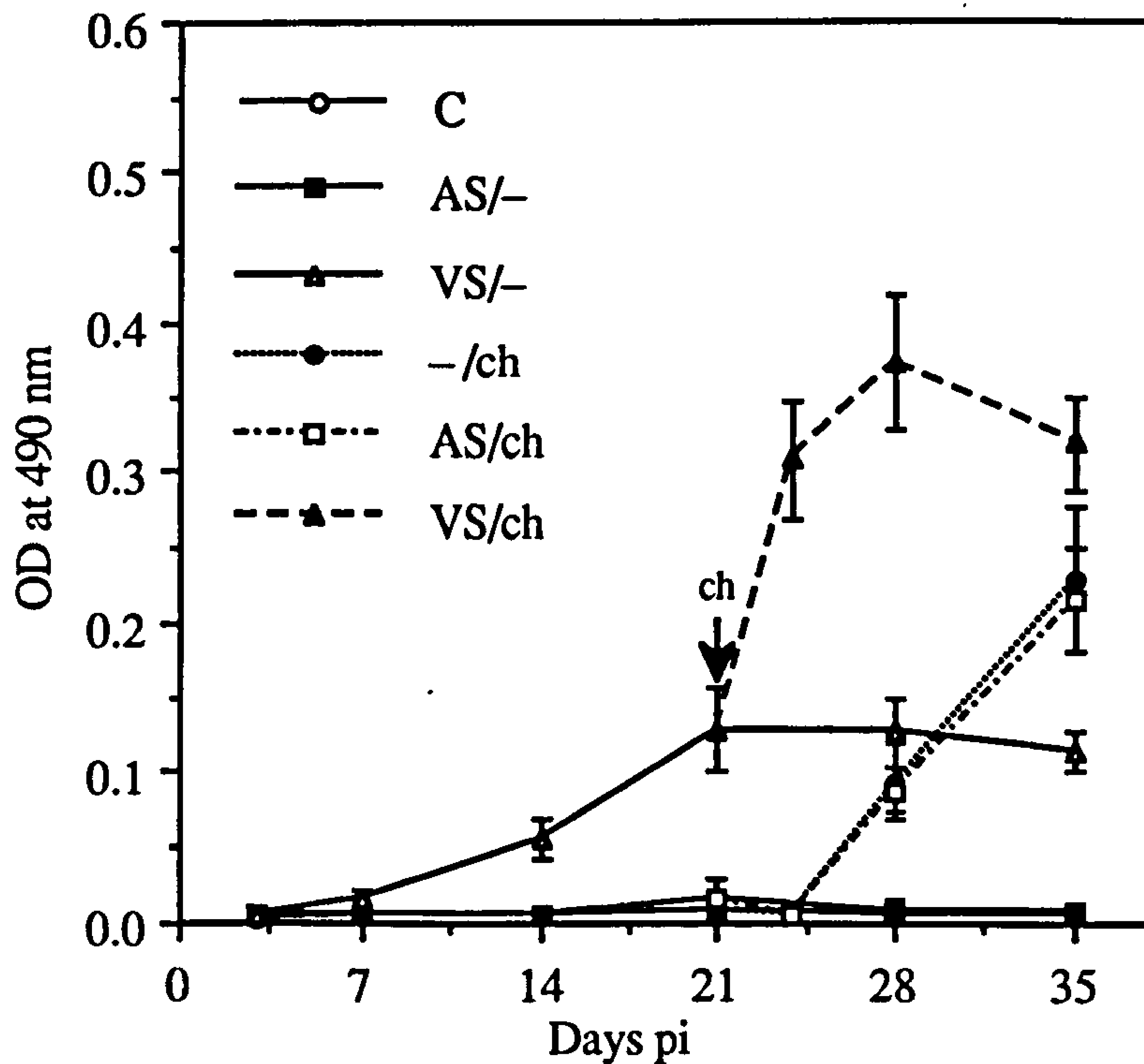

Fig. 4.24. Virus-specific IgG antibody (mean ± sd) in serum of chicks following priming and challenge.

4.5.6. Virus neutralising antibodies

Following priming of chicks with the VS, lachrymal fluid (Fig. 4.25) and serum (Fig. 4.26) showed VN activity from days 7 to 35 pi with highest titres on day 28 pi. Titres were similar in both lachrymal fluid and serum, but on day 7 pi, they were about 1.0 log_2 higher in lachrymal fluid. Following challenge, titres rose on days 3 and 7 pc but with about 2.0 log_2 higher titres in lachrymal fluid than serum. After priming with the AS, no VN activity could be demonstrated either in lachrymal fluid (Fig. 4.25) or serum (Fig. 4.26) and following challenge, there was no difference from unprimed challenged birds.

No VN antibodies were detected in tracheal washes following either priming or challenge (data not shown).

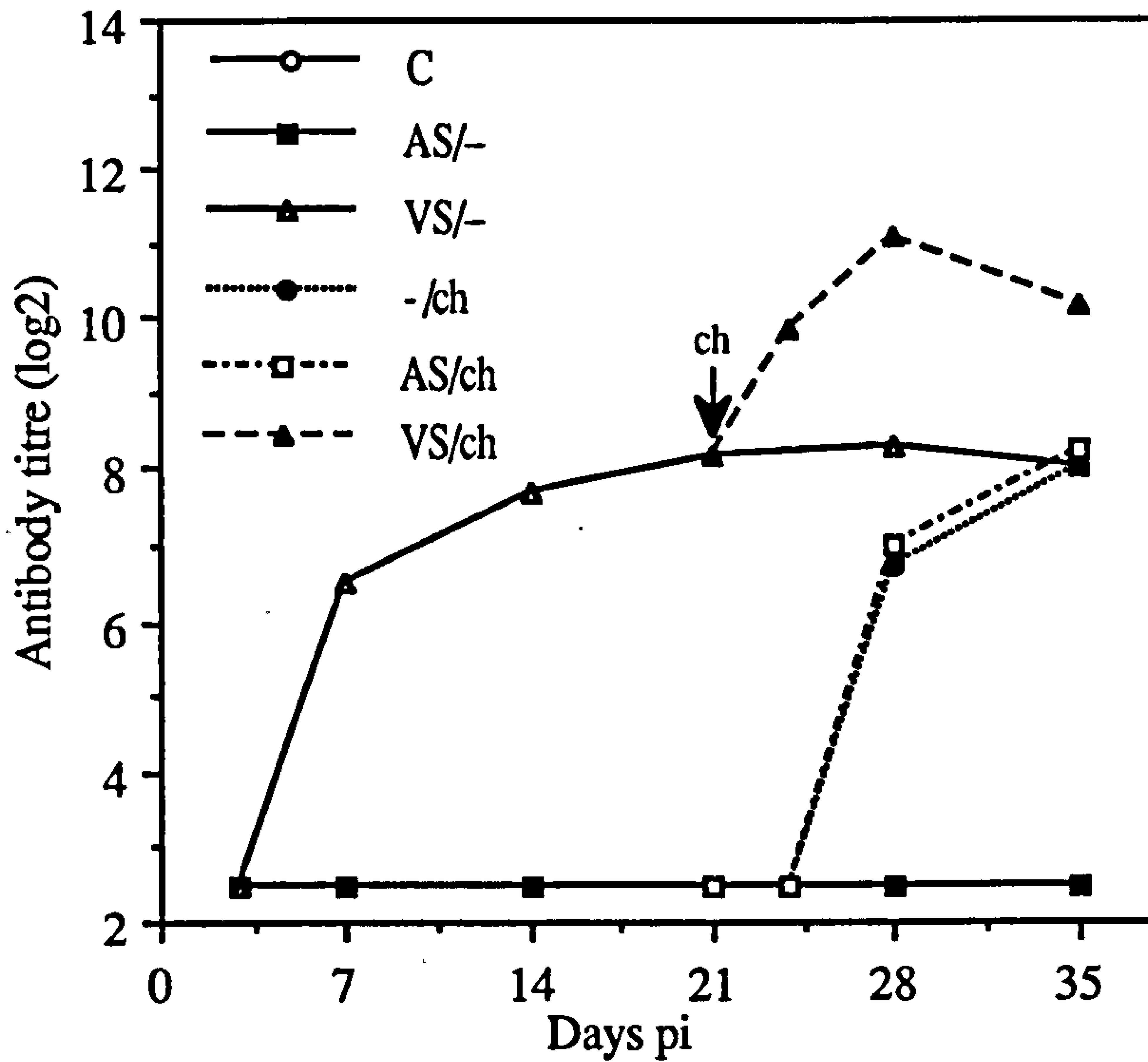

Fig. 4.25. VN antibody in pooled samples of lachrymal fluid of chicks following priming and challenge. Lowest detectable level <2.5 log_2.

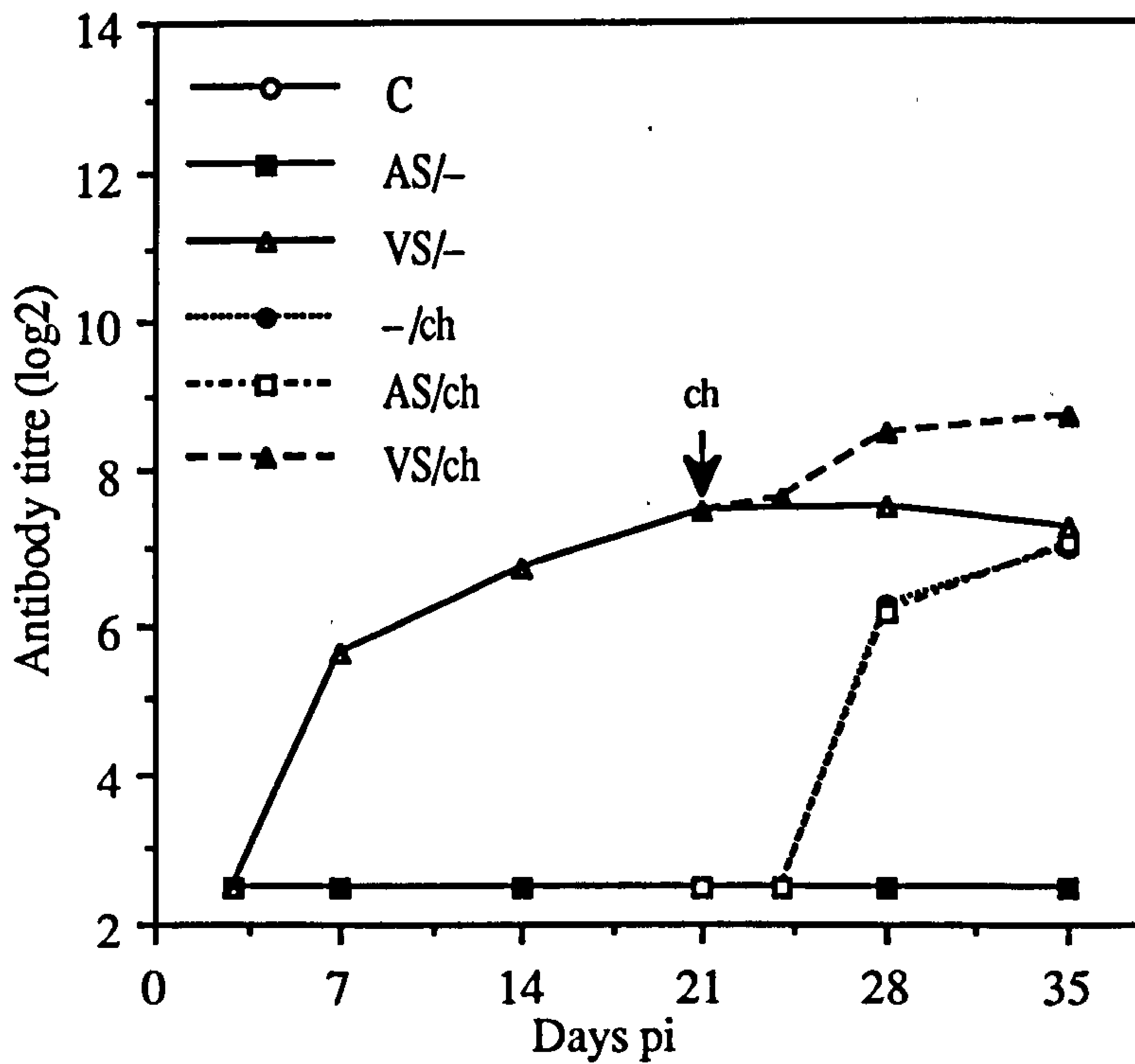

Fig. 4.26. VN antibody in pooled samples of serum of chicks following priming and challenge. Lowest detectable level <2.5 log_2.

Experiment 2. Poults

4.6. MATERIALS AND METHODS

4.6.1. Poults

Turkey poults free of MA to APV were obtained from a commercial source. They were reared in strict isolation with food and water *ad libitum.*

4.6.2. Experimental design

A comparison of virulent and attenuated APVs in relation to Ig profile and protection against challenge was also made in poults by an experimental trial identical to that for chicks. All procedures were the same as in Experiment 1 with the exception of the ELISA. The mouse Mab against chicken IgA and IgG used in Experiment 1 were found to react weakly with samples from turkeys. Turkey anti-isotype specific Mab or Ig were not available. For detection of turkey IgA and IgG, an ELISA using goat-raised chicken anti-isotype specific Ig (Nordic Immunologicals Labs., Tilburg, Netherlands) was tested and found to be satisfactory. The procedure for this ELISA is described below.

4.6.3. Enzyme-linked immunosorbent assay

ELISA plates (Falcon 3912) were coated overnight at 4°C with purified antigen diluted 1: 50 in CBC buffer. The wells were coated alternatively with antigen and CBC buffer. Following day, the wells were blocked with PBS containing 3 % BSA (Sigma). Based on checkerboard titration, the samples were tested at single dilution with tracheal washes at 1: 10, lachrymal fluid at 1: 20 and serum at 1: 100, each was diluted in PBS containing 0.05% tween-80 (dilution buffer). Ig against chicken IgA and IgG were used as primary antibodies at a dilution of 1: 200 followed by affinity-purified rabbit anti-goat IgG-peroxidase conjugate (Nordic) at a dilution of 1: 1000. The enzyme substrate OPD was added and the reaction stopped after 20 minutes by adding 25 µl of 5 N sulphuric acid. The plates were read at 490 nm (test filter 3) with a reference reading at 630 nm (reference filter) in the micro-ELISA reader. Volumes of 50 µl were used for each step except for the substrate where 100 µl was used. The plates were washed three times after each step except for the blocking step and washing buffer was the same as dilution buffer.

For lachrymal fluid, tracheal washes and serum, the baselines were established from mean plus twice the standard deviation of the absorbance values of twenty normal samples collected from 2 and 4 week old poults free of MA to APV.

4.7. RESULTS

4.7.1. Clinical signs

Primary inoculation of poults with the VS resulted in clinical signs from days 2 to 12 pi, but no signs were observed in poults inoculated with AS or unprimed controls (data not shown). Following challenge, no signs were observed in poults primed with either strain whereas in the unprimed group signs were recorded from day 3 to 10 pc (Fig. 4.27). These comprised clear to turbid nasal exudate, eye discharge, coughing, sneezing and occasional infraorbital swelling.

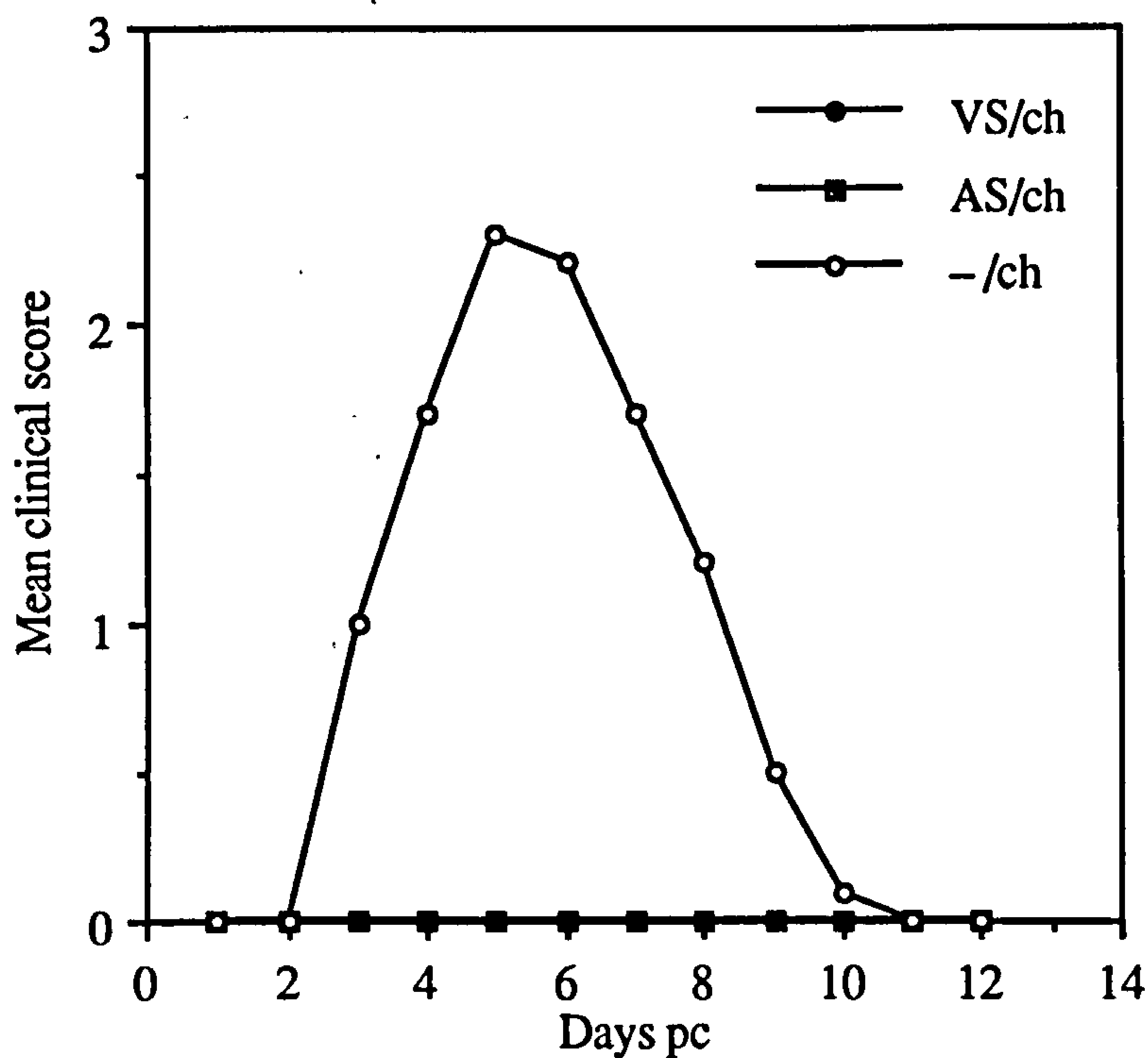

Fig. 4.27. Mean clinical scores following challenge of poults primed with VS or AS.

4.7.2. Virus isolations

Following priming of poults with VS, virus was recovered on days 3 and 5 pi from HG and turbinates, and on days 3, 5 and 7 pi from tracheas but not from the tissues of those primed with the AS or unprimed controls (not shown). After challenge, no virus was isolated from any tissues of poults which were primed with either strain indicating complete protection (Table 4.3). In the case of unprimed challenged control, APV was isolated between days 3 and 7 pc (Table 4.3).

Table 4.3. Virus isolations from tissues of poults after challenge

Days pc	Tissues	Group		
		-/ch	VS/ch	AS/ch
3	HG	5*	0	0
	Turbinates	5	0	0
	Trachea	4	0	0
5	HG	4	0	0
	Turbinates	4	0	0
	Trachea	5	0	0
7	HG	0	0	0
	Turbinates	0	0	0
	Trachea	1	0	0

*: No. positive of 5 samples tested each time.

4.7.3. IgA antibody in lachrymal fluid, tracheal washes and serum

In lachrymal fluid, IgA rose significantly between days 3 and 28 following priming of poults with either strain and was sustained above the baseline (0.06) at least until day 35 pi (Fig. 4.28). OD values for the AS were lower than those for VS. IgA levels elicited by the AS rose more slowly than for VS and did not reach significance until day 21 pi. After challenge, an elevation in secondary IgA was noted on day 3 pc and which was significant by day 7 pc in both the primed groups (Fig. 4.28). The secondary IgA response was consistently higher in poults primed with VS than those primed with AS.

In tracheal washes, although no significant levels (baseline = 0.05) of IgA were elicited in response to either priming, challenge induced small but significant levels in both groups by day 7 pc (Fig. 4.29).

In serum, IgA was detectable in small amounts (baseline = 0.05) in poults primed with either strain (Fig. 4.30). Challenge resulted in a significant rise by day 7 pc in both primed groups.

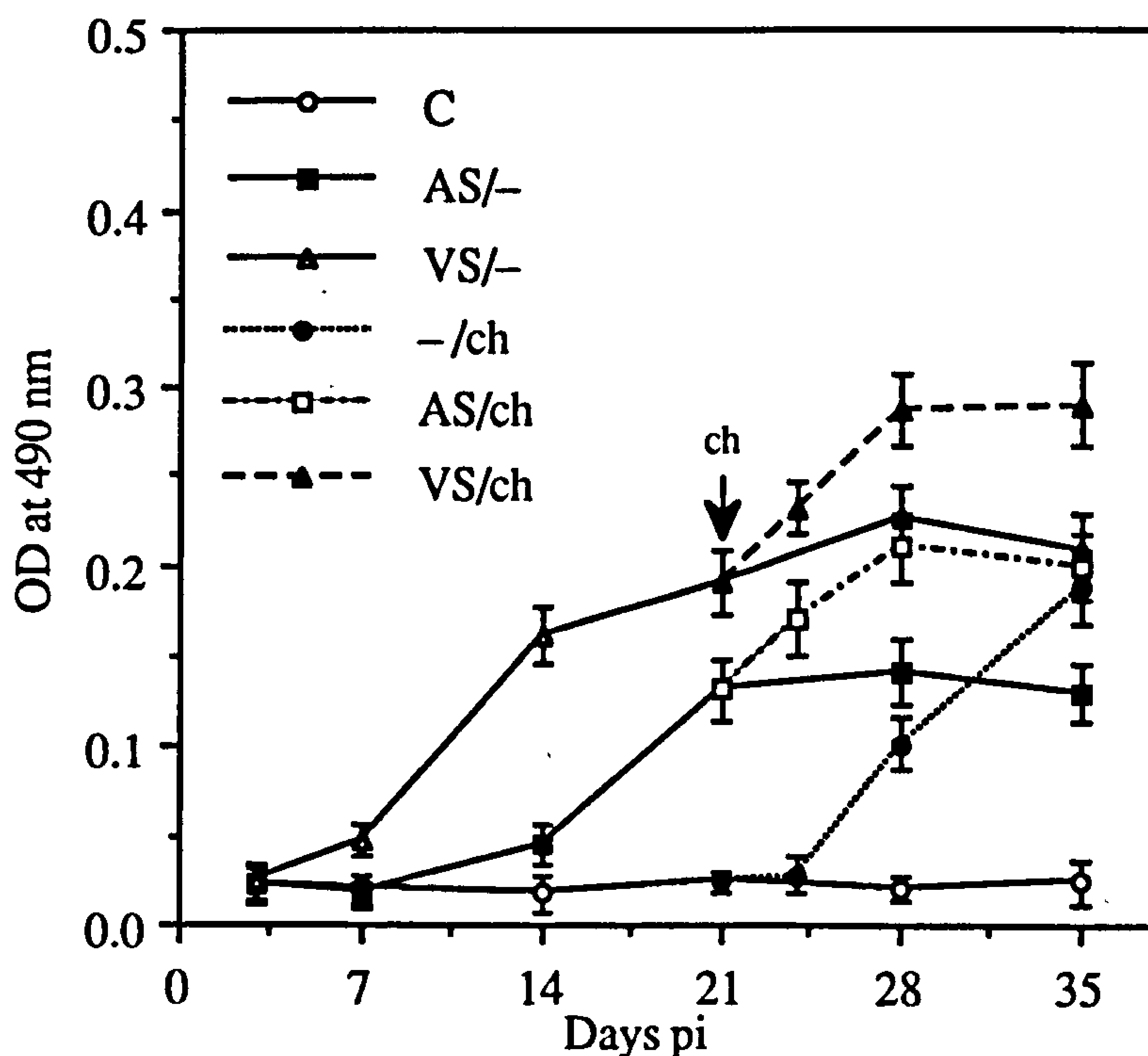

Fig. 4.28. Virus-specific IgA antibody (mean ± sd) in lachrymal fluid of poults following priming and challenge.

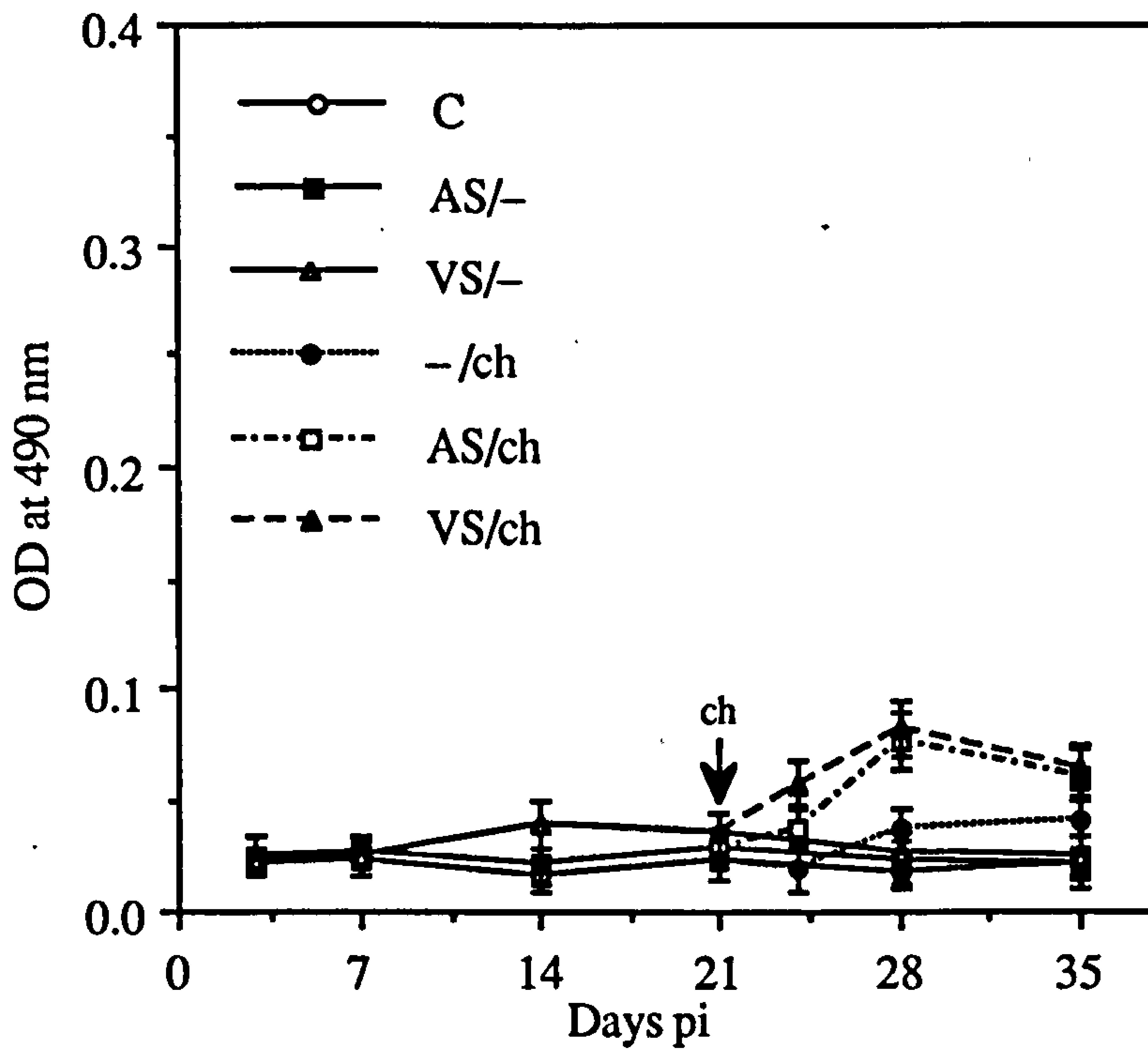

Fig. 4.29. Virus-specific IgA antibody (mean ± sd) in tracheal washes of poults following priming and challenge.

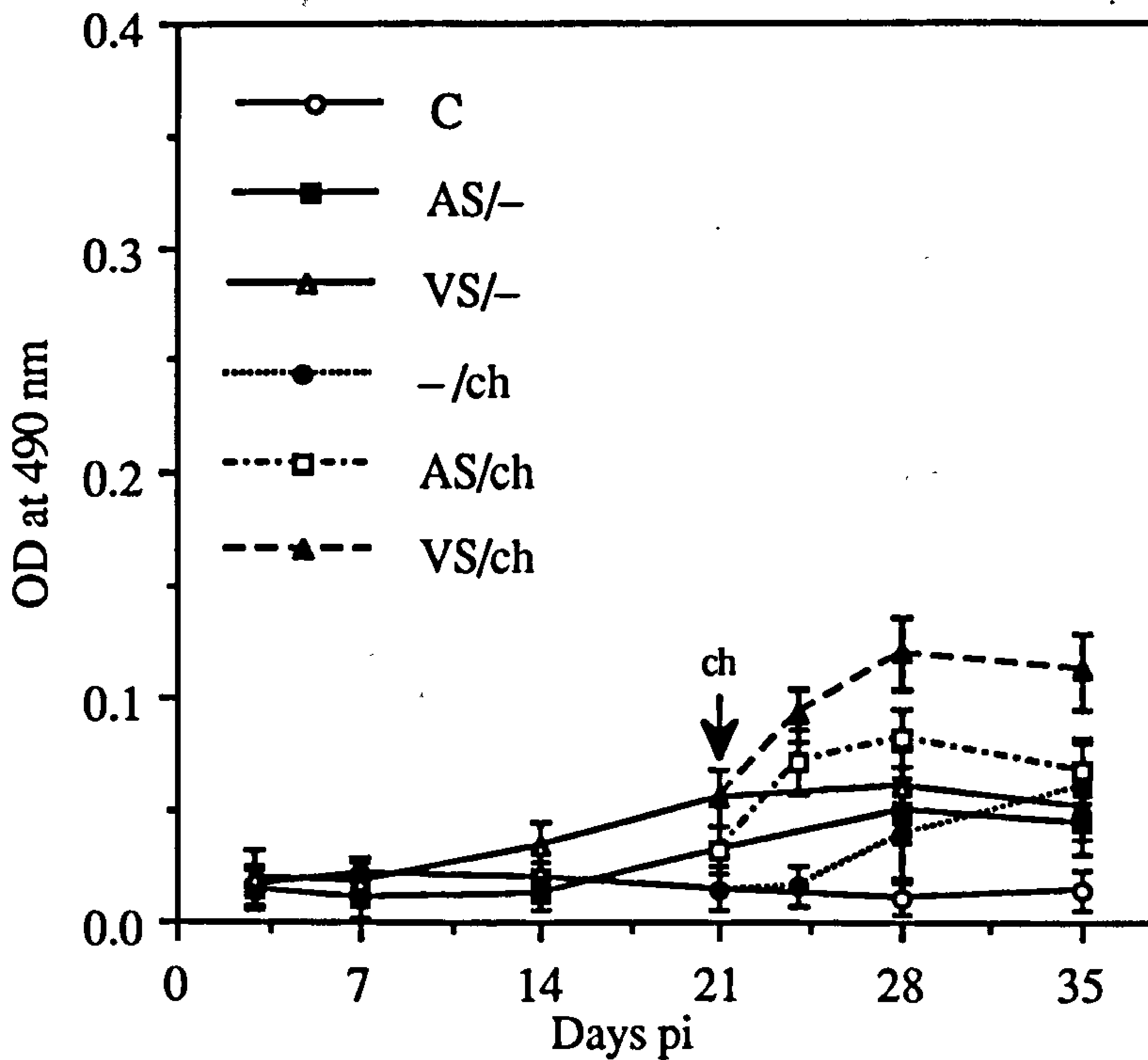

Fig. 4.30. Virus-specific IgA antibody (mean ± sd) in serum of poults following priming and challenge.

4.7.4. IgG antibody in lachrymal fluid, tracheal washes and serum

In lachrymal fluid (Fig. 4.31), there was a significant rise in IgG levels between days 3 and 28 pi followed by an insignificant decline on day 35 pi. IgG levels elicited by AS were lower and rose more slowly than for VS and did not reach significance (baseline = 0.06) until day 21 pi. Following challenge, a significant increase in IgG levels was detected on days 7 and 14 pc in both the primed groups.

There was no significant (baseline = 0.05) IgG antibody detectable in tracheal washes from poults primed with either strain (Fig. 4.32). However challenge resulted in a significant elevation by days 7 pc in both primed groups.

In serum (Fig. 4.33), IgG antibody (baseline = 0.05) rose rapidly and significantly between days 3 and 28 after VS priming and were sustained between days 28 to 35 pi. The response to AS was slower and the peak at day 28 pi much lower. Challenge caused a significant boost in levels by day 7 pc in both the primed groups.

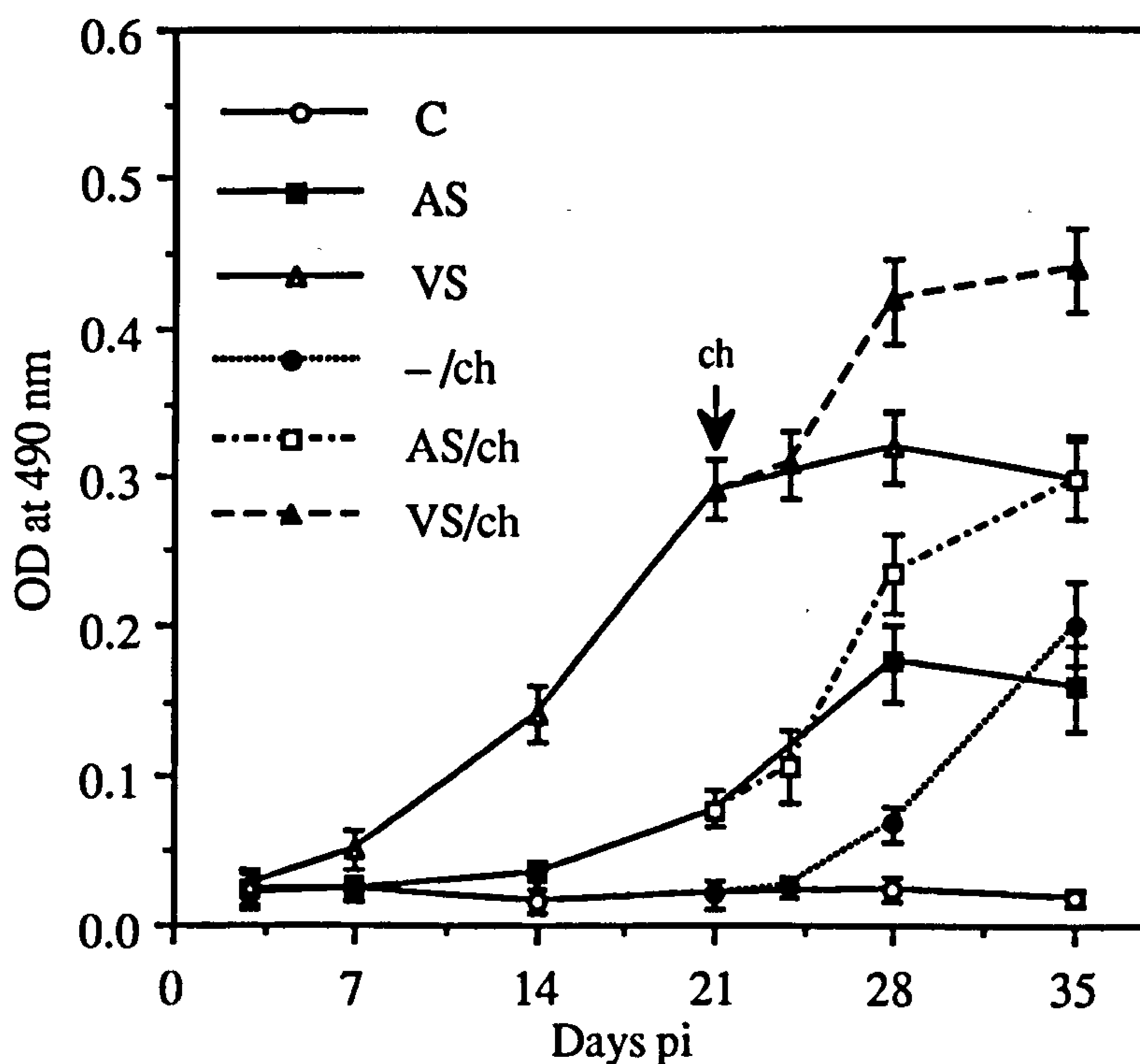

Fig. 4.31. Virus-specific IgG antibody (mean ± sd) in lachrymal fluid of poults following priming and challenge.

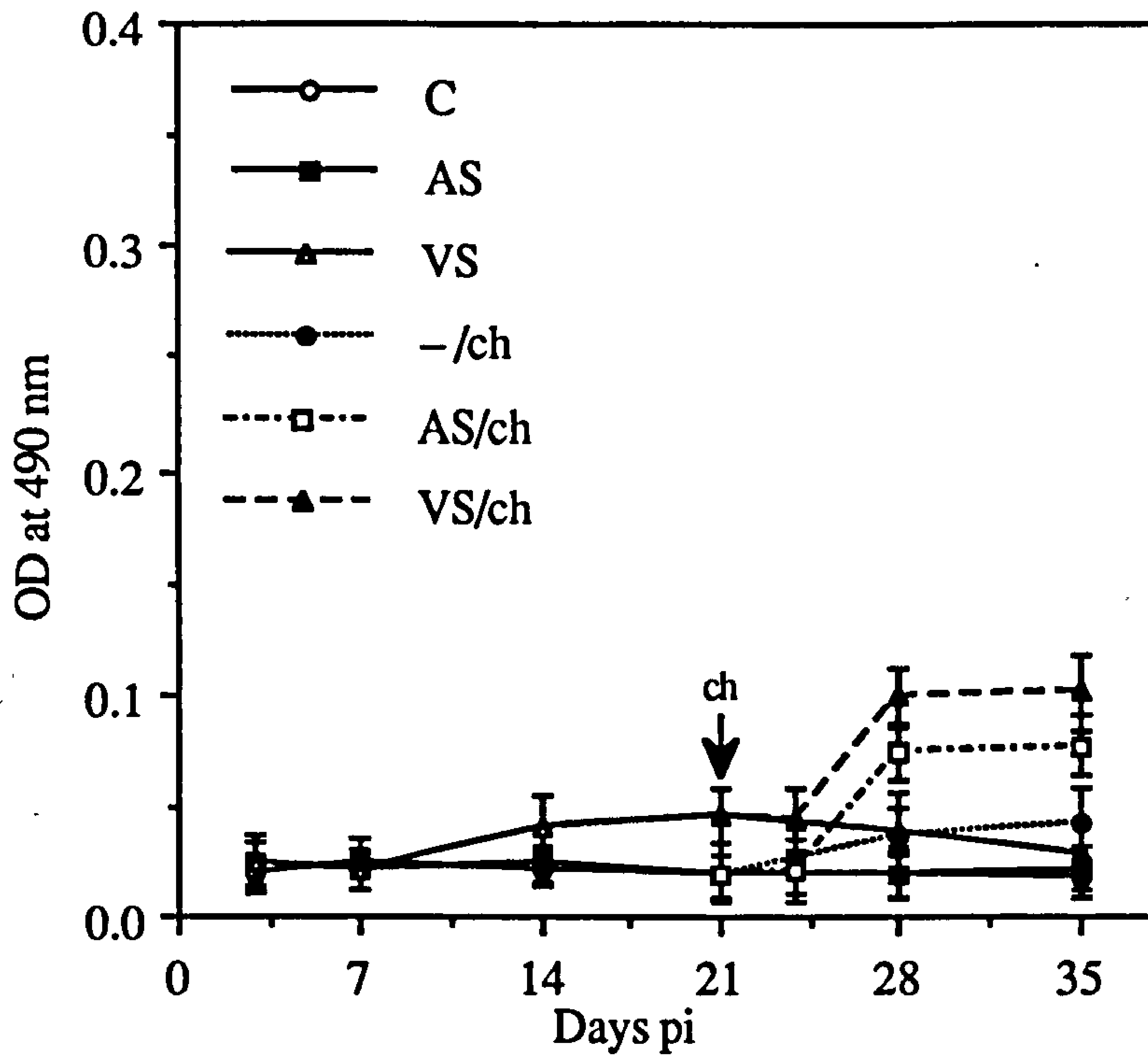

Fig. 4.32. Virus-specific IgG antibody (mean ± sd) in tracheal washes of poults following priming and challenge.

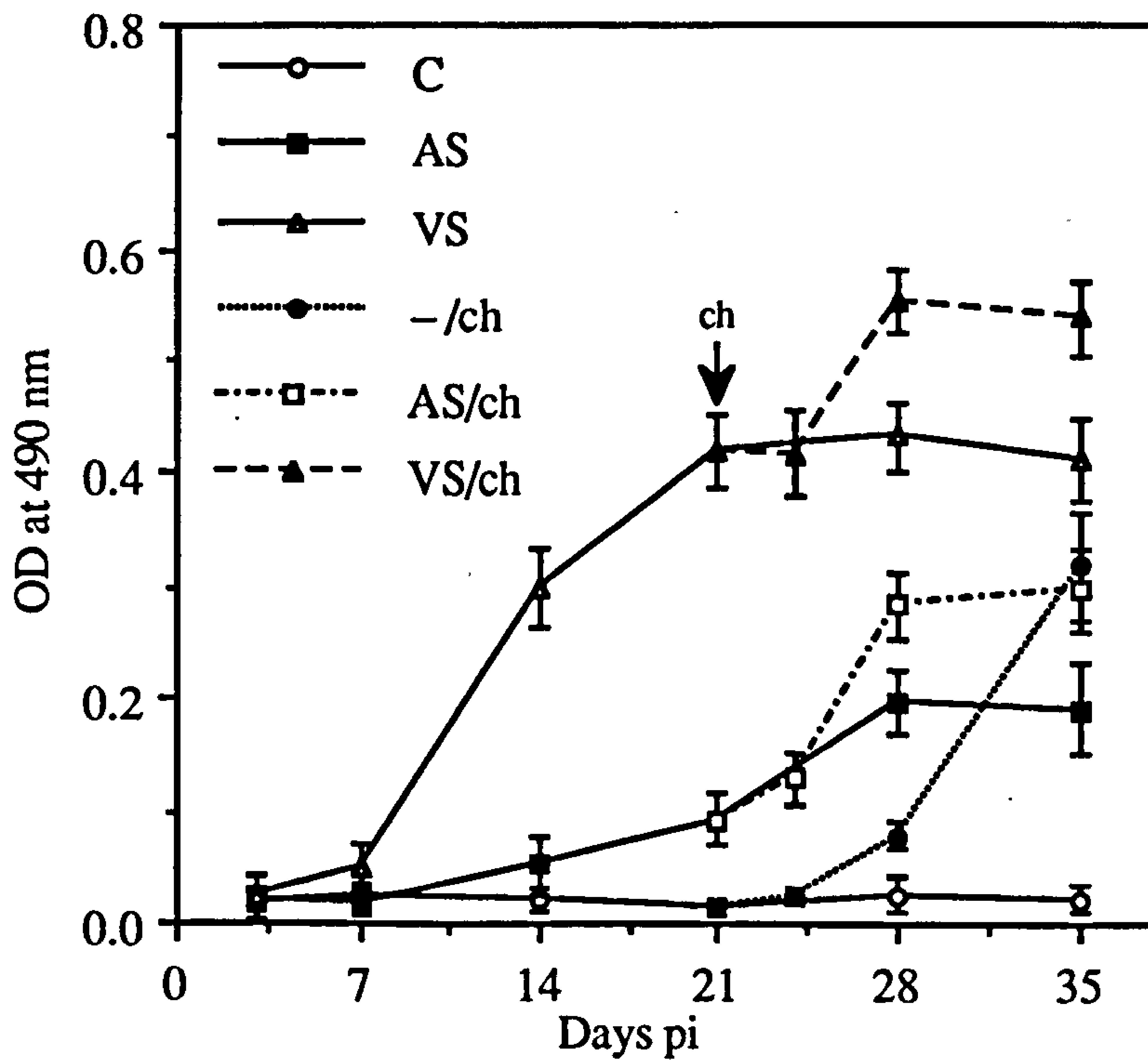

Fig. 4.33. Virus-specific IgG antibody (mean ± sd) in serum of poults following priming and challenge.

4.7.5. Virus neutralising antibodies

A rise in VN antibodies between days 3 and 28 pi occurred in lachrymal fluids of poults primed with either strain but with substantially higher titres in the case of VS (Fig. 4.34). Following challenge, VN antibodies were elevated by day 3 pc in both the primed groups.

There was no VN activity in tracheal washes after priming of poults with either of strains (Fig. 4.35) and following challenge only trace amounts were demonstrable.

Following priming of poults with either strain, VN antibodies in serum also showed a rise in levels between days 3 and 28 pi (Fig. 3.36). Again, the antibody titres for the VS were markedly higher than that for AS. Challenge resulted in elevation in VN antibodies by day 7 pc in both primed groups unlike lachrymal fluid where elevation occurred by day 3 pc.

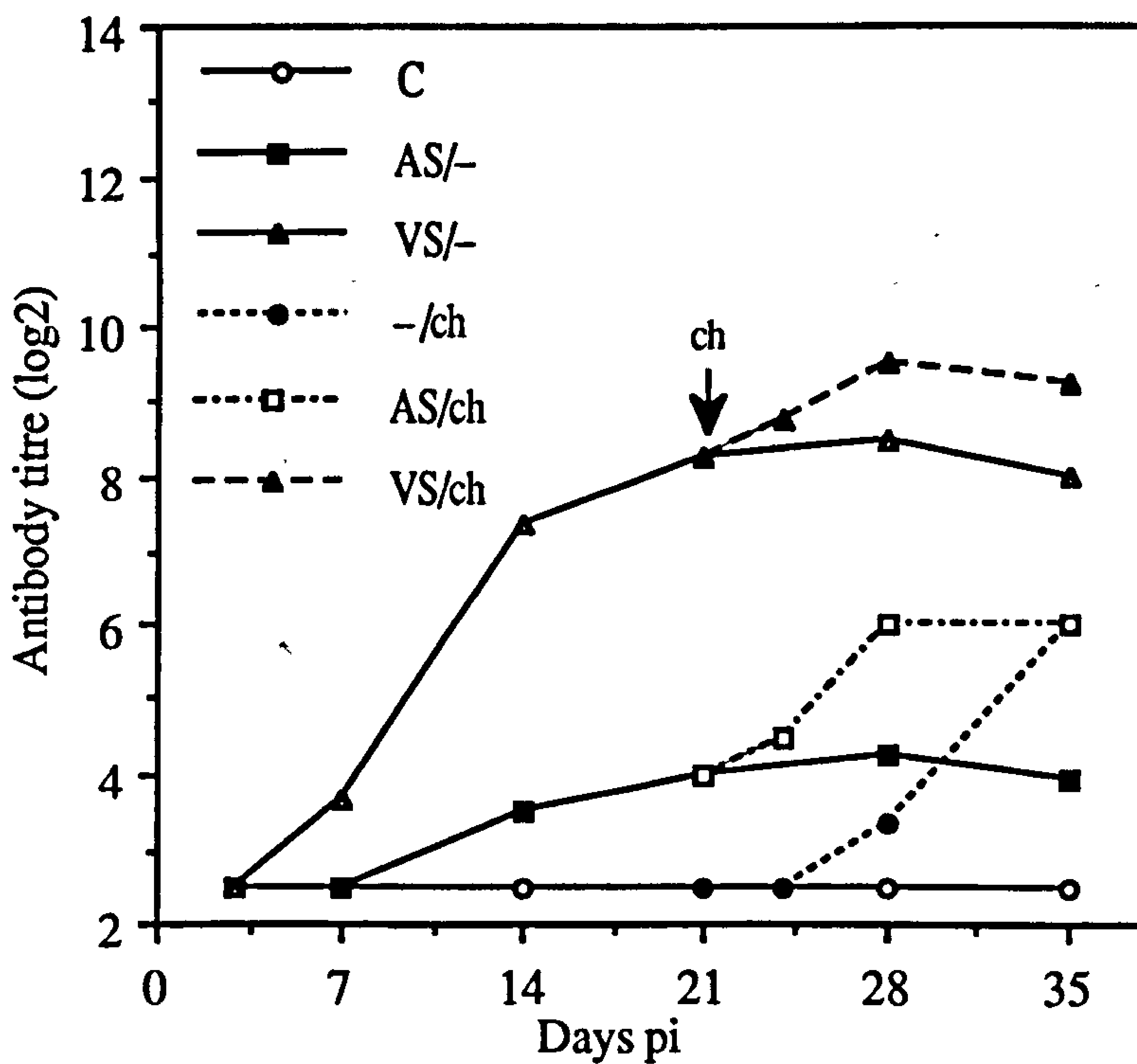

Fig. 4.34. VN antibody in pooled samples of lachrymal fluid of poults following priming and challenge. Lowest detectable level <2.5 log_2.

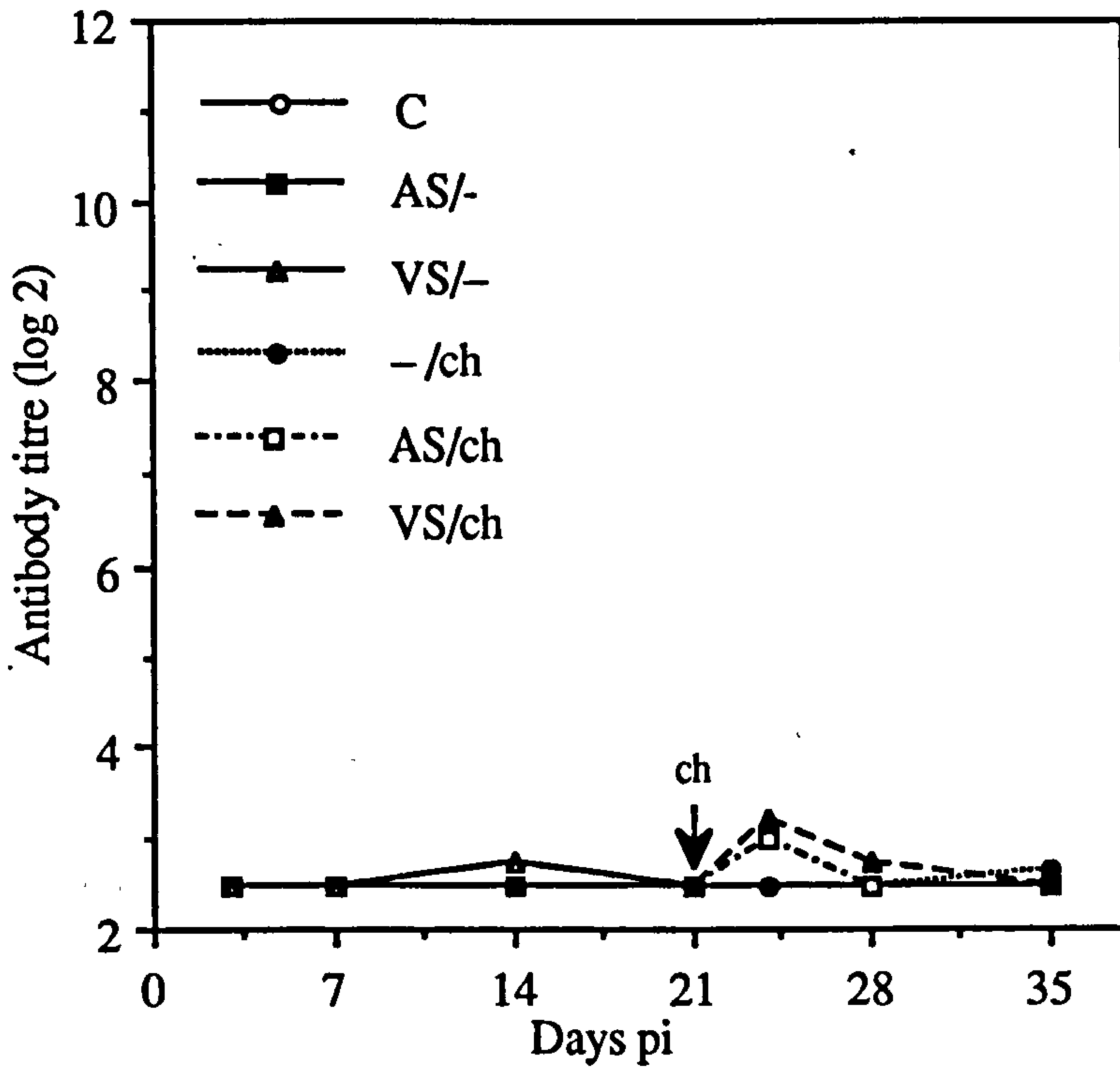

Fig. 4.35. VN antibody in pooled samples of tracheal washes of poults following priming and challenge. Lowest detectable level <2.5 log_2.

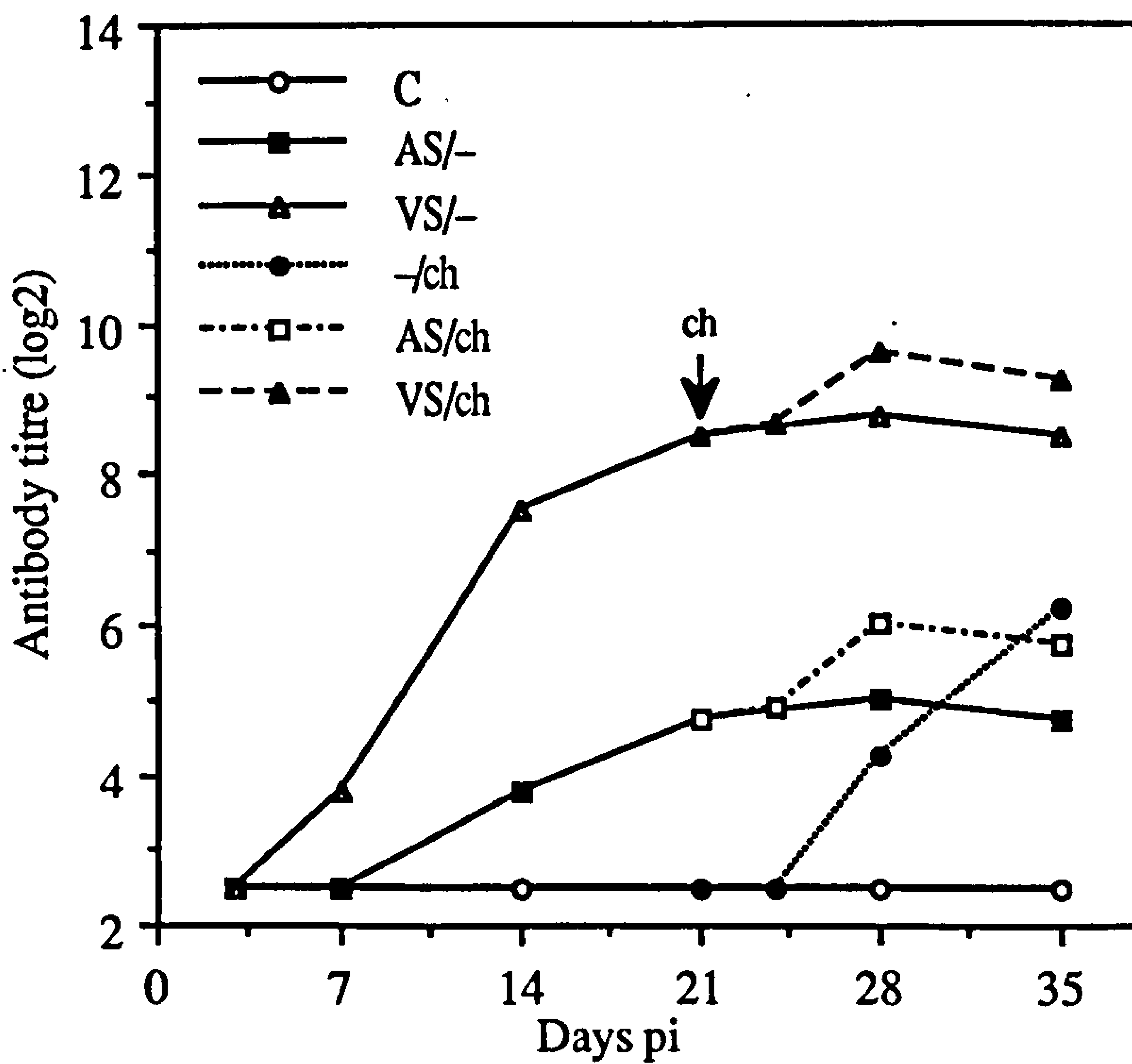

Fig. 4.36. VN antibody in pooled samples of serum of poults following priming and challenge. Lowest detectable level <2.5 log_2.

4.4. DISCUSSION

The first part of the present study revealed local production of both IgA and IgG antibody to a larger extent in the HG than the tracheas of 4-week old chickens following APV infection. A temporal relationship between appearance of lachrymal and serum antibodies, and clearance of virus from the tissues following primary infection was also observed.

APV is known to replicate for a relatively short duration in the upper respiratory tract of chickens and turkeys, with trachea and turbinates being the main target organs (Jones et al., 1988; Cook et al., 1991; Cook et al., 1993a). In the present study, this was confirmed by isolation of VS from turbinates and tracheas of chickens and turkeys between days 3 and 7 pi only. Attenuated APV was not recovered from the tissues of either species. However, Williams et al. (1991b) reported the reisolation of this AS in turbinates and trachea of poults between days 2 to 4 pi. From HG, only virulent APV was isolated for up to day 5 pi in both species. Although APV isolation from the HG has not been reported previously, Majo et al. (1994) have demonstrated APV in epithelial cells lining the duct of the HG of poults by immunoperoxidase staining.

Following eyedrop inoculation of 4-week old chickens with attenuated APV, local production of IgA antibody but not IgG was demonstrated in the HG on days 14 and 21 pi, corresponding with its presence in lachrymal fluid. Following inoculation of chickens with VS, *in vitro* local synthesis of both Igs was demonstrated in the HG on days 7, 14 and 21 pi with corresponding secretion of these antibodies in the lachrymal fluid. In lachrymal fluid, both antibodies first appeared on day 7 pi and reached high levels between day 14 and 21 pi. The HG is known to manufacture most of the lachrymal antibody and all the lachrymal IgA (Baba et al., 1988). Although it is generally accepted that IgA is produced locally in the HG and there is transport of IgG into the lachrymal fluid from serum (Toro et al., 1993), some in *vitro* local IgG production (about a third to a half of IgA) in the HG has also been demonstrated previously (Baba et al., 1990).

IgA or IgG antibody was not detected in tracheal washes in response to either virus strain and only small quantities of these antibodies were demonstrable in the trachea by *in vitro* culture on day 14 pi in response to the VS. Only IgG antibody response was detectable in the serum from chickens infected with VS, which to some extent corresponded with *in vitro* synthesis of these antibodies from the spleen. The assay used for detecting active local secretion of antibodies (Zigtermann et al., 1993) was useful except for the splenic tissue where reduction in OD at 4°C for IgG antibody could not be established as being statistically significant. This might have been due to

large amounts of extra-cellular antibody, which perhaps was not removed fully after repeated washings from the tissue pieces and would have been preserved at 4°C.

Since antibody was collected from the tracheal mucosa by washing, direct quantitative comparison of antibody in tracheal washes with that in lachrymal fluid or serum should be interpreted with caution but lower or negligible level of antibodies in the trachea might be due to the paucity of lymphoid tissue.

Following infection of 4 week old chickens with the VS, virus titres in tissues rapidly declined between days 5 and 7 pi and this was accompanied by a rise in IgA and IgG antibody to significant levels in lachrymal fluid and IgG in serum. A rise in VN antibodies in lachrymal fluid and serum at the time of virus clearance from tissues was also demonstrable. Moreover, VN antibody titres in lachrymal fluid were higher than in serum, particularly at 7 days pi. It is not known what contributed to the higher titres of VN antibodies in lachrymal fluid, but higher titres of VN antibodies in lachrymal fluid than serum on day 7 pi, suggests an important relationship with the clearance of virus from tissues of chickens after primary infection.

In the second part of this study, priming of one-week old chicks with the VS elicited significant IgA and IgG levels in lachrymal fluid at the same time between days 7 and 14 pi, that peaked around days 21 to 28 pi and were sustained until at least day 35 pi. Serum IgG levels were significant between days 14 to 21 pi and sustained up to day 35 pi. IgA in serum and both Igs in tracheal washes never reached levels of significance. VN antibodies in both lachrymal fluid and serum were detectable as early as day 7 pi, with a peak between days 21 to 28 pi and remained at high levels at least until day 35 pi.

In general, in one-week old chicks primed with the VS, the IgA, IgG or VN antibody levels were lower and/ or delayed compared to those in 4-week old chickens indicating the influence of age at infection. Seto (1981) have described age-related delay in the onset of immunocompetence in chicks which was thought to relate to insufficient numbers of T helper cells in peripheral lymphoid organs in early days of life post-hatching. In the HG, age-related increase in the percentage of plasma cells and T-cells has also been reported (Albini et al., 1974).

The priming of 1 week-old chicks with the AS induced neither detectable IgA nor IgG antibody nor VN antibodies in lachrymal fluid, tracheal washes or serum. However, infection of 4-week old chickens, with a higher dose of this strain had shown some IgA in lachrymal fluid, and neutralising antibodies in lachrymal fluid and serum. It is not

known if age at infection or dose rate influenced the IgA and VN antibody response but the lack of IgG antibody in response to this strain was consistent and was not influenced by these factors.

Following challenge, complete protection was seen in chicks primed with the VS but only partial protection was induced by the AS. The complete protection of chicks was accompanied by a rapid and significant elevation of secondary IgA and IgG levels in lachrymal fluid and IgG in serum but this did not occur in the group with partial protection. No significant secondary IgA or IgG antibody response was evident in tracheal washes from either group. A rise in secondary VN antibodies was also detected in lachrymal fluid and serum after challenge of chicks primed with the VS. On days 3 and 7 pc, VN antibody titres in lachrymal fluid were higher than in serum, suggesting a relative significance of the lachrymal antibodies in protection.

The reason for the lack of complete protection in chicks primed with the AS is not clear. It could be related to undetectable levels of both local and serum antibodies at time of challenge or absence of memory antibody responses. In a previous study, Cook et al. (1995) observed good protection against virulent APV challenge of chicks vaccinated with another commercial vaccine, but there is no published work describing a correlation of even circulating antibodies with the protection of chicks.

Different Ig isotypes of turkeys were isolated and quantified in serum and secretions (Goudswaard et al., 1977, 1978) nearly two decades ago, but unfortunately, antibodies which specifically recognise the different isotypes of turkey Ig are still not readily available. Van Nerom et al. (1997) reported that Mabs and polyclonal antibodies to chicken Ig isotypes specifically detect turkey Ig isotypes. In the present study, the available Mabs to chicken IgA and IgG were found to reacted poorly with turkey counterparts in the ELISAs employed for testing chicken samples. Instead, the ELISAs based on polyclonal antibody to chicken isotypes were used for detecting turkey IgA and IgG antibodies. Thus any comparison between chicken and turkey IgA and IgG antibody responses should bear this difference in mind.

The priming of poults with the VS elicited significant IgA and IgG levels in lachrymal fluid and IgG in serum at the same time between days 7 and 14 pi, that peaked around day 28 pi and were still high until at least day 35 pi. Priming of poults with the AS also induced significant IgA in lachrymal fluid between days 14 and 21 pi, and IgG in both lachrymal fluid and serum on day 21 pi. The peak levels for both antibodies in either lachrymal fluid or serum were attained on day 28 pi and followed by an insignificant decline on day 35 pi. IgA or IgG antibody in tracheal washes in response to either

strain did not reach the level of significance. IgA response to either strain in serum was markedly lower than in lachrymal fluid.

The lower IgA response in the serum of poults and its undetectable levels in the serum of chicks compared to lachrymal fluid might have been due to local replication of APV in the HG presenting more antigenic load to its lymphoid cells, leading to higher IgA levels in the lachrymal fluid. Russell (1993) has demonstrated that the local replication of Newcastle disease virus in the HG of chickens stimulates lachrymal IgA.

VN antibodies in both the lachrymal fluid and serum were detectable as early as day 7 pi in poults primed with the VS and by day 14 pi in those primed with the AS. VN antibodies in both groups showed a peak on day 28 pi and maintained high levels at least until day 35 pi. The overall IgA, IgG or VN antibody response to the AS was lower and delayed compared to the VS. It is likely that the stimulus evoked by infection with the VS was stronger and more persistent owing to greater multiplication of infectious virus.

Consistent with earlier reports (Cook et al., 1989b; Williams et al., 1991b; Naylor et al., 1997a), the present study also showed a complete protection after challenge of poults either primed by virulent or attenuated APV strain. This was accompanied by a rise in secondary IgA antibody on day 3 pc followed by IgG on day 7 pc in lachrymal fluid, tracheal washes or serum from poults primed with either of strains. Challenge also resulted in elevation in VN antibodies in lachrymal fluid as early as day 3 pc and in serum on day 7 pc, and in minute quantities in tracheal washes from poults primed with either strain. The secondary antibody levels in poults primed with the AS were always lower compared to those primed with the VS.

The results of experiments on chicks and poults have broadly shown that IgA and IgG antibodies in lachrymal fluid and IgG in serum are associated with virus clearance following primary infection as well as with resistance against APV infection. However, following challenge of poults, there was a boost in virus-specific IgA prior to IgG both locally in lachrymal fluid and tracheal washes, and serum indicating that IgA might have a initial role in inhibition of virus replication. Secondary boost in VN antibodies in lachrymal fluid occurred prior to that in serum suggesting the importance of local antibodies.

In chickens, following challenge, both IgA and IgG antibodies elevated at the same time but there were higher levels of VN antibodies in lachrymal fluid than in serum which also suggest their relative significance in protection to APV infection. Protection

against APV infection like other viral infections may involve different specific and non specific immune mechanism and certainly not only due to the lachrymal or humoral antibodies. In addition, to establish a statistical correlation between lachrymal antibody levels and resistance to APV infection, further work is required involving larger data and monitoring lachrymal antibody response from individual birds in relation to protection against challenge.

CHAPTER 5

EFFECT OF T-CELL SUPPRESSION BY CYCLOSPORIN A ON AVIAN PNEUMOVIRUS INFECTION IN CHICKENS AND TURKEYS

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CHAPTER 5

EFFECT OF T-CELL SUPPRESSION BY CYCLOSPORIN A ON AVIAN PNEUMOVIRUS INFECTION IN CHICKENS AND TURKEYS

5.1. INTRODUCTION

APV vaccination of turkeys has been shown to elicit humoral antibody response and protect against virulent challenge (Cook et al., 1989b; Williams et al., 1991b). In an experimental study, Jones *et al.* (1992) found that chemically (cyclophosphamide) bursectomised vaccinated turkey poults were protected against virulent challenge of APV, despite the fact that there were no significant enzyme-linked immunosorbent assay (ELISA) antibodies detectable in the serum.

In another long term-study, turkey hens given live APV vaccine at 12 days of age were protected against challenge at 22 weeks even though they had no significant levels of ELISA antibodies at the time of challenge (Williams et al., 1991b). Conversely, the presence of high titres of maternal antibodies (MA) did not prevent development of respiratory disease after virulent APV challenge of turkey poults (Naylor et al., 1997b). These investigations suggested that humoral antibodies to APV correlate poorly with protection.

However, the results of Chapter 4 showed a relationship between the presence of the lachrymal and serum antibodies and resistance to APV infection in poults and chicks. Protection against APV infection like other viral infections may involve different specific and non-specific immune mechanisms. The role of cell-mediated immunity (CMI) in resistance to APV infections has not been documented in the literature. In this chapter, attempts were made to study the role of CMI in APV infections by use of a T-cell suppressant drug cyclosporin A (CSA).

CSA has been shown to produce a selective T-lymphocyte suppression in chickens and turkeys (Nowak *et al.*, 1982; Suresh & Sharma, 1995). It acts by preventing the synthesis of cytokines by T-cells (Schreiber & Crabtree, 1992; Ho et al., 1996). CSA has been extensively used to study the role of CMI in various infections of poultry such as those due to coccidia (Lillehoj, 1987), reovirus (Hill *et al.*, 1989), hemorrhagic enteritis virus (HEV) (Suresh & Sharma., 1995), and infectious bronchitis virus (IBV) (Dhinakar Raj & Jones, 1997). In the present study, the role of T-lymphocytes in (i) primary infection in chickens and (ii) primary and secondary APV infections in turkeys was investigated by comparing the pathogenesis of pneumoviral disease in normal and CSA-induced T-cell suppressed birds.

5.2. MATERIALS AND METHODS

5.2.1. Experimental birds

SPF chicken eggs and turkey poults free of MA to APV were obtained from separate commercial sources. The chicken eggs were hatched in our laboratory. Both the chicks and poults were maintained under conditions of strict isolation with food and water *ad libitum.*

5.2.2. Virus

Virulent APV strain #8544 (Wilding *et al.*, 1986) was used for both primary and secondary infection. For primary infection, each chick or poult was inoculated oculonasally with 3.5 log_{10} CD_{50}/0.1 ml of APV at 15 days of age. For secondary infection, each poult was similarly re-inoculated with 3.5 log_{10} CD_{50}/0.1 ml of APV four weeks after primary infection.

5.2.3. T-cell suppression

T-cell suppression was induced by CSA treatment ('Sandimmun', Sandoz Pharmaceuticals, Surrey, England). The drug was administered intramuscularly at a dose rate of 100 mg/kg body weight every three days (Nowak et al., 1982). For primary infection in chicks or poults, CSA treatment was started from 11 days of age (i.e. four days before primary infection), then every three days until day 10 post primary infection (ppi). For reinfection in poults, CSA treatment was commenced from day 39 of age (i.e. four days before secondary infection), then every three days until day 10 post secondary infection (psi).

5.2.4. Experimental designs

The effect of T-cell suppression on APV pathogenesis was studied in (i) chicks after primary infection only (Experiment 1), and (ii) poults after both primary and secondary infections (Experiment 2).

Experiment 1. Effect of T-cell suppression on primary infection in chicks

Ninety-two chicks were randomly divided into four experimental groups (Table 5.1) and housed separately. Two groups were treated with CSA starting from 11 days of age (i.e. four days before primary infection) and continued until day 10 ppi (see section 5.2.3). One CSA-treated and an untreated group were infected with APV on day 15 of age (see section 5.2.3). One CSA-treated group and another group of uninfected and untreated birds were kept as controls. Heparinised whole blood samples were collected from 8 birds each in CSA treated and untreated group on day 15 of age i.e. before

infection and were used in mitogenic assay to assess lymphoproliferative responses to a T-cell mitogen con A.

Following infection, 10-15 chicks in each group were examined daily for presence of clinical signs. Birds were euthanased and sampled at regular intervals. Pieces of nasal turbinates, trachea and lungs were collected from five birds from each infected group and from three birds from each uninfected groups on days 3, 5, 7, and 10 ppi. Virus recovery was attempted in TOC. Pieces of tracheas were also collected by snap-freezing for IF staining and in 10% formalin for histopathology. Sera collected from 8 birds in each group on day 7 and 10 ppi were examined for virus-specific antibody by ELISA and serum neutralisation test (SNT).

Table 5.1. Experimental design for studying effect of CSA on primary APV infection in chicks/poults

Group	APV	CSA
C	–	–
CSA	–	+
APV	+	–
APV+CSA	+	+

Experiment 2. Effect of T-cell suppression on primary and secondary infection in poults

Primary infection

A total of 158 birds were used for studying the effect of T-cell suppression on both primary and secondary infection in poults. For primary infection, a trial identical to that described for chicks was performed and all procedures were the same as for the chicks. For secondary infection, additional groups of poults were raised and treated as required (Table 5.2) during the primary stage.

Secondary infection

Three weeks following primary infection, poults were divided into five experimental groups (Table 5.2) and housed in separate pens. One group was kept as uninfected and untreated control (C), a second was CSA treated during primary and secondary stages (1* & 2* CSA), a third infected at the secondary stage only (2* APV), a fourth infected at primary and secondary stages (1* & 2* APV), and a fifth CSA treated and infected both during primary and secondary parts (1* & 2* APV+CSA). CSA treatment was re-

started on day 39 of age (i.e. four days before reinfection) in the respective groups and was continued until day 10 psi. Virus infection was given at 43 day of age i.e. 4 weeks after primary infection.

Following infection, 10-15 poults were examined daily for clinical signs. Birds were euthanased for sampling. Pieces of nasal turbinates, trachea and lungs were collected from five birds from each infected group and two birds from each uninfected group on days 4, 7, and 10 psi for virus isolation. Pieces of tracheas were also collected by snap-freezing for IF staining and in 10% formalin for histopathology. Sera collected from 6 birds in each group on day 28 ppi (i.e. before secondary infection) and on day 7 psi were examined for virus-specific antibody by ELISA and SNT.

Table 5.2. Experiment groups for studying effect of CSA on secondary APV infection in poults

Group	Primary infection		Secondary infection	
	APV	CSA	APV	CSA
C	–	–	–	–
1* & 2* CSA	–	+	–	+
2* APV	–	–	+	–
1* & 2* APV	+	–	+	–
1* & 2* APV+CSA	+	+	+	+

1*: Primary stage, 2*: Secondary stage.

5.2.5. Mitogenic assays of whole blood

Chicken blood

Mitogenic assay with the chicken blood was performed following the procedure of Talebi et al. (1995) with slight modifications. Briefly, the heparinised blood was diluted 1 in 40 in RPMI 1640 medium containing L-glutamine and HEPES (Life Technologies, Paisley, Scotland) supplemented with 100 IU penicillin and 100 µg streptomycin per ml. Volumes of 200 µl of the diluted blood were added to six wells of 96-well flat-bottom tissue culture microplates (Nunc, Denmark). Three wells each received an additional 20 µl of medium containing con A (Sigma) to yield a concentration of 2 µg per well. The remaining three wells received medium without mitogen.

Turkey blood

Mitogenic responses of turkey blood cells were assessed following the method of Sharma and Belzer (1992) with slight modifications. Heparinised blood was diluted 1 in 20 in RPMI 1640 medium containing L-glutamine and HEPES (Life Technologies), and supplemented with 7.5 % normal turkey serum and antibiotics (100 IU penicillin and 100 μg streptomycin per ml). Volumes of 100 μl of this diluted blood were added to six wells of a 96 well flat-bottom microtitre plate (Nunc). Three wells received 100 μl medium containing 24 μg of con A (Sigma) and the remaining 3 wells received the same volume of medium without mitogen.

Mitogenic assay

Both chicken and turkey blood cultures were incubated at 37.5°C for 56 hours in a humidified atmosphere containing 5% CO_2. They were pulsed with 0.05 μCi of ^3H thymidine (Amersham Life Sciences, Amersham, UK) for 16 hours prior to harvesting onto filter mats (Skatron Instruments, Suffolk, UK) using a cell harvester (Skatron, Herbyen, Norway). The discs were punched out and placed into disposable scintillation tubes and mixed with 2 ml of scintillation fluid (Optiphase 'Safe', Wallac UK, Milton Keynes, England). Radioactivity was measured in counts per minute (cpm) using a scintillation counter (Packard Tricarb, Berks, England). The stimulation index (SI) for each sample was calculated according to the following formula:

$$SI = \frac{\text{Mean cpm of stimulated cultures}}{\text{Mean cpm of unstimulated cultures}}$$

5.2.6. Clinical signs

The severity of clinical signs was scored on a 0 to 3 scale by the method of Jones *et al.* (1992) as described in Chapter 3.

5.2.7. Virus isolation and titration

Aseptically-collected tissues from euthanased birds were processed and used for virus isolations in tracheal organ cultures (TOC) according to the methods described in Chapter 3. A minimum of three passages was given and the ciliostatic virus was identified by immunofluorescence (IF) staining (Chapter 3). All the samples positive for virus were titrated individually in TOC (Cook *et al.*, 1976) as described in Chapter 3.

5.2.8. Immunofluorescence staining

This technique was employed both for detecting virus-specific antigens on unfixed tracheal rings (Bhattacharjee *et al.*, 1994) for confirmation of viral isolates as well as for demonstration of viral antigens in frozen tracheal tissue using hyperimmune serum against APV strain #8544. All these procedures are detailed in Chapter 3.

5.2.9. Histopathology

Samples of tracheas collected in 10% neutral buffered formalin were processed by conventional methods and the sections stained with haematoxylin and eosin.

5.2.10. Serology

Sera were titrated for specific antibodies to APV using ELISA (Naylor *et al.*, 1992) and SNT (Cook et al., 1988) as described in Chapter 3. Serum samples were tested individually by ELISA but for SNT, pooled samples were used.

5.2.11. Statistical analysis

Mean clinical scores and the total numbers of virus isolations in different groups were compared by chi-square test ($p<0.05$). The SI values for mitogenic assays, virus titres in tissues and ELISA results between experimental groups were compared by student's t-test ($p<0.05$).

5.3. RESULTS

5.3.1. Effect of cyclosporin A on mitogenic responses

There were no adverse effects associated with CSA administration in any of the injected chicks in Experiment 1 or poults in Experiment 2. CSA treatment resulted in T-cell suppression in both chicks and poults as shown by the results of mitogenic responses (Table 5.3). The SI values to con A were significantly lower in CSA-treated chicks or poults compared to untreated controls.

Table 5.3. Mitogenic responses of whole blood of chicks or poults 4 days post-initiation of CSA treatment

Group	Stimulation index (mean ± sd)	
	Chicks	Poults
Control	$24.0^{a} \pm 2.2$	$16.6^{a} \pm 3.0$
CSA	$1.0^{b} \pm 0.2$	$0.95^{b} \pm 0.2$

Values with different superscripts between two groups differ significantly ($p<0.05$).

5.3.2. Clinical signs

Experiment 1. Primary infection in chicks

Following APV infection, mild clinical signs were recorded in both CSA-treated and untreated chicks from days 3 to 9 ppi (Fig. 5.1). These included the presence of clear to turbid nasal exudate expressed after gentle beak squeezing behind the nostrils. The mean clinical scores (MCS) of the CSA-treated group on days 3, 6 and 7 ppi were higher than for the untreated group but without significant differences (Fig. 5.1). No signs were observed in uninfected chicks (not shown).

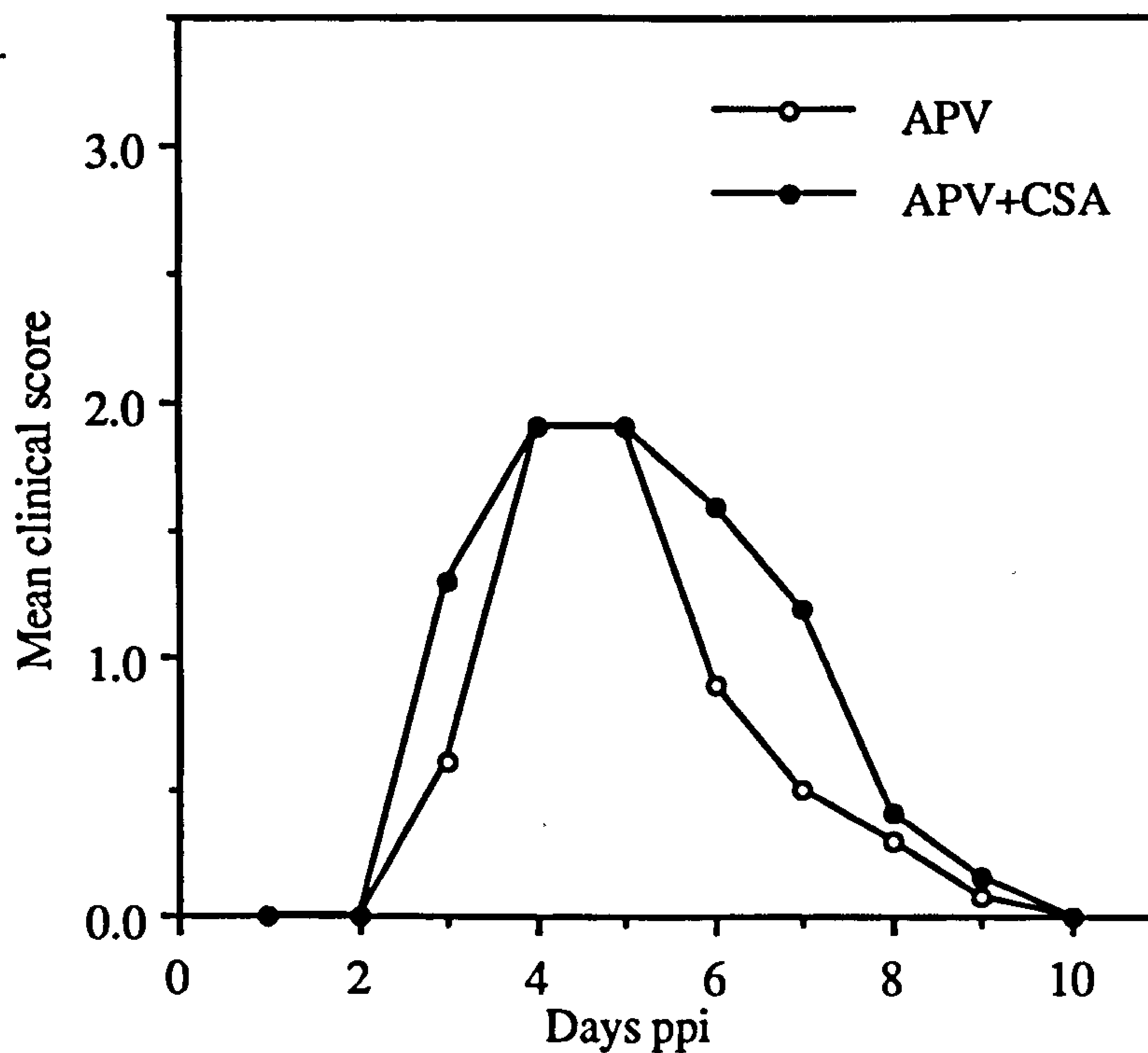

Fig. 5.1. Mean clinical scores of CSA-treated and untreated chicks after primary APV infection.

Experiment 2.
Primary infection in poults
In poults, APV infection resulted in a more severe clinical disease compared to the infection in chicks. There were differences between CSA-treated and untreated poults in terms of both the duration and severity of clinical signs (Fig. 5.2). In untreated poults, clinical signs were recorded from days 2 to 11 ppi, whereas in CSA-treated birds they persisted until day 14 ppi despite the fact that CSA treatment was stopped on day 10 ppi due to economic reasons. It is not known how long the disease could have continued if CSA treatment had not been curtailed.

The MCS of CSA-treated poults were significantly higher than for the untreated birds on days 7 to 12 ppi (Fig. 5.2). Signs in both the groups included clear to turbid nasal exudate, conjunctivitis with frothy eye-discharge, coughing and sneezing. In the CSA-treated group, however, more severe signs including infra-orbital swelling in 5-10% of poults, and highly frothy and copious eye discharge in 30-50% birds were recorded between days 7 and 11 ppi. In addition, one bird each on days 4 and 7 ppi was found dead. Necropsy revealed presence of a thick mucous plug in the trachea, with congestion of lungs.

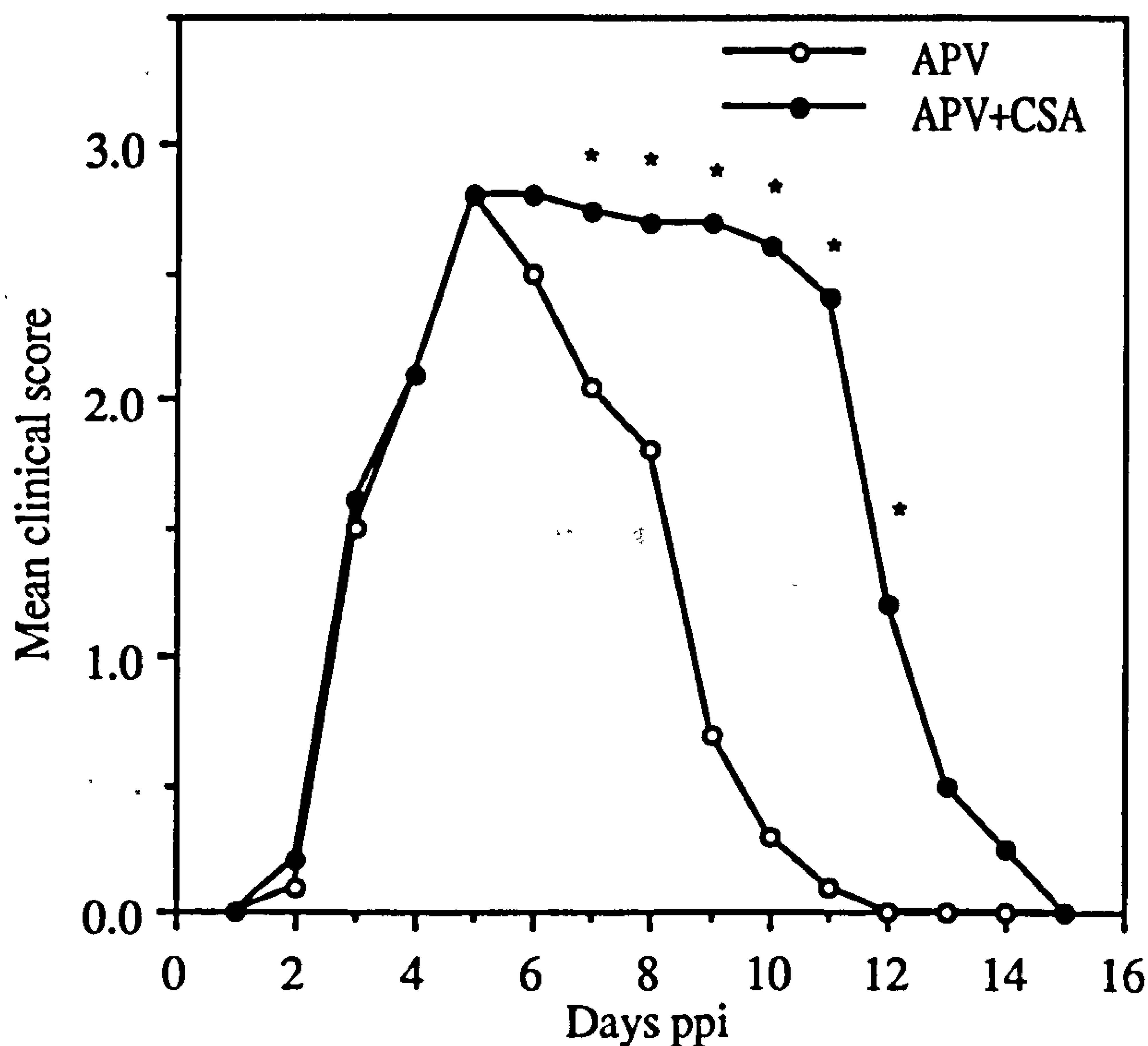

Fig. 5.2. Mean clinical scores of CSA-treated and untreated poults after primary APV infection. *: Significantly higher ($p<0.05$) scores in CSA-treated poults. CSA treatment was discontinued on day 10 ppi.

Secondary infection in poults

Following reinfection of untreated poults, no clinical signs were observed and the group which was CSA re-treated and reinfected showed the presence of clear nasal exudate in only 5-10% poults between days 5 to 7 psi (Fig. 5.3). In infected controls (2* APV) which had not had primary infection or CSA treatment, the clinical disease was severe and signs were recorded between days 4 and 9 pi. The MCS of CSA-treated poults after reinfection was significantly lower compared to that of unprimed but infected controls on days 5 to 8 psi (Fig. 5.3).

No signs were recorded in uninfected poults in either the primary or secondary stage (not shown).

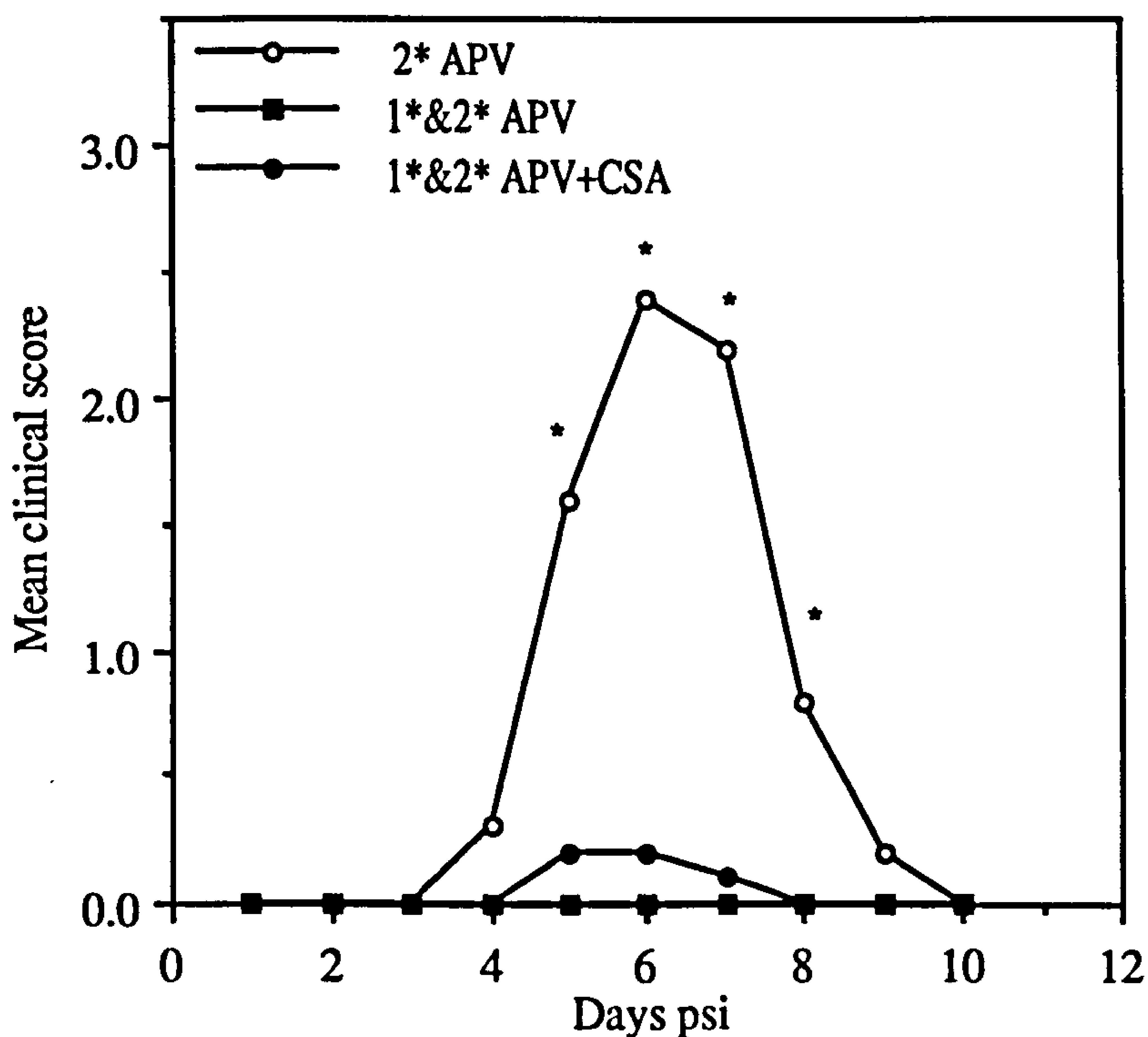

Fig. 5.3. Mean clinical scores of poults following secondary infection. *: Significantly higher ($p<0.05$) scores in unprimed but infected group.

5.3.3. Virus isolations

Experiment 1. Primary infection in chicks

Following infection, APV was recovered from turbinates and trachea of both CSA-treated and untreated chicks on days 3 and 5 ppi only (Table 5.4). There were no significant differences in the total numbers of isolations between these two groups. No virus was isolated from lungs on any occasion from either of the infected groups, nor from the tissues of uninfected chicks.

Table 5.4. Virus isolations from tissues of CSA-treated and untreated chicks following primary APV infection

Tissue	Group	Days ppi				
		3	5	7	10	Total
Turbinates	APV	4*	5	0	0	9
	APV+CSA	5	5	0	0	10
Tracheas	APV	4	5	0	0	9
	APV+CSA	4	5	0	0	9
Lungs	APV	0	0	0	0	0
	APV+CSA	0	0	0	0	0

*: No. positive of 5 samples tested each time.

Experiment 2.

Primary infection in poults

Following primary infection, APV was isolated from turbinates on days 3 and 5 ppi in the untreated group but from days 3 to 7 ppi in the CSA-treated group (Table 5.5). Similarly, virus isolations were made for longer period in tracheas from days 3 to 10 ppi and in lungs from days 5 to 10 ppi in CSA-treated poults compared to untreated birds where isolations in tracheas were made from days 3 to 7 ppi and in lungs on days 5 and 7 ppi. The total numbers of virus isolations from turbinates, tracheas and lungs were insignificantly higher in CSA-treated poults compared to untreated birds (Table 5.5). The two birds from the CSA-treated group that died during the experiment yielded APV from turbinates, trachea, lungs and also kidneys. No virus isolations were made from tissues of uninfected poults (not shown).

Table 5.5. Virus isolations from tissues of CSA-treated and untreated poults following primary APV infection

Tissue	Group	Days ppi				
		3	5	7	10	Total
Turbinates	APV	5*	5	0	0	10
	APV+CSA	5	5	2	0	12
Trachea	APV	3	5	1	0	9
	APV+CSA	3	5	4	1	13
Lungs	APV	0	3	1	0	4
	APV+CSA	0	4	4	1	9

*: No. positive of 5 samples tested each time.

Secondary infection in poults

Following reinfection, no isolations were made from either the untreated or CSA re-treated group (Table 5.6). In infected controls (2* APV) which were not primed, virus was recovered on day 4 pi only from turbinates and trachea but not from the lungs (Table 5.6). No virus was isolated from the tissues of uninfected poults (not shown).

Table 5.6. Virus isolations from tissues of poults after secondary infection

Tissue	Group	Days psi			Total
		4	7	10	
Turbinates	2*APV	4*	0	0	4
	1* & 2* APV	0	0	0	0
	1* & 2* APV+CSA	0	0	0	0
Trachea	2*APV	4	0	0	4
	1* & 2* APV	0	0	0	0
	1* & 2* APV+CSA	0	0	0	0
Lungs	2*APV	0	0	0	0
	1* & 2* APV	0	0	0	0
	1* & 2* APV+CSA	0	0	0	0

*: No. positive of 5 samples tested each time.

5.3.4. Virus titrations

Experiment 1. Primary infection in chicks

The mean virus titres in the turbinates (Fig. 5.4) and trachea (Fig. 5.5) of CSA-treated chicks were higher than those of untreated birds with a significant difference on day 5 ppi.

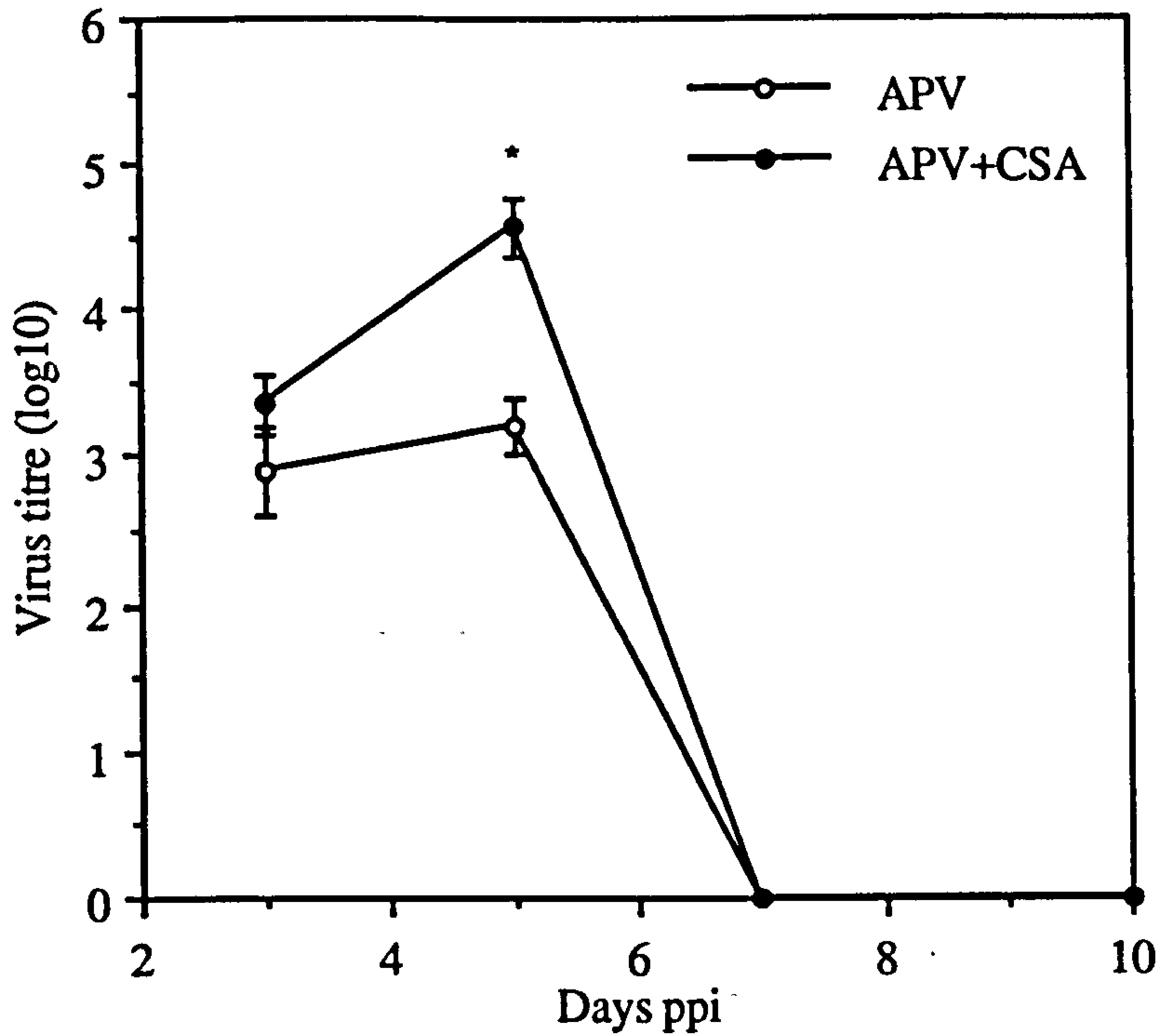

Fig. 5.4. Virus titres (mean ± sd) in turbinates of CSA-treated and untreated chicks after primary APV infection. Titres expressed as CD_{50} log_{10}/g tissue. *: Significantly higher ($p<0.05$) titres in CSA-treated chicks.

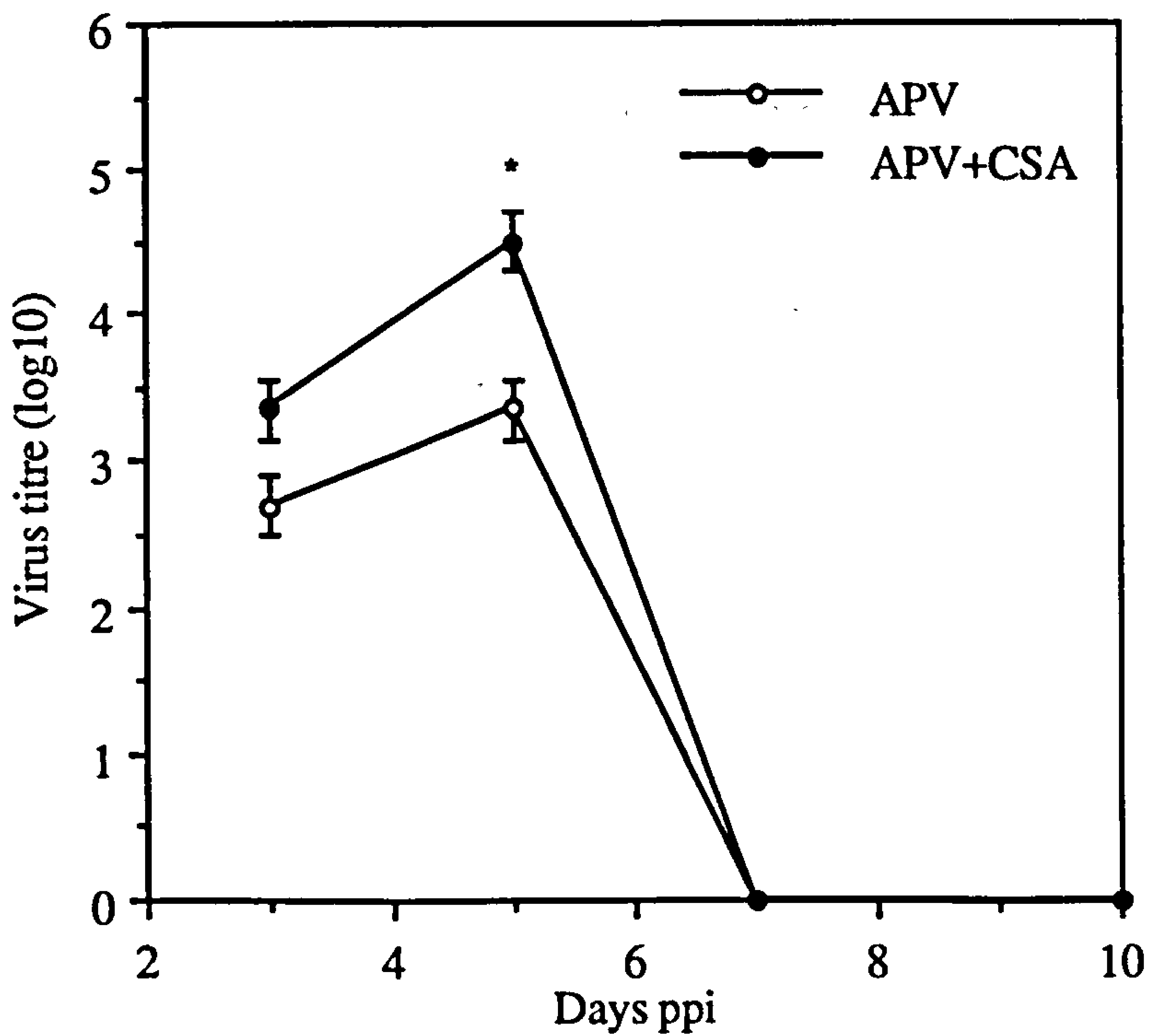

Fig. 5.5. Virus titres (mean ± sd) in tracheas of CSA-treated and untreated chicks after primary APV infection. *: Significantly higher ($p<0.05$) titres in CSA-treated chicks.

Experiment 2. Primary infection in poults

Virus titres in the turbinates (Fig. 5.6), trachea (Fig. 5.7) and lungs (Fig. 5.8) of CSA-treated poults were generally higher than those of untreated birds. These differences was more marked on day 7 (all tissues) or 10 (trachea & lungs) ppi with about 1.5 to 3.0 $\log_{10}$ higher titres, suggestive of delay in virus clearance from tissues of CSA-treated poults. Statistical comparisons by student's t-test were not possible because of either no value or only one value for titres in respective tissues.

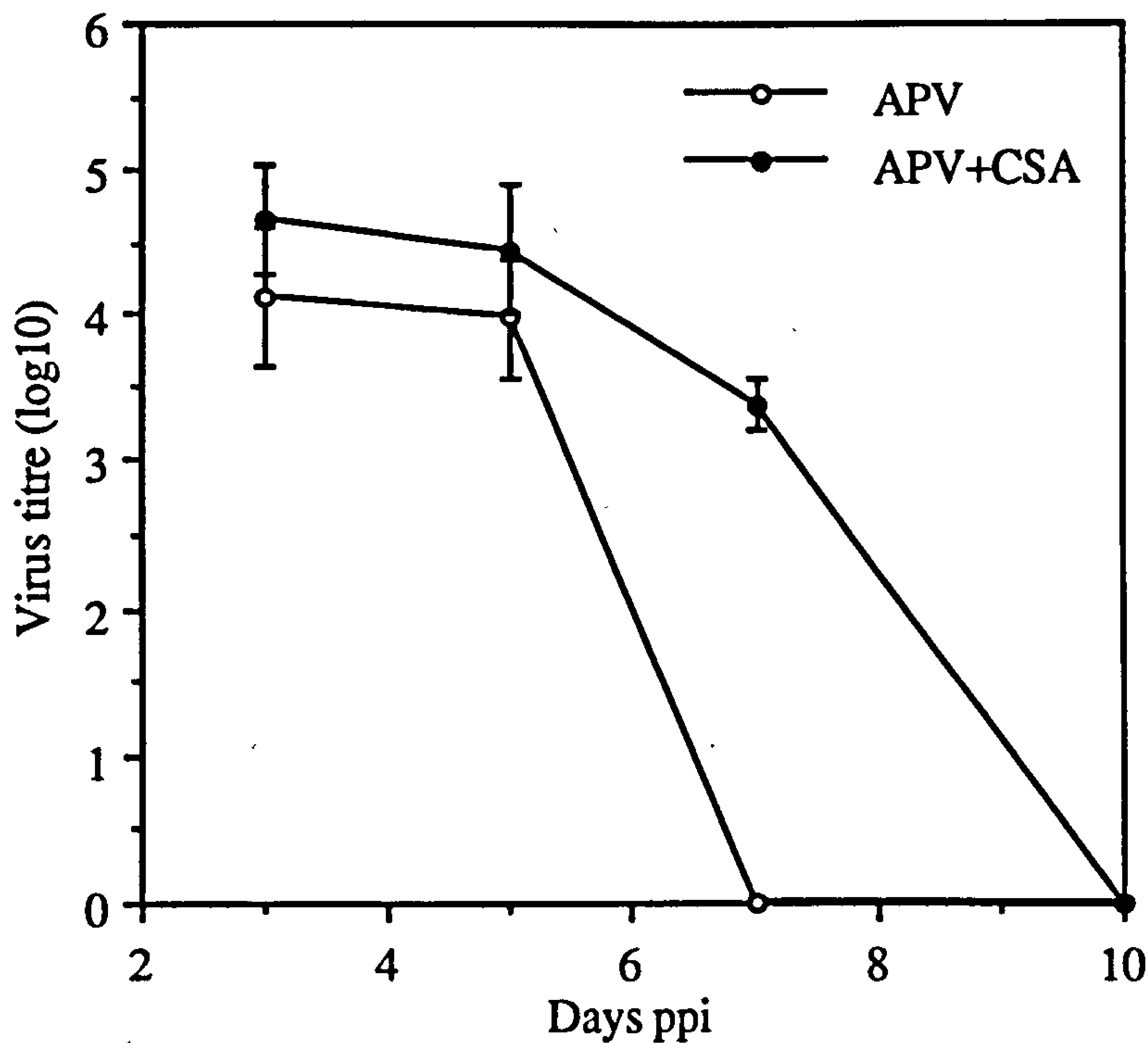

Fig. 5.6. Virus titres (mean ± sd) in turbinates of CSA-treated and untreated poults after primary APV infection. Titres expressed as CD_{50} $\log_{10}$/g tissue.

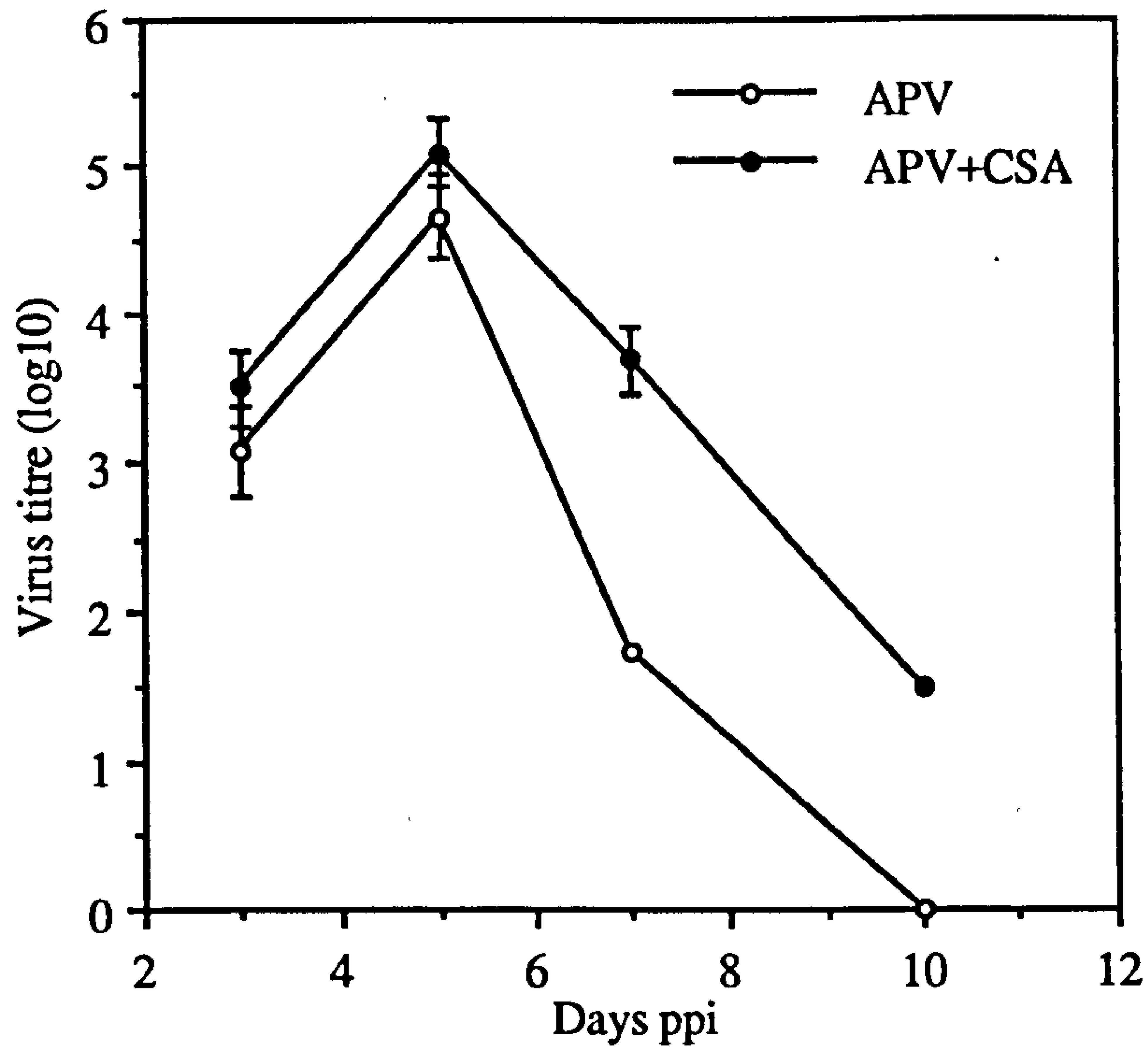

Fig. 5.7. Virus titres (mean ± sd) in tracheas of CSA-treated and untreated poults after primary APV infection. Titres expressed as CD_{50} log_{10}/g tissue.

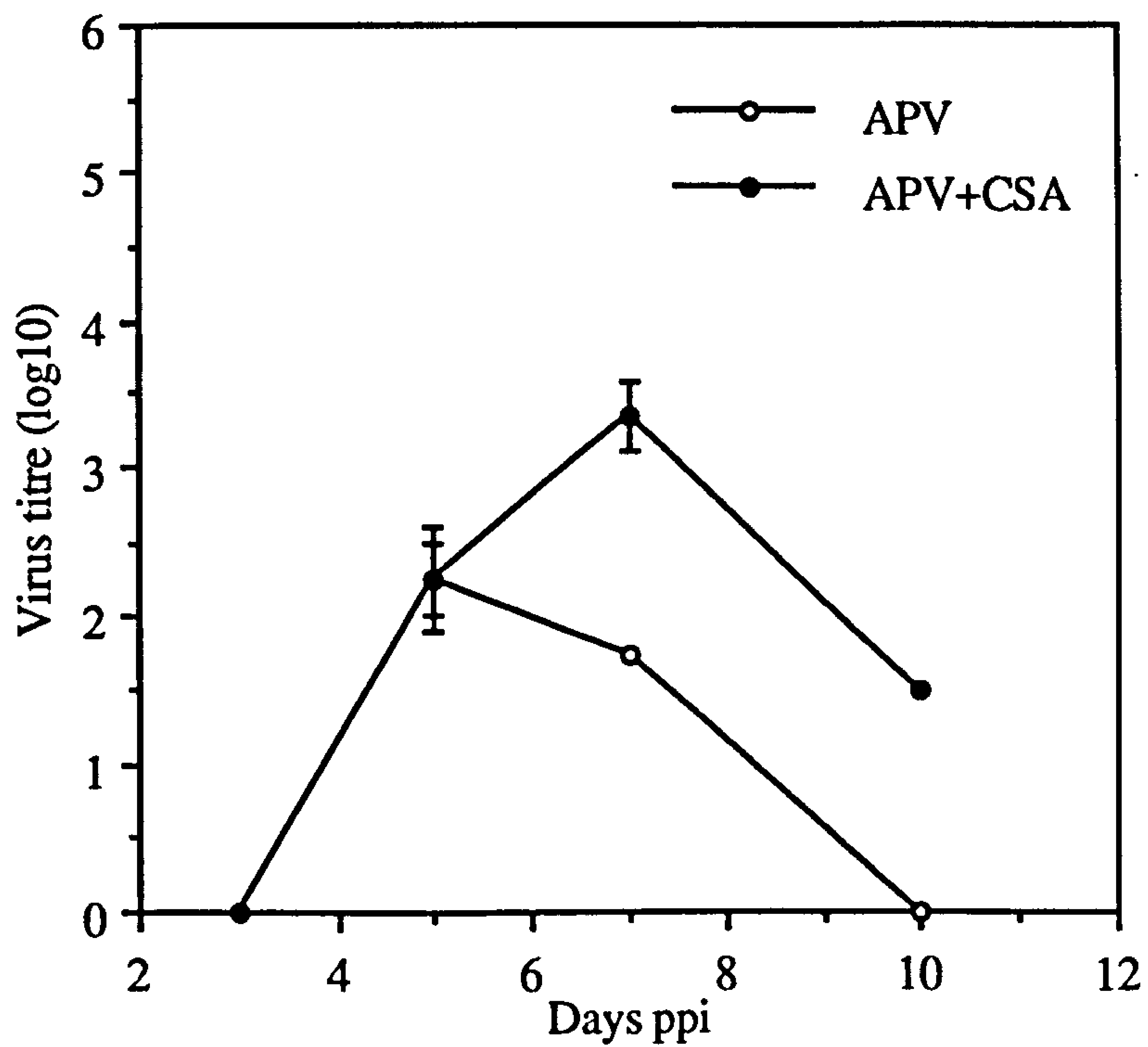

Fig. 5.8. Virus titres (mean ± sd) in lungs of CSA-treated and untreated poults after primary APV infection. Titres expressed as CD_{50} log_{10}/g tissue.

5.3.5. Immunofluorescence staining

IF staining of tracheas from chicks or poults reflected the virus isolation results. Following primary infection of chicks (Experiment 1), APV-specific antigens were detected in the tracheal epithelium on days 3 and 5 ppi in both the CSA-treated and untreated groups, without difference in total number of IF-positive tracheas (Table 5.7).

Following primary infection of poults (Experiment 2), viral antigens were detected in tracheal epithelium on days 3 and 5 ppi in the untreated group but from day 3 to 7 ppi in the CSA-treated poults (Table 5.7). The total number of IF-positive tracheas was higher in the CSA-treated poults.

Following reinfection of poults (Experiment 2), no viral antigen could be detected in either untreated or CSA re-treated group but in the unprimed infected group (2* APV), APV was detected on day 4 pi (data not shown). There was no specific IF staining in tracheas of uninfected chicks or poults.

Table 5.7. Immunofluorescence staining of trachea of CSA-treated and untreated chicks or poults following primary APV infection

Species	Group	Days ppi				
		3	5	7	10	Total
Chicks	APV	3*	3	0	0	6
	APV+CSA	3	3	0	0	6
Poults	APV	3	5	0	0	8
	APV+CSA	3	5	3	0	11

*: No. positive of 5 samples tested each time.

5.3.6. Histopathology

Experiment 1. Primary infection in chicks

Mild histopathological lesions were recorded in the tracheas of chicks infected with APV. The lesions seen in the CSA-treated group were similar to those for the untreated group. These changes included slight vascular congestion and thickening of mucosa, focal loss of cilia and occasional mononuclear cell infiltration between day 3 and 7 ppi. No tracheal damage was seen in uninfected chicks.

Experiment 2

Primary infection in poults

The severity of the tracheal lesions in poults was greater than observed in chicks. Following infection of the untreated poults, the tracheas between days 3 and 5 ppi (Fig. 5a) showed loss of cilia, epithelial exfoliation, inflammatory exudate in the lumen, mucous gland depletion, vascular congestion and mild infiltration of heterophils and mononuclear cells. On day 7 ppi, epithelial hypertrophy with mononuclear cell infiltration was seen. By day 10 ppi, there was reappearance of ciliated epithelium (Fig. 5c). In the CSA-treated group (Fig. 5b), the microscopic changes between days 3 and 7 ppi were similar to those in untreated poults. However, the inflammatory and degenerative changes persisted until day 10 ppi (Fig. 5d). The epithelial deciliation, mucous gland depletion, and heterophilic and mononuclear cell infiltration in submucosa of tracheal epithelium were still evident on day 10 ppi. There was no tracheal damage in uninfected poults (Fig. 5e).

Secondary infection in poults

Following reinfection, there was no evidence of epithelial damage either in untreated (Fig. 5f) or CSA-treated groups (Fig. 5g). However, both the groups (Figs. 5h & 5i) showed lymphoid follicles in the submucosa of epithelium. In unprimed infected poults (2* APV), the tracheal lesions were similar to those described for untreated poults after primary infection (Fig. 5j). There was no tracheal damage in uninfected poults.

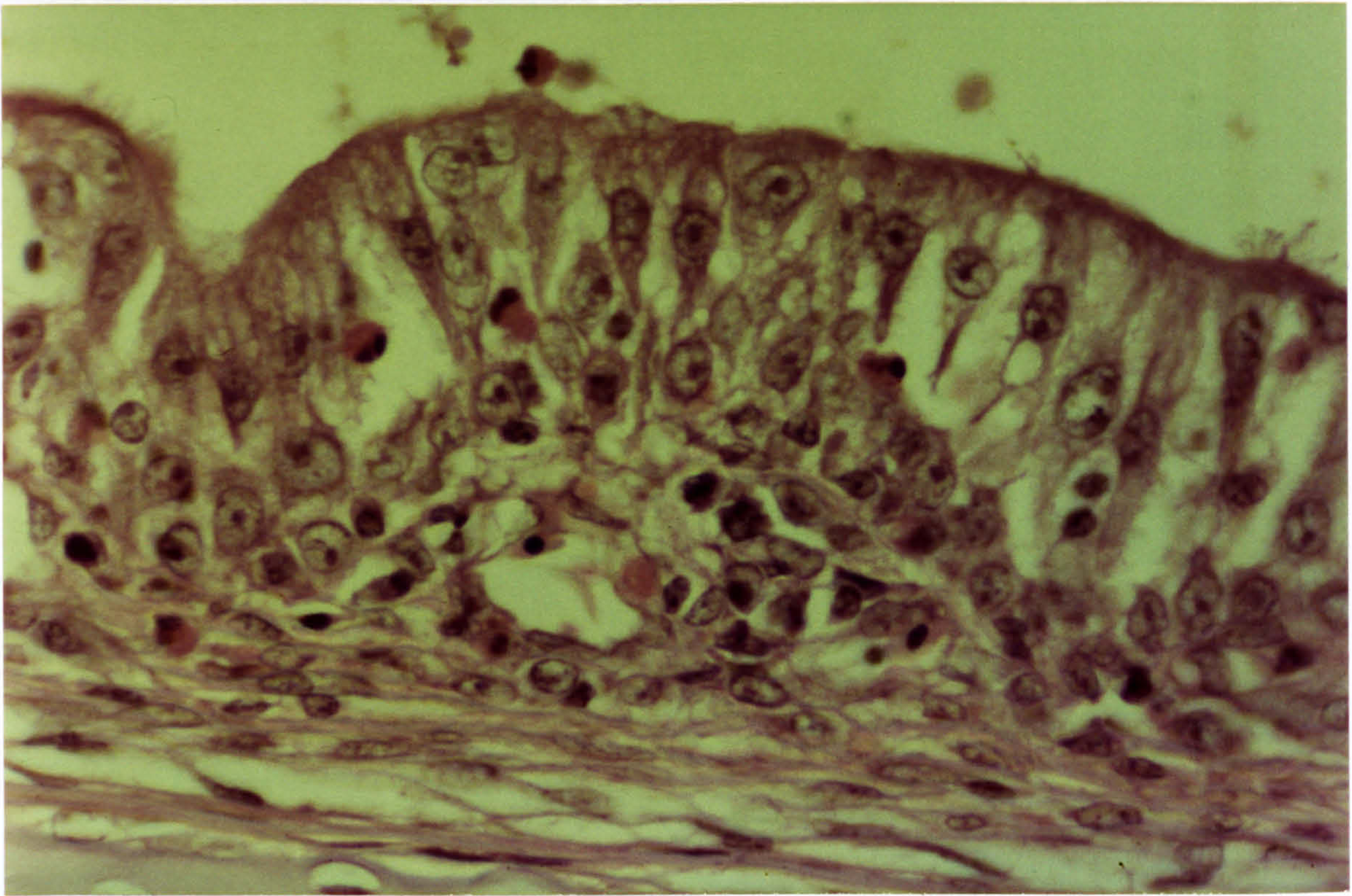

Fig. 5a. Section of trachea from an untreated APV-infected poult on day 5 ppi showing degenerative and inflammatory changes. Magnification x675.

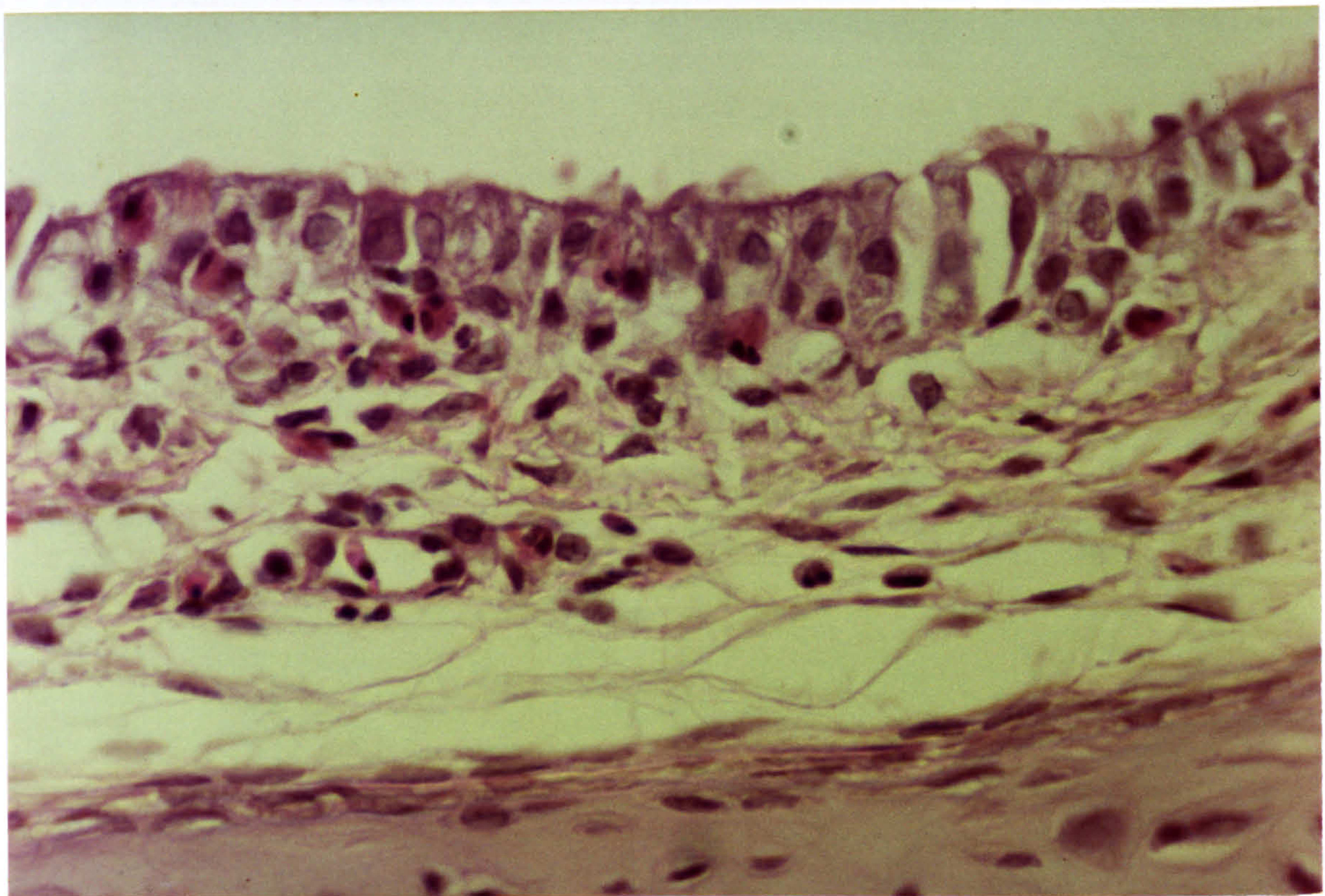

Fig. 5b. Section of trachea from a CSA-treated APV-infected poult on day 5 ppi showing degenerative and inflammatory changes. Magnification x675.

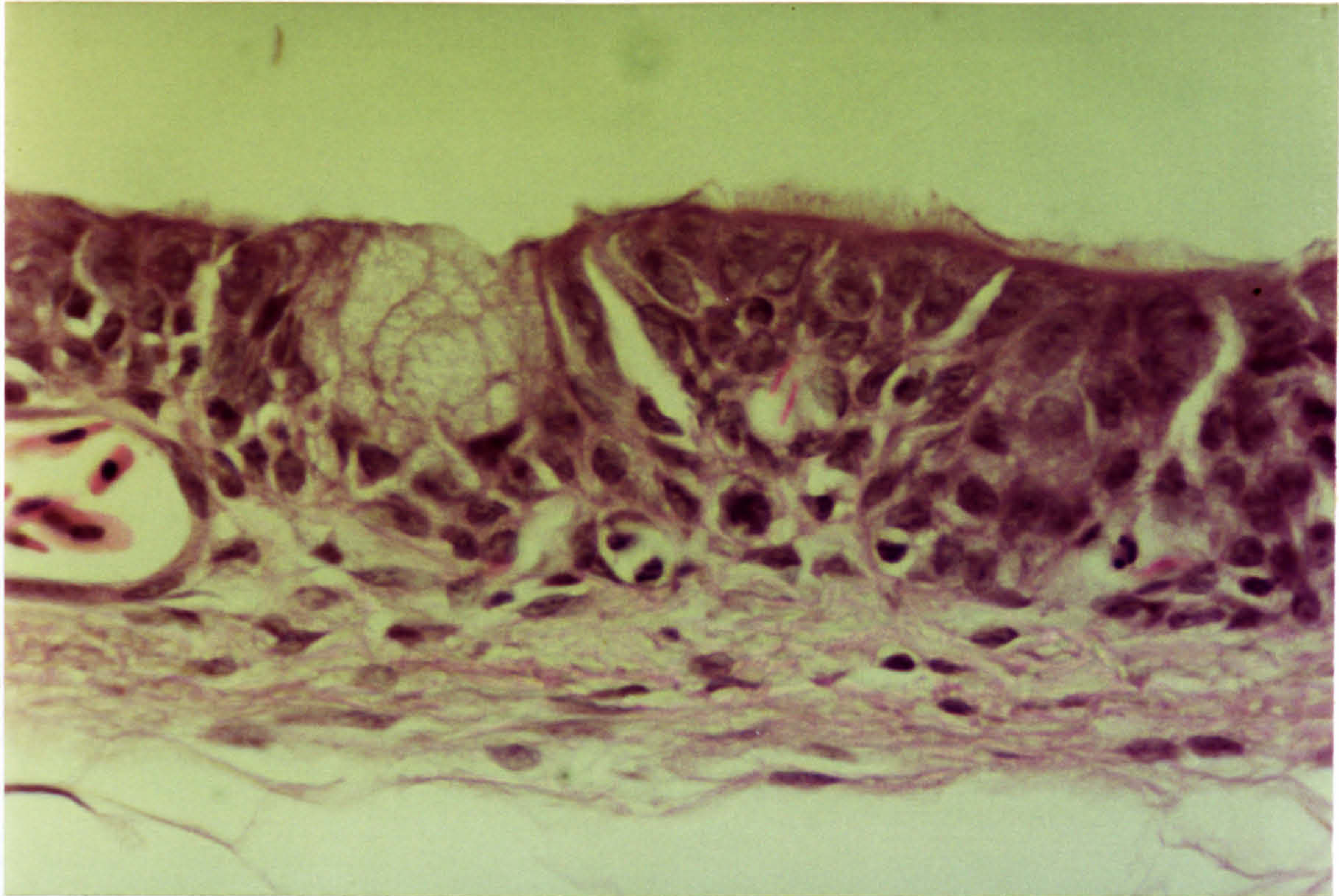

Fig. 5c. Section of trachea from an untreated APV-infected poult on day 10 ppi in the process of regeneration. Magnification x675.

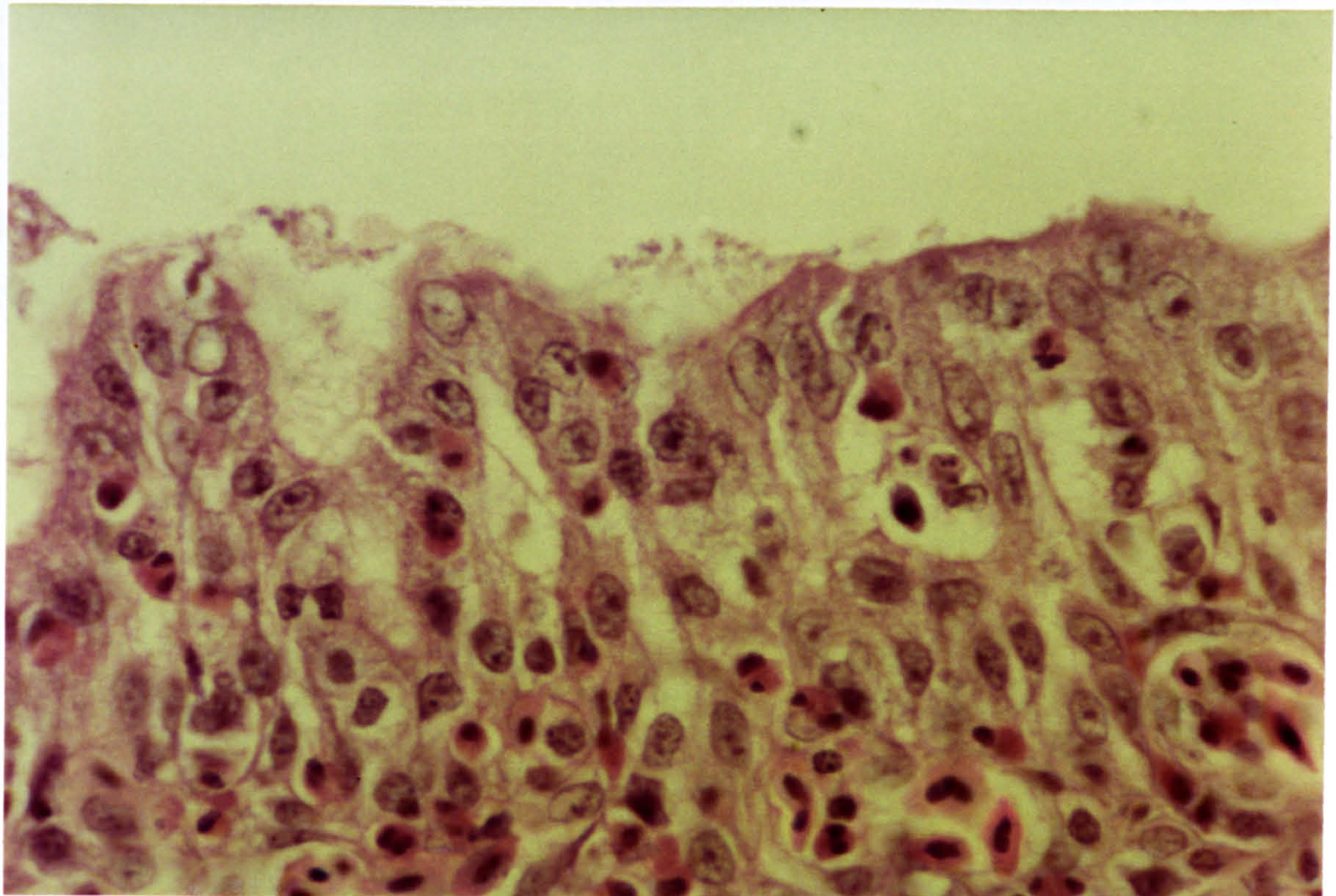

Fig. 5d. Section of trachea from a CSA-treated APV-infected poult on day 10 ppi showing persistence of degenerative and inflammatory changes. Magnification x675.

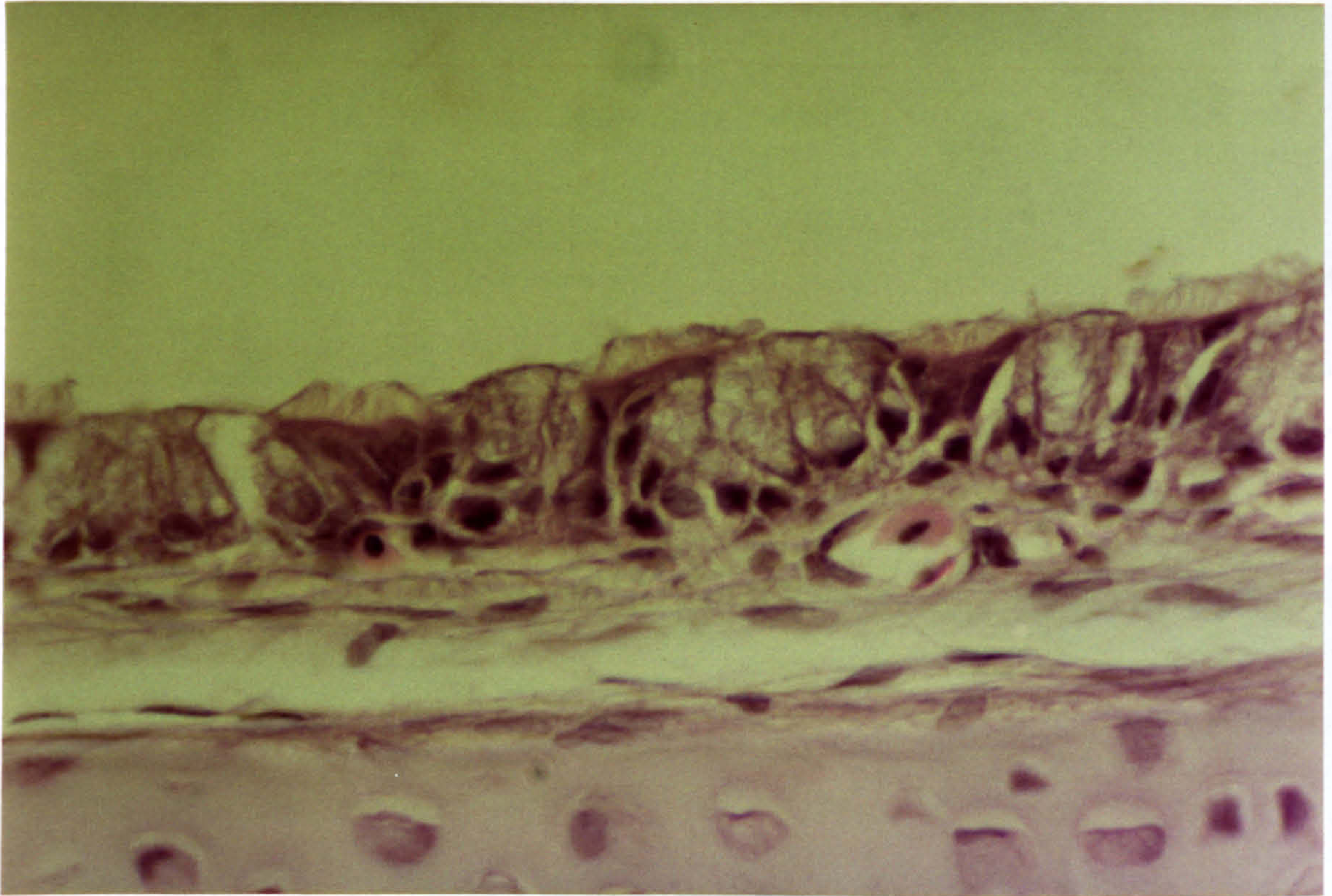

Fig. 5e. Section of normal trachea from a 20-day old poult. Magnification x675.

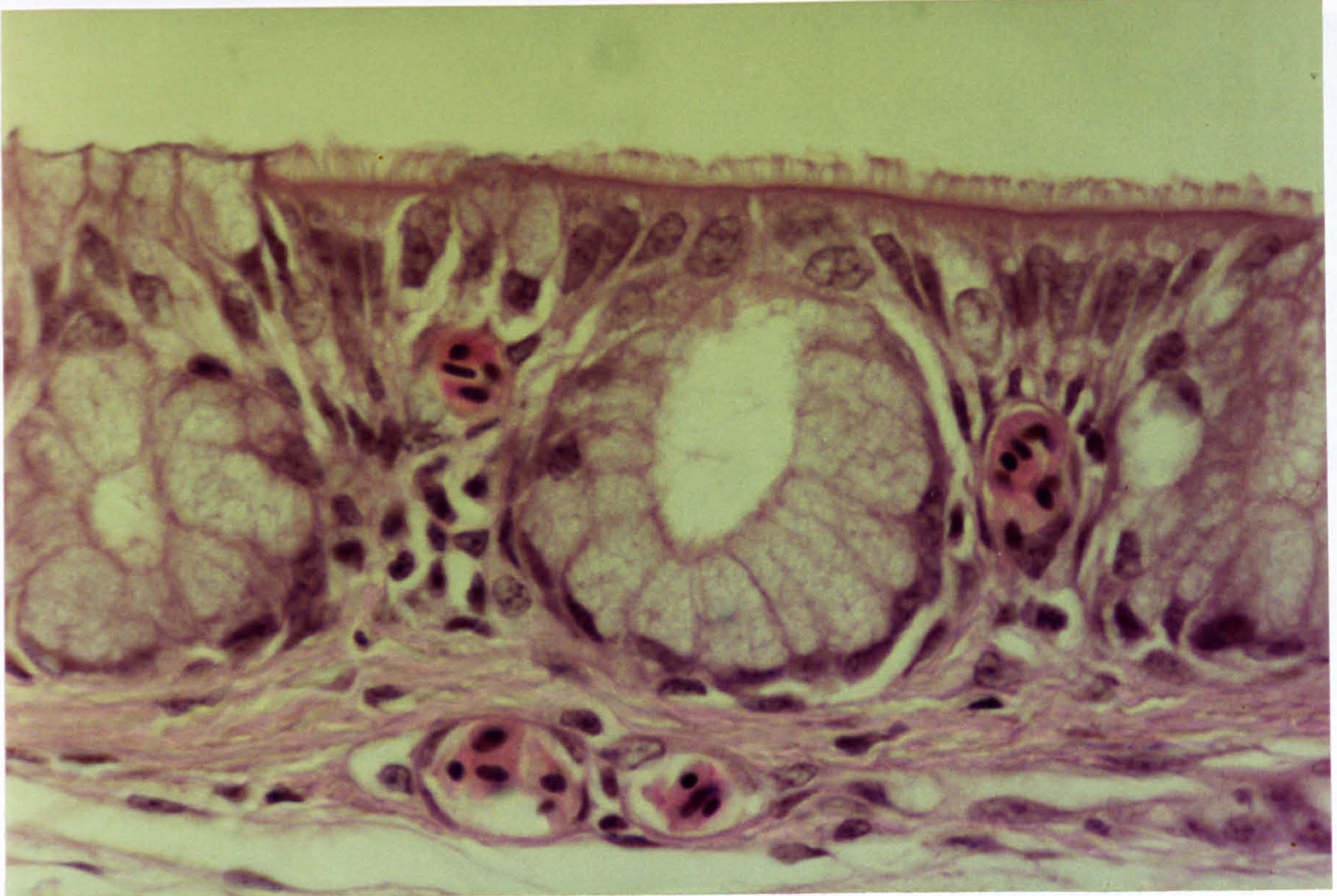

Fig. 5f. Section of trachea from an untreated APV-reinfected poult on day 4 psi showing mild lymphocytic infiltration and congestion but no epithelial damage. Magnification x675.

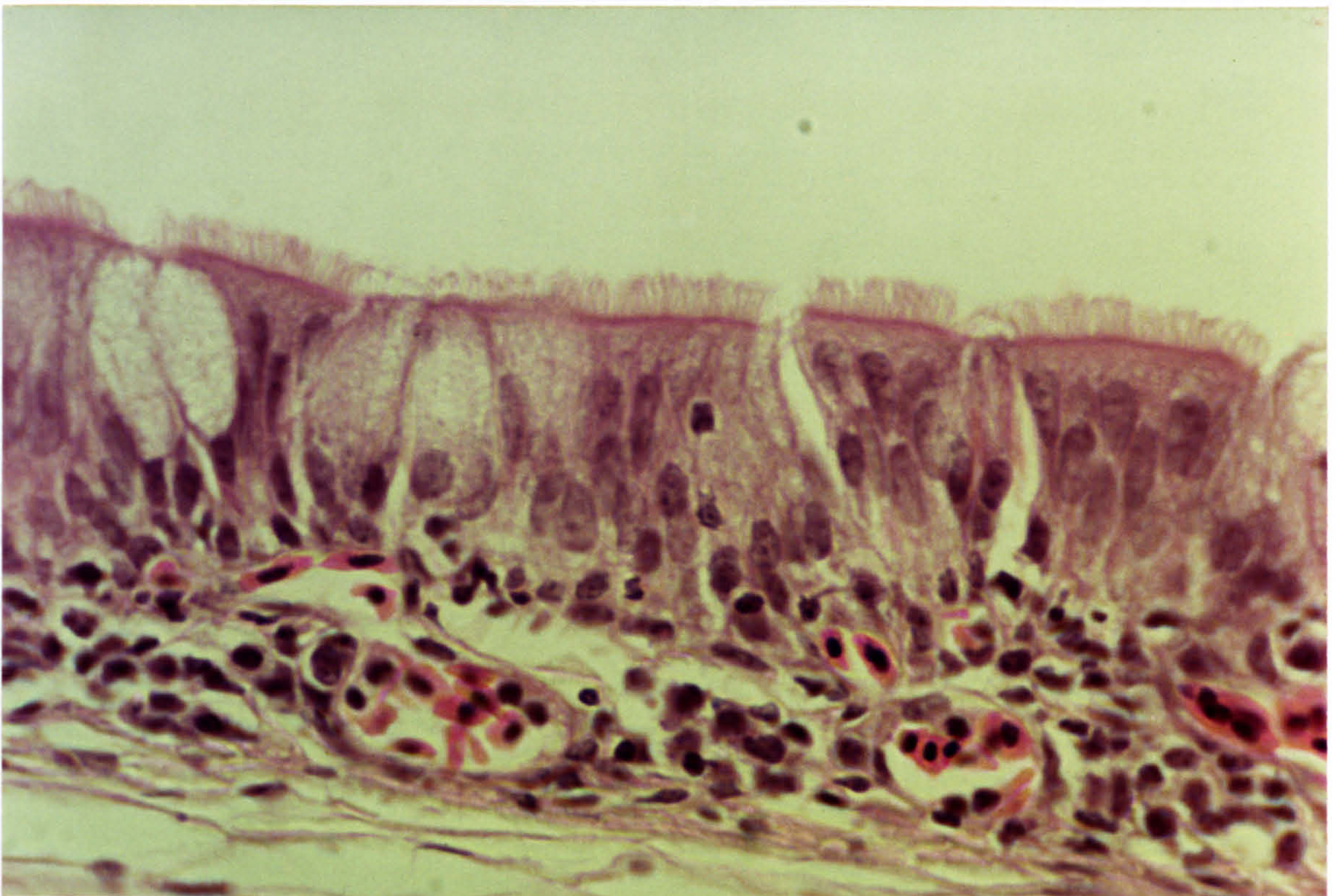

Fig. 5g. Section of trachea from an CSA-treated APV reinfected poult on day 4 psi showing marked lymphocytic infiltration and congestion but no epithelial damage. Magnification x675.

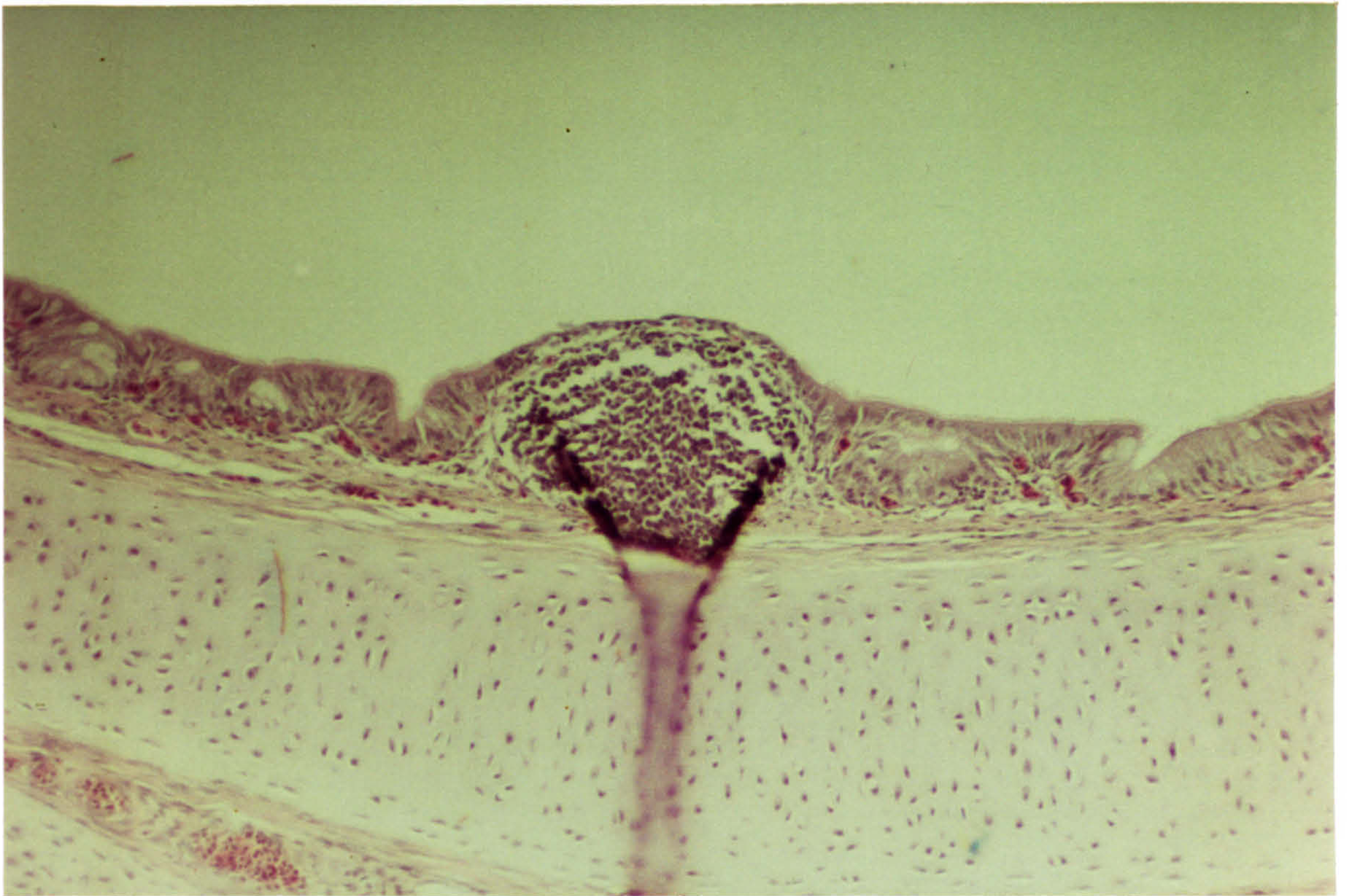

Fig. 5h. Section of trachea from an untreated APV-reinfected poult on day 7 psi showing a large lymphoid follicle and congestion but no epithelial damage. Magnification x125.

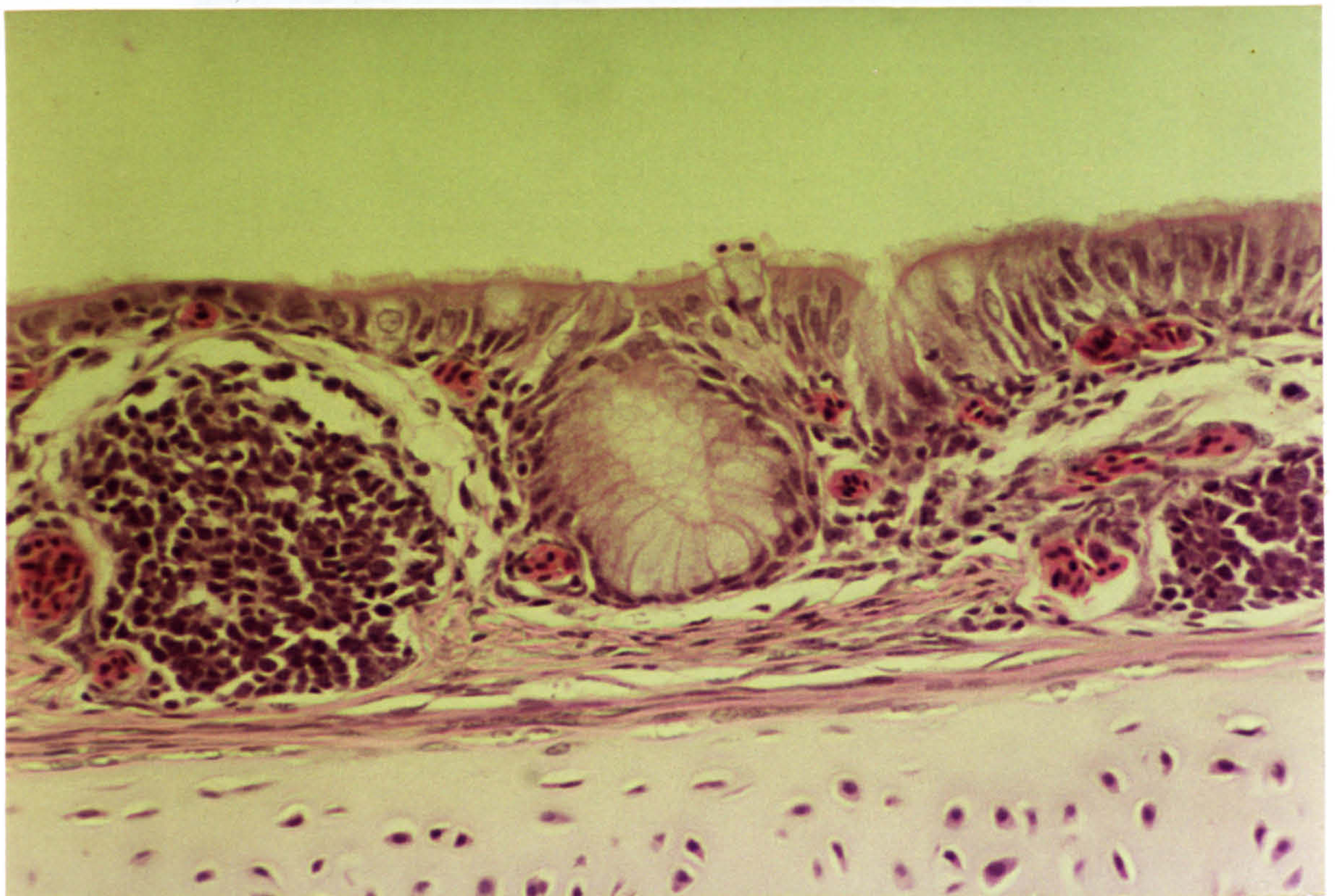

Fig. 5i. Section of trachea from an CSA-treated APV-reinfected poult on day 7 psi showing two lymphoid follicles and congestion but no epithelial damage. Magnification x312.5.

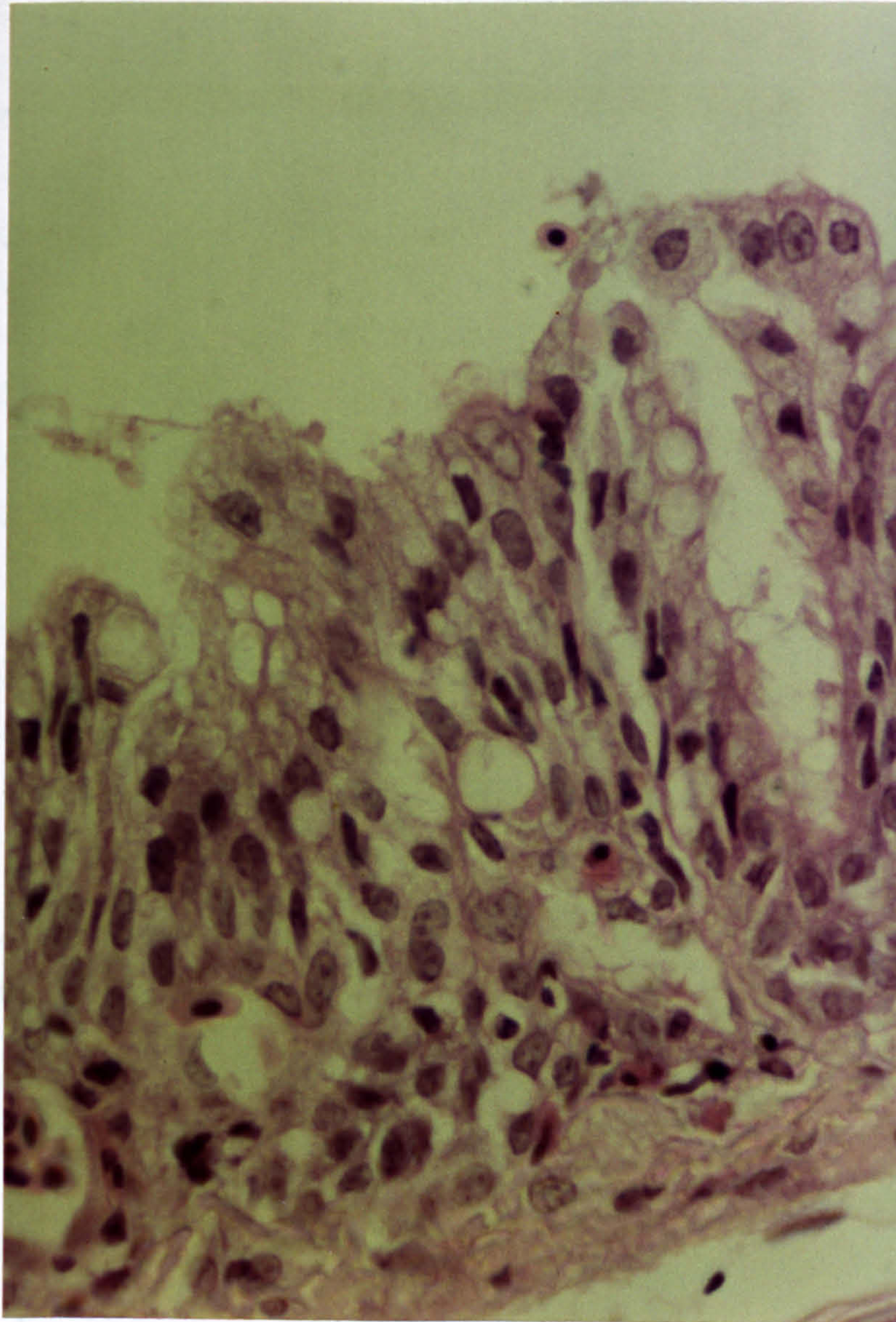

Fig. 5j. Section of trachea from an un-primed and untreated but APV-infected poult on day 4 psi showing degenerative and inflammatory changes. Magnification x675.

5.3.7. Serology

Experiment 1. Primary infection in chicks

Following APV infection of chicks, ELISA antibodies (baseline >6.1 log_2) were detected both in CSA-treated and untreated groups on days 7 and 10 ppi without any significant differences in their levels (Fig. 5.9). VN antibodies titres (Fig. 5.10) were similar in both groups on day 7 ppi, but on day 10 ppi they were about 1.0 log_2 higher in the CSA-treated group.

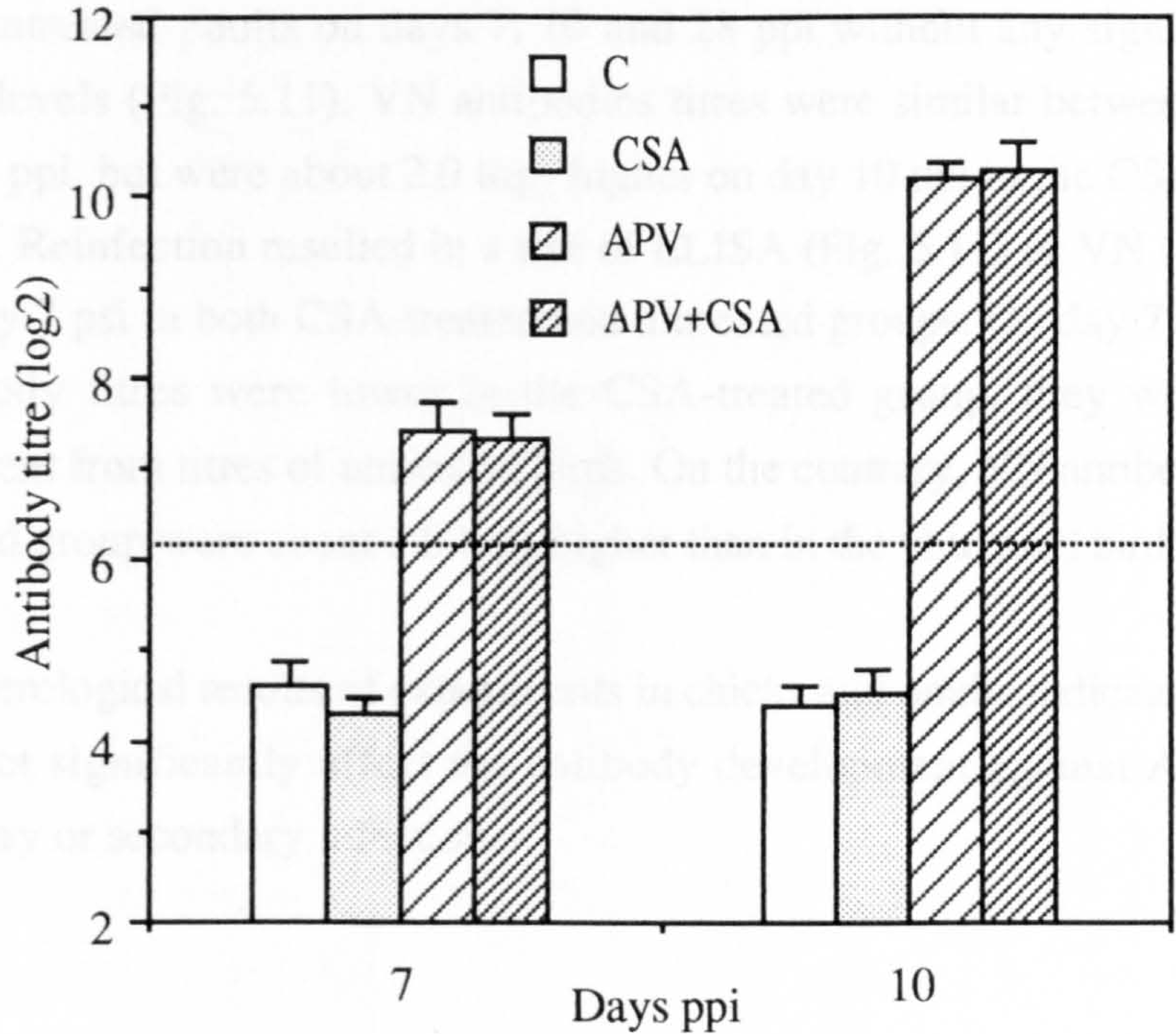

Fig. 5.9. ELISA antibodies (mean ± sd) in serum of chicks following primary APV infection. Level of significance > 6.1 log_2.

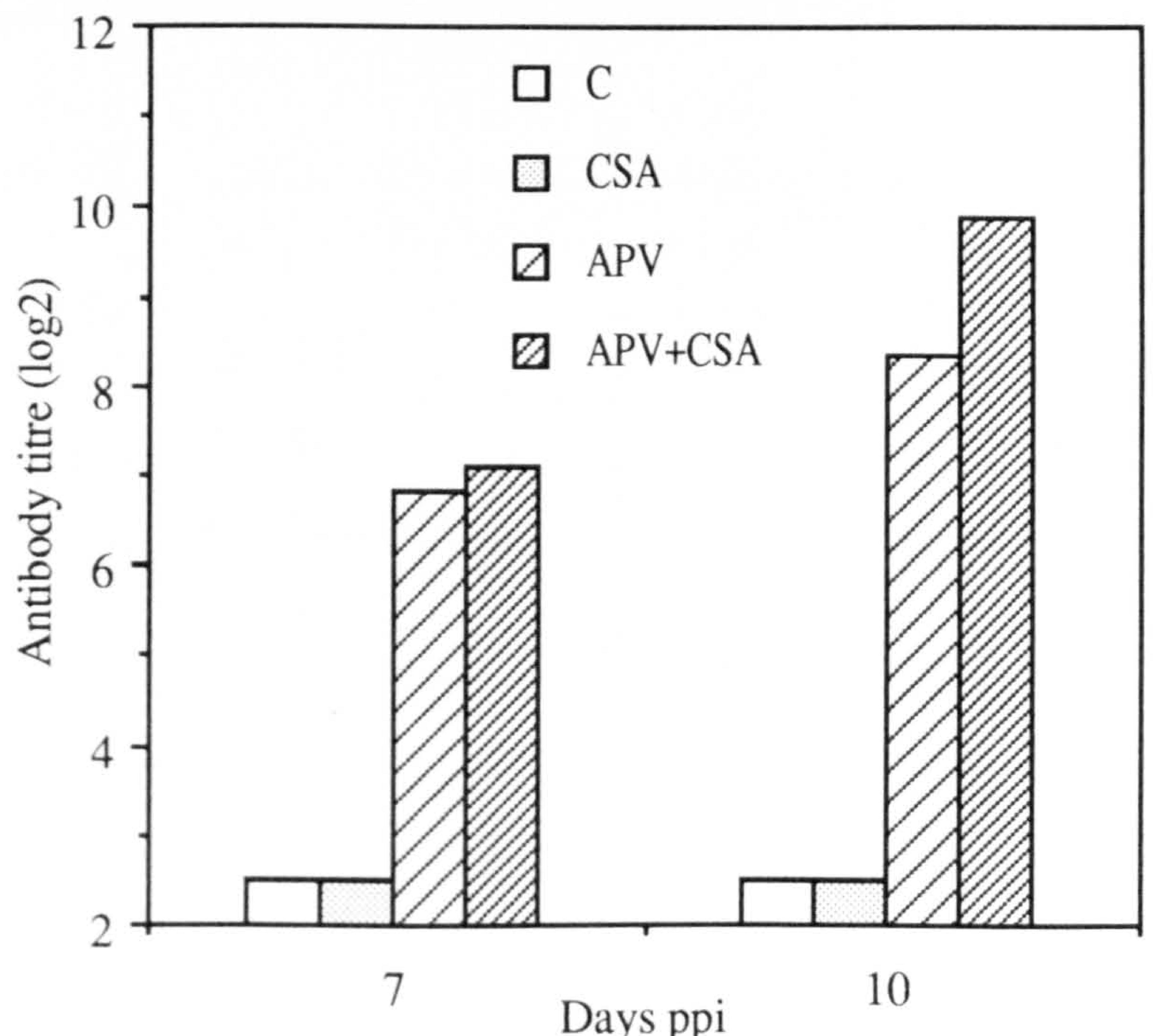

Fig. 5.10. VN antibodies in pooled sera from chicks following primary APV infection. Lowest detectable level <2.5 log_2.

Experiment 2. Primary and secondary infection in poults

Following primary infection, ELISA antibodies were demonstrated both in CSA-treated and untreated poults on days 7, 10 and 28 ppi without any significant differences in their levels (Fig. 5.11). VN antibodies titres were similar between the two groups on day 7 ppi, but were about 2.0 $\log_2$ higher on day 10 ppi in the CSA-treated group (Fig. 5.12). Reinfection resulted in a rise of ELISA (Fig. 5.11) or VN (Fig. 5.12) antibodies on day 7 psi in both CSA-treated and untreated groups. On day 7 psi, although ELISA antibody titres were lower in the CSA-treated group, they were not significantly different from titres of untreated birds. On the contrary, VN antibody titres in the CSA-treated group were about 1.0 $\log_2$ higher than in the untreated birds.

The serological results of experiments in chicks and poults indicated that CSA treatment did not significantly affect the antibody development against APV following either primary or secondary infection.

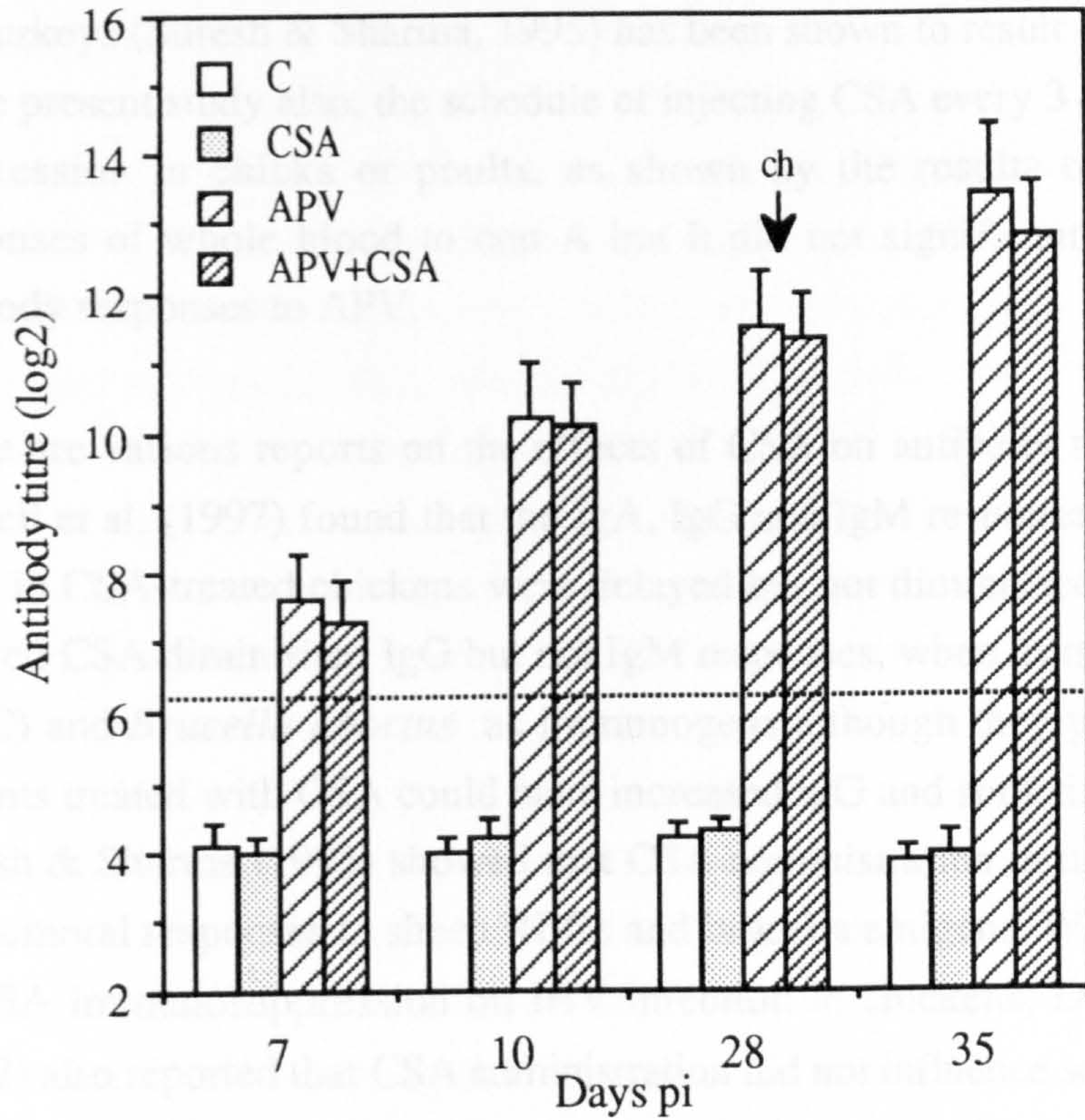

Fig. 5.11. ELISA antibodies (mean ± sd) in sera from poults following primary and secondary APV infection. Level of significance >6.1 $\log_2$.

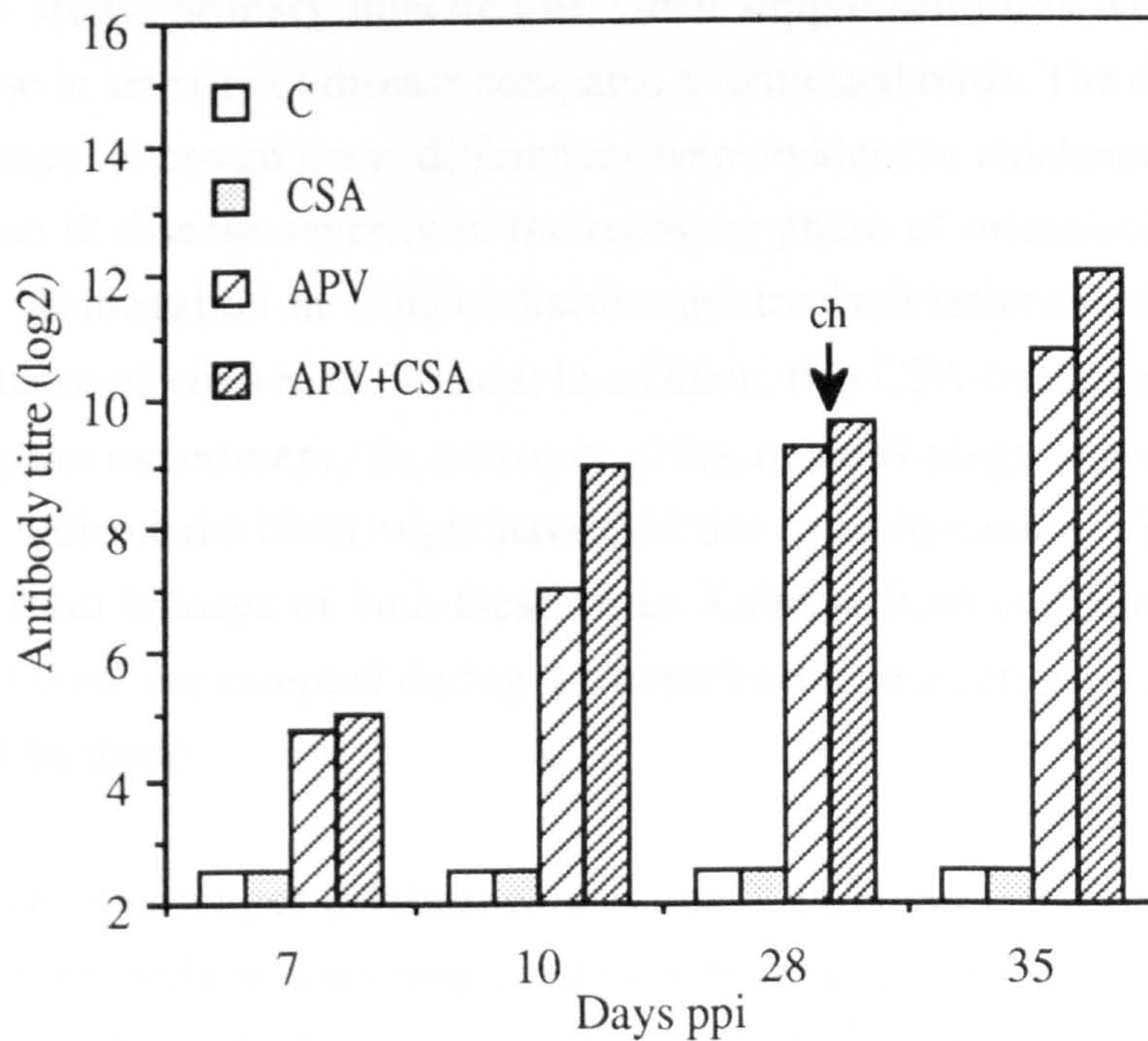

Fig. 5.12. VN antibodies in pooled sera from poults following primary and secondary APV infection. Lowest detectable level <2.5 $\log_2$.

5.4. DISCUSSION

Intramuscular administration of CSA every 3 days in chickens (Nowak et al., 1982) and turkeys (Suresh & Sharma, 1995) has been shown to result in a T-cell suppression. In the present study also, the schedule of injecting CSA every 3 days induced the T-cell suppression in chicks or poults, as shown by the results of lymphoproliferative responses of whole blood to con.A but it did not significantly diminish the serum antibody responses to APV.

There are various reports on the effects of CSA on antibody response. In one study, Russell et al. (1997) found that the IgA, IgG and IgM responses to Newcastle disease virus in CSA-treated chickens were delayed but not diminished. Nowak et al. (1982), showed CSA diminished IgG but not IgM responses, when using sheep red blood cells (RBC) and *Brucella abortus* as immunogens although they pointed out that human patients treated with CSA could have increased IgG and sometimes IgM. Furthermore, Suresh & Sharma (1995) showed that CSA administration in turkeys did not decrease the humoral responses to sheep RBCs and brucella antigens. While studying the effect of CSA immunosuppression on IBV infection in chickens, Dhinakar Raj and Jones (1997) also reported that CSA administration did not influence serum antibody response to IBV. The effects of CSA on antibody is therefore variable but in the work presented here the reduction in T-cell activity was not associated with a significant influence on antibody response to APV.

In this study, primary infection of T-cell suppressed birds with APV resulted in an increase in severity of disease compared to untreated birds. The effect was more marked in turkeys, although some differences were evident in chickens. In turkeys, there was increase in disease severity in the recovery phase of infection. In other words, there was a prolongation in clinical disease and tracheal lesions accompanied by a longer persistence of virus in the tissues. In addition, two CSA-treated and infected poults died during the experiment. At necropsy, thick mucous plugs were seen in their tracheas which indicate the birds might have died due to asphyxiation. Virus isolation were also made from kidneys of both these birds. Kidneys from untreated birds or other CSA-treated were not sampled during the experiment, so a comparison between two groups cannot be made.

However, the longer persistence of virus in turbinates, tracheas and lungs of T-cell suppressed birds was accompanied by a decrease in rate of virus clearance from these tissues as shown by higher virus titres towards recovery phase of disease. Virus titres were also significantly higher in turbinates and tracheas of T-cell suppressed chicks when the disease was clearing, although there was no prolonged virus persistence as

seen in poults. The results of IF staining of tracheas of CSA-treated poults following primary infection also depicted longer persistence of viral antigens compared to untreated poults.

The role of T-cells in the recovery of calves from infection with another pneumovirus, bovine RSV, has been recently shown (Taylor et al, 1995). A delay in clearance of bovine RSV from the nasopharynges and lungs of calves with increase in the severity of pneumonic consolidation was demonstrated following the depletion of $CD8^+$ T-cells by administration of monoclonal antibodies (Mabs). Similar effects of delayed virus clearance with increase in severity of clinical signs and tracheal lesions were also observed in the present study in CSA-treated poults following primary APV infection. Thus, the possibility of a role of CD8+ T-cells in the recovery of birds from APV infection can also be speculated.

Lillehoj (1987) successfully demonstrated the use of CSA in studying the role of CMI in protective immunity against *Eimeria tenella* infection in chickens. It was shown that oral administration of CSA in chickens for 1-week beginning 1-day before primary infection and for 2-weeks beginning 1-day before secondary infection abrogated their resistance to reinfection at 5 weeks ppi, despite the presence of high levels of coccidia-specific secretory IgA and serum IgG. Groups of chickens that were not treated with CSA during both the primary and secondary infections were resistant to challenge infection.

In the present work, intramuscular administration of CSA similarly at the time of both primary and secondary infections was not found to abrogate protective immunity to APV infection in turkeys and was accompanied by high levels of ELISA and VN antibodies to APV. Indeed, VN antibody titres in reinfected CSA-treated poults were higher than in untreated birds. Higher titres of VN antibodies in CSA-treated chicks or poults were also observed following primary APV infection. Dhinakar Raj and Jones (1997) reported a similar increase in VN antibody titres in CSA-treated chickens following primary infection with IBV.

It was suggested that higher levels of VN antibodies to IBV could have been elicited by the higher virus titres in tissues of T-cell suppressed birds. While this seems to be a likely explanation for our observation of higher VN antibodies following primary infection with APV, the higher levels of VN antibodies following reinfection might have been due to greater memory responses to neutralising epitopes of APV. Following reinfection, since CSA-treated poults were protected in the presence of high levels of ELISA or VN antibodies, it appears that antibodies might have played a role in

protective immunity to APV infection. However, it is not known if some other immune mechanism might also have been responsible in conferring protection of CSA-treated poults.

The only published report on the effects of immunosuppression on APV infection is that of Jones et al. (1992). They treated poults with cyclophosphamide (CP) (primarily a B-cell suppressant drug) for three days post-hatching and then vaccinated them with an attenuated strain of APV at 10 days of age. These poults had no circulating antibodies to APV detectable by ELISA. However, when they were challenged at 23 days post-vaccination with a virulent virus, they were all completely protected. These findings suggested the importance of CMI in protection against APV infection.

Jones et al. (1992) also reported an additional observation relating to the reaction of CP-treated but unvaccinated poults to virulent challenge. Compared to untreated and unvaccinated birds, this group experienced more severe respiratory disease and higher virus titres were recorded from tracheal swabs. In this instance, antibodies appeared to play a role in limiting the severity of primary infection. The role of CMI in recovery from primary infection with APV in both species was also evident in the present study, using CSA-induced T-cell immunosuppression. Thus, from our present state of knowledge, it seems that both B-cell and T-cell immunity are involved in limiting primary APV infection. Similarly, findings from the earlier report (Jones et al., 1992) and the work presented here indicate that for protective immunity against APV challenge, both arms of the adaptive immune response are necessary.

Both attempts at immunosuppression were done in a relatively simple way and it is worthwhile discussing their possible limitations. The immunosuppression afforded by these drugs might have been incomplete. Indeed, it is well documented that CP has a transient effect on T-cells also (Elmubrak et al., 1981). In the CSA work, the drug was given at the time of both primary and secondary infections but not between, a similar protocol used successfully by Lillehoj (1987) for studying the role of CMI in the protective immunity to an avian coccidia. It is not known whether continuous administration of CSA throughout the experiment would have produced different results. More precise immune depletion could be achieved by treatment with selective Mabs against T- and B-lymphocytes.

CHAPTER 6

A PRELIMINARY INVESTIGATION OF AVIAN PNEUMOVIRUS PERSISTENCE IN POULTS AND CHICKS USING CYCLOSPORIN A IMMUNOSUPPRESSION

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CHAPTER 6

A PRELIMINARY INVESTIGATION OF AVIAN PNEUMOVIRUS PERSISTENCE IN POULTS AND CHICKS USING CYCLOSPORIN A IMMUNOSUPPRESSION

6.1. INTRODUCTION

A knowledge of modes of transmission and maintenance of reservoir of infection in nature is essential to adopt most accurate measures for disease control (Thrusfield, 1995). In the case of APV infection, horizontal transmission through direct contact has been experimentally demonstrated (Giraud et al., 1986b; Cook et al., 1991). Due to rapid spread of APV infection, airborne transmission is thought to be important but it has not been established. Under experimental conditions, Cook et al. (1991) reported that the virus did not transfer from infected to uninfected poults placed in the same room even though the air flow was favourable.

The maintenance of a reservoir of APV infection in nature is not completely understood. Some of the important strategies for maintenance of viral agents include extension of host range, a rapidly-in, rapidly-out strategy or persistence within the host (Thrusfield, 1995). In addition to the common hosts, chickens and turkeys, APV can also infect pheasants and guinea fowl (Picault et al., 1987; Gough et al., 1988) and perhaps ostriches (Cadman et al., 1994) where only seroconversion to APV has been reported. It is not known if these birds are capable of maintaining and transmitting APV infection to chickens and turkeys.

Many viruses of the upper respiratory tract are capable of adopting a rapidly-in, rapidly out strategy by entering the host, replicating and leaving very quickly (Thrusfield, 1995). The strategy requires a continuous supply of susceptible hosts. It is likely that APV being an upper respiratory tract pathogen of chickens and turkeys could adopt this strategy for maintenance of infection.

The role of persistently infected birds in APV transmission has not been investigated though in our present state of knowledge, it seems to be less likely as infected birds shed APV for relatively short periods of time, usually from 3 to 9 or 10 days after infection (Cook et al., 1991; Cook et al., 1993a). However, in an experimental study, following infection of laying turkeys, APV-specific antibodies were found to persist for a long period of at least 89 days (Jones et al. 1988). Hegele et al. (1994) demonstrated persistence of another pneumovirus, human respiratory syncytial virus (RSV) following acute bronchiolitis in guinea pigs. It was shown that RSV protein and

genomic RNA can persist in the lungs of experimentally inoculated guinea pigs for at least 60 days after infection. In this chapter, an attempt was made to investigate the possibility of APV persistence in chickens and turkeys.

The persistence of viruses within the host in other respiratory viral infections of poultry such as infectious bronchitis (IB) (Cook, 1968; Alexander et al., 1978; Bhattacharjee et al., 1995) or infectious laryngotracheitis (ILT) (Hughes et al., 1987 & 1989) has been studied by either long term experimental studies or by inducing viral re-excretion on subjection to stress factors like onset of lay, re-housing and administration of immunosuppressive agents including cyclosporin A (CSA). CSA is known to cause a T-cell suppression in chickens (Nowak et al., 1982) and turkeys (Suresh & Sharma, 1995). In the previous chapter, use of CSA showed that T-cells play a significant role in clearance of APV following primary infection. CSA-based T-cell suppression was therefore selected as one way of exploring APV persistence.

The objective of present study was to investigate the possibility of long term persistence of APV in the two important host species, turkeys and chickens. CSA immunosuppression was used in an attempt to induce re-excretion of APV.

6.2. MATERIALS AND METHODS

6.2.1. Experimental birds

Turkey eggs free of maternal antibodies to APV and SPF chicken eggs were obtained from separate commercial sources and were hatched in our laboratory. The birds were maintained in complete isolation with food and water *ad libitum.*

6.2.2. Virus

APV strain #8544 (Wilding et al., 1986) was used. Each poult or chick was inoculated oculonasally with 3.5 $\log_{10}$ CD_{50}/0.1ml of APV at day-one of age in the case of poults and at 2-weeks of age in the case of chicks, in two separate experimental trials.

6.2.3. Cyclosporin A administration

CSA (Sandimmun) was administered intramuscularly at a dose rate of 100 mg/kg bodyweight every three days (Nowak et al., 1982) for 12 days starting from 3 weeks post infection (pi) in poults and from 4 weeks pi in chicks.

6.2.4. Experimental designs

Two experiments were performed, one each in poults (Experiment 1) and chicks (Experiment 2).

Experiment 1. One day-old poults

Eighty-two poults were divided into 2 groups comprising of one infected and other uninfected group. APV infection was given at one-day of age and following infection, 10 birds were examined for clinical signs. On days 3, 6, 9, 13, 17, 21, 24, 27, 30 and 33 pi, three birds from each group were euthanased and samples of harderian gland (HG), turbinates, trachea, lungs, kidneys and cloacal swabs were collected for virus isolation.

At 3 weeks pi, birds from the infected group were further divided into two groups and housed separately. One infected group and the uninfected was sampled at regular intervals as mentioned above. The poults in the other infected group were treated with CSA every three days for 12 days, in an attempt to induce re-excretion of virus. On days 5 and 11 post-CSA treatment, heparinised blood was collected from 6 birds in each group for mitogenic assay to assess T-cell suppression. Tracheal and cloacal swabs were collected from 5 birds in the CSA-treated group each time on days 1, 3, 5, 7, 9 and 11 post-CSA treatment for virus isolation. Birds in the CSA-treated group were euthanased and pieces of HG, turbinates, trachea, lungs and kidneys were collected from three birds each time on days 3, 6, 9 and 12 post-CSA treatment. Serum samples from five birds in each group were also collected at weekly intervals following infection for assessment of antibody responses by ELISA.

Experiment 2. Two week-old chicks

One hundred and two chicks were divided into two groups, one uninfected and a second infected at two-weeks of age. Following infection, 10 birds were examined for signs and three birds from each group were euthanased each time on days 3, 5, 7, 10, 14, 18, 21, 25, 28, 31, 34, 37 and 40 pi. Samples of HG, turbinates, trachea, lungs, kidneys and cloacal swabs were collected for virus isolations.

At 4 weeks pi, chicks in the infected group were further divided into two groups and one was subjected to CSA treatment. Thereafter, chicks in all the groups were sampled for mitogenic assay, virus isolations and serology using the same protocol as described for poults in Experiment 1.

6.2.5. Mitogenic assays

The assessment of mitogenic responses in whole blood from either poults or chicks to a T-cell mitogen con A was done. Mitogenic assay for the turkey blood was performed following the method of Sharma and Belzer (1992) and that for the chicken blood was performed using the procedure of Talebi et al. (1995), and the results were expressed in stimulation index (SI) values. Both the methods have been described in detail in the previous chapter.

6.2.6. Clinical signs

The severity of clinical signs was scored on 0 to 3 scale following the method of Jones et al. (1992) as described in Chapter 3.

6.2.7. Virus isolation

Aseptically collected tissues and swabs from birds were processed and used for virus isolations in tracheal organ cultures (TOC) following the method described in Chapter 3. A minimum of three passages was given and the presence of virus assessed by ciliostasis. Ciliostatic virus was identified by immunofluorescence (IF) staining of unfixed TOC using hyperimmune serum against APV strain #8544 (Chapter 3).

6.2.8. Serology

Sera were titrated for specific antibodies to APV using an ELISA (Naylor et al., 1992) as described in detail in Chapter 3.

6.2.9. Statistical analysis

The SI values for mitogenic assays and antibody titres for ELISA were compared by student's t-test ($p<0.05$).

6.3. RESULTS

6.3.1. Mitogenic assays

CSA treatment of both poults (Experiment 1) and chicks (Experiment 2) induced a T-cell suppression as indicated by the results of mitogenic assays performed on days 5 and 11 post-CSA treatment (Table 6.1). The SI values of con A-induced mitogenic responses of whole blood of either CSA-treated poults or chicks were significantly lower compared to untreated groups.

Table 6.1. Mitogenic responses of whole blood of poults and chicks following CSA treatment

Days post CSA treatment	Group	Stimulation index (mean ± sd)	
		Poults	Chicks
5	Control	15.7 ± 1.5^a	28.7 ± 3.7^a
	APV	16.2 ± 1.9^a	30.3 ± 2.1^a
	APV+CSA	1.1 ± 0.3^b	1.0 ± 0.1^b
11	Control	19.3 ± 1.7^a	32.7 ± 3.3^a
	APV	18.1 ± 2.1^a	30.6 ± 2.9^a
	APV+CSA	1.3 ± 0.1^b	1.1 ± 0.2^b

Values with different superscripts differ significantly between groups ($p<0.05$).

6.3.2. Clinical signs

Experiment 1. Poults infected at one day of age

In infected poults, clinical signs were observed from days 2 to 13 pi and the severity of clinical signs scored as mean clinical score is shown in Fig. 6.1. The signs included presence of clear to turbid nasal exudate on gentle beak squeezing, frothy eye discharge, swollen infra-orbital sinuses and difficulty in breathing.

Experiment 2. Chicks infected at 2-weeks of age

Following infection, chicks exhibited clinical signs from days 3 to 9 pi (Fig. 6.2). The signs in chicks were milder as compared to turkeys, only presence of clear to turbid nasal exudate on beak squeezing was observed.

No clinical signs were recorded either in the infected group of poults and chicks that were given CSA treatment or in the uninfected controls (data not shown).

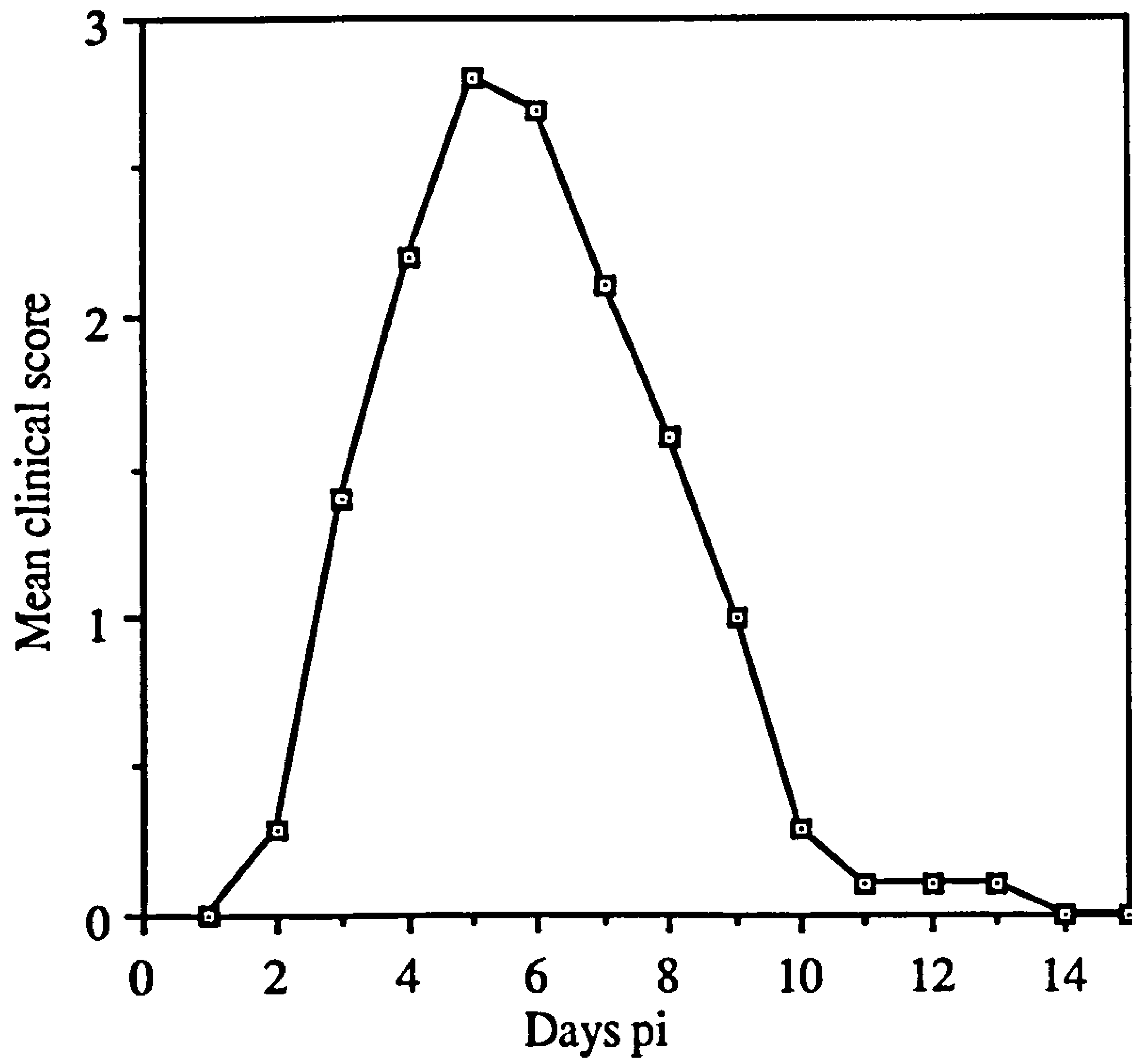

Fig. 6.1. Mean clinical scores of poults following APV infection at day old.

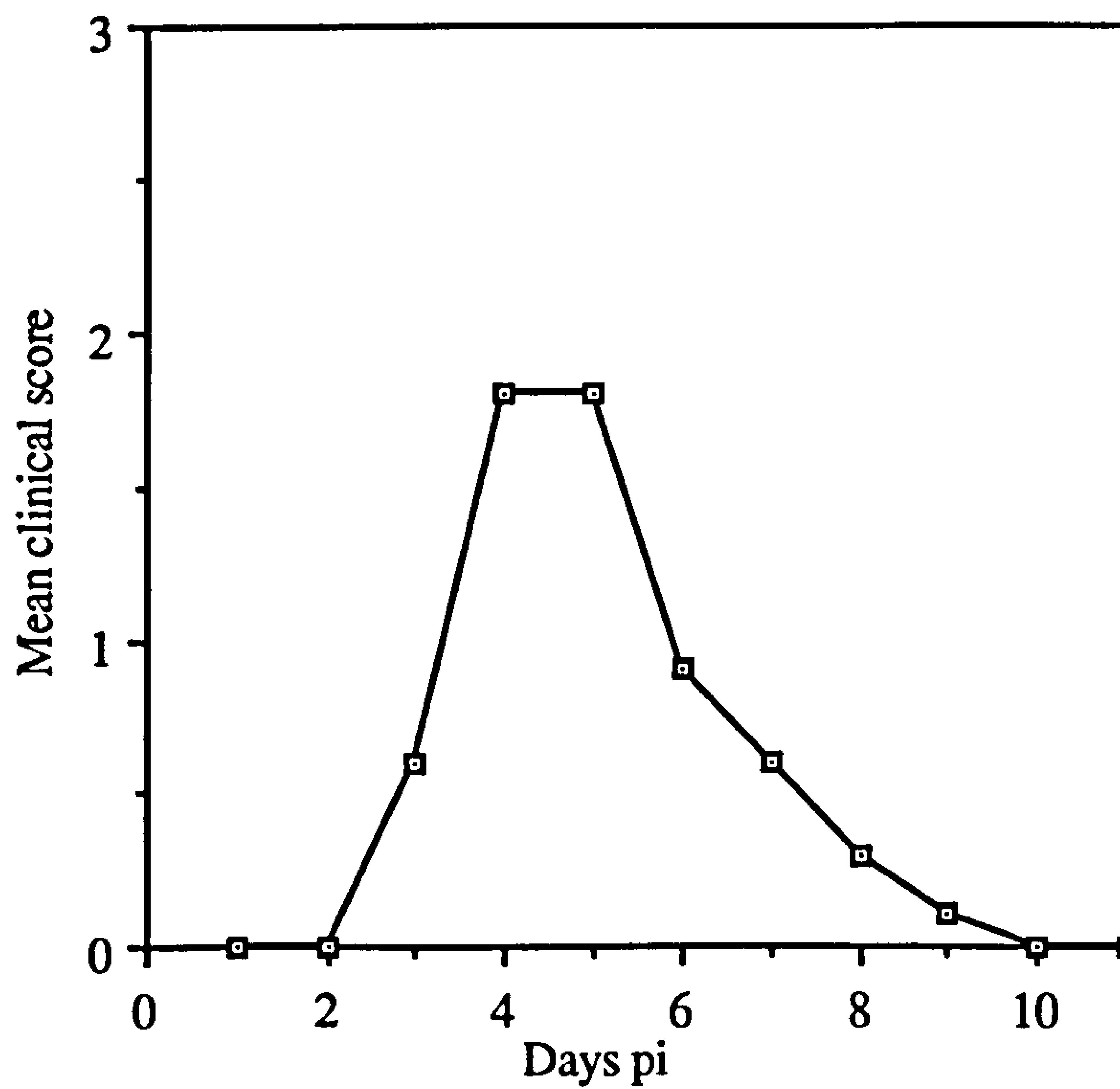

Fig. 6.2. Mean clinical scores of chicks following APV infection at 2 week of age.

6.3.3. Virus isolations

Table 6.2 depicts the results of virus isolations from both poults (Experiment 1) and chicks (Experiment 2). Following infection of poults, APV was isolated from HG and turbinates on days 3 and 6 pi, from tracheas on days 3, 6 and 9 pi, and from lungs and kidneys on days 6 and 9 pi but not from cloacal swabs. After day 9 pi, no virus isolations were made from any of the samples up to day 33 pi i.e. the end of the experiment. APV could not be isolated on any sampling occasion from either the infected group of poults which received CSA treatment from day 21 pi or from the uninfected group (not shown).

In infected chicks, APV isolations were made from HG, turbinates and trachea only on days 3 and 5 pi, but not thereafter up to day 40 pi i.e. the end of the experiment (Table 6.2). No virus isolation was made from either infected group given CSA treatment from day 28 pi or from the uninfected controls (not shown).

Table 6.2. Virus isolations from poults or chicks following APV infection

Sample	Days post-infection							
	Poults infected 1 day-old				Chicks infected 2 weeks-old			
	3	6	9	13-33*	3	5	7	10-40*
HG	3[a]	3	0	0	2	3	0	0
Turbinates	3	3	0	0	2	3	0	0
Tracheas	2	3	1	0	2	3	0	0
Lungs	0	3	1	0	0	0	0	0
Kidneys	0	2	2	0	0	0	0	0
Cloacal swabs	0	0	0	0	0	0	0	0

a: No. positive of 3 samples tested each time. *: Sampled at 3 or 4 day interval.

6.3.4 Serology

Experiment 1. Poults infected at one day old

APV-specific antibodies following infection of poults rose to a significant level (above 6.1 $\log_2$) on day 14 pi, reached peak titres on day 28 pi, and were maintained at least up to day 33 pi, i.e. the end of the experiment (Fig. 6.3). CSA treatment of infected poults from day 21 pi had no significant effect on antibody titres on days 28 and 33 pi compared to untreated but infected poults.

Experiment 2. Chicks infected at 2-weeks of age

In infected chicks, antibodies rose to the significant level as early as day 7 pi, peaked on day 28 pi, were still high on day 40 pi (Fig. 6.4). As in poults, CSA treatment of infected chicks from day 28 pi had no significant effect on antibody levels compared to untreated but infected chicks.

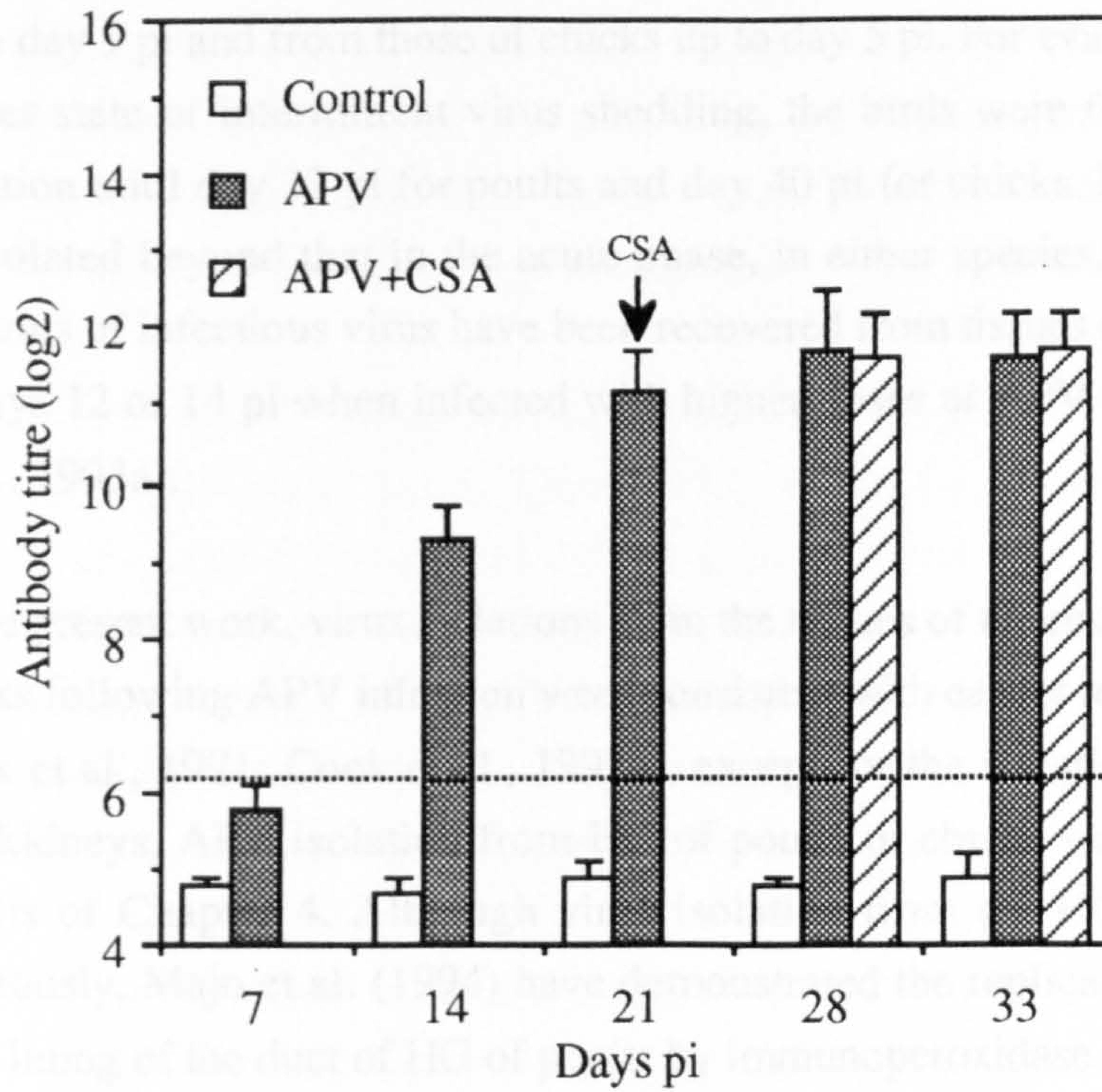

Fig. 6.3. ELISA antibodies (mean ± sd) in serum of poults following infection at one day-old. Level of significance >6.1 log_2.

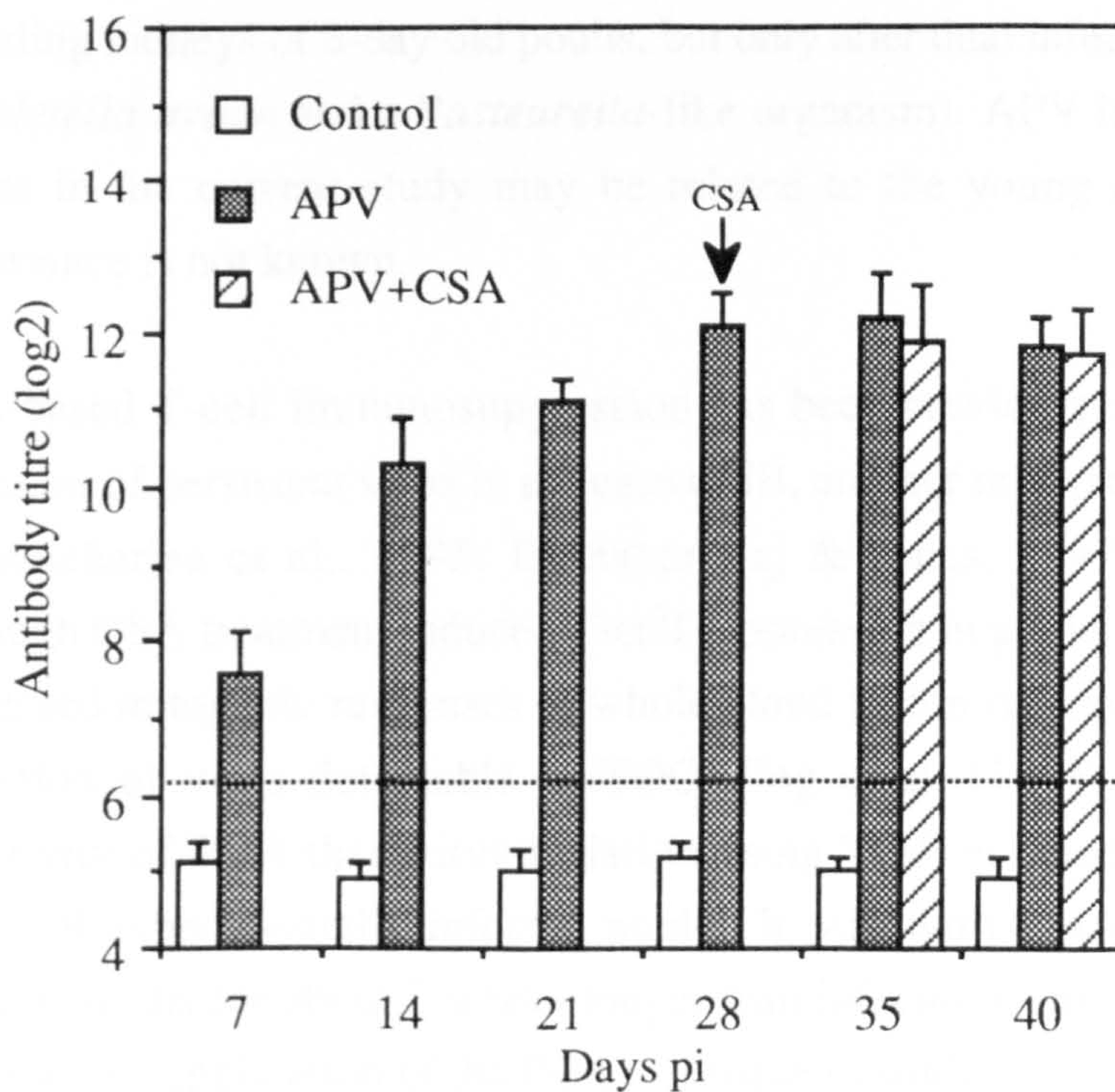

Fig. 6.4. ELISA antibodies (mean ± sd) in serum of chicks following infection at 2 weeks of age. Level of significance >6.1 log_2.

6.4. DISCUSSION

During the acute phase of infection, APV was recovered from various tissues of poults up to day 9 pi and from those of chicks up to day 5 pi. For evaluation of a possibility of carrier state or intermittent virus shedding, the birds were further sampled for virus isolation until day 33 pi for poults and day 40 pi for chicks. However virus could not be isolated beyond that in the acute phase, in either species. In earlier studies, small amounts of infectious virus have been recovered from tissues of turkeys or chickens up to days 12 or 14 pi when infected with higher doses of APV (Cook et al., 1991; Cook et al., 1993a).

In the present work, virus isolations from the tissues of the respiratory tract of poults or chicks following APV infection were consistent with earlier reports (Jones et al., 1988; Cook et al., 1991; Cook et al., 1993a), except for the isolation of APV from the HG and kidneys. APV isolation from HG of poults or chicks was in agreement with the results of Chapter 4. Although virus isolation from the HG has not been reported previously, Majo et al. (1994) have demonstrated the replication of APV in epithelial cells lining of the duct of HG of poults by immunoperoxidase staining.

The isolation of APV from the kidneys of 1-day old poults on days 6 and 9 pi was unexpected, as APV infection is normally regarded as disease of the upper respiratory tract. Cook et al. (1991) were able to isolate APV from various internal organs including kidneys of 8-day old poults, but only after dual infection of APV and bacteria (*Bordetella avium* and a *Pasteurella*-like organism). APV isolation from kidneys of poults in the current study may be related to the young age at infection, but its importance is not known.

CSA-based T-cell immunosuppression has been previously used to demonstrate re-excretion of persistent virus in the case of IB, another respiratory infection of chickens (Bhattacharjee et al., 1995; Dhinakar Raj & Jones, 1997). In the present study, although CSA treatment induced T-cell suppression in poults or chicks as indicated by depressed mitogenic responses of whole blood to con A, there was no evidence of re-excretion of virus detectable in TOC. Jing et al. (1993) demonstrated a greater sensitivity of PCR than virus isolation using TOC in detecting APV from tracheal swabs of experimentally infected poults. It was shown that PCR could detect viral RNA in swabs for about 2-weeks longer than infectious virus detectable by TOC. It is possible that application of the PCR technique in studying APV persistence could have reflected relatively a clearer picture.

In a previous study, Bhattacharjee et al. (1995) reported that CSA treatment of chickens infected with IBV, resulted in virus re-excretion accompanied by the appearance of IBV-specific serum IgM, with an anamnestic boost in IgG levels. In the present study, although class-specific antibodies were not measured, there was no anamnestic boost in the total APV-specific ELISA antibodies following CSA treatment of either poults or chicks. Such an effect might have retrospectively indicated viral re-excretion.

Age at infection of chickens has been found to play an important role for IBV persistence (Dhinakar Raj & Jones, 1997). It was found that CSA treatment of IBV infected chickens at day-old resulted in virus re-excretion but not when the birds were infected at two weeks of age. In the present work, CSA treatment of day-old poults did not reveal persistence of APV. There may be several reasons for this. Firstly, perhaps APV does not persist. Secondly, if it persists, the persistence is not under T-cell control and third, that it persists in other sites not examined in this study.

Before drawing a final conclusion, additional studies are required to investigate APV persistence using other types of stressors such as re-housing, onset of lay or cyclophosphamide B-cell immunosuppression. In addition to this, other factors like a higher challenge dose, virus strain difference and use of PCR technique along with routine virus isolation method may also be considered.

CHAPTER 7

IN VITRO AND *IN VIVO* STUDIES ON PATHOGENICITY OF AVIAN PNEUMOVIRUS FOR THE CHICKEN REPRODUCTIVE TRACT

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CHAPTER 7

IN VITRO AND *IN VIVO* STUDIES ON PATHOGENICITY OF AVIAN PNEUMOVIRUS FOR THE CHICKEN REPRODUCTIVE TRACT

7.1. INTRODUCTION

APV causes respiratory disease in turkeys and chickens (see Naylor & Jones, 1993). It is also known to infect the reproductive tract of the turkey (Jones et al., 1988; O'Loan & Allan, 1990) and cause a drop in egg production in breeders that follows the respiratory infection (Stuart, 1989; Cook et al., 1996). However, little is known about its effects on the reproductive tract of chickens. There is continued speculation in the field that the virus can cause reduced egg production and perhaps poor egg shell quality as it does in turkeys, but whether it is capable of infecting the reproductive tract of chickens is unknown.

The present study was therefore conducted to investigate pathogenicity of APV for the chicken reproductive tract in two ways:

1. *In vitro* effects of APV on oviduct organ cultures prepared from either oestrogen-induced precocious oviducts of young chicks or normal oviducts of adult chickens.
2. *In vivo* studies involving APV infection of young chicks with precocious oviducts and adult female chickens with normal oviducts.

Oviduct organ cultures (OOC) prepared from precocious oviducts have been used effectively for studying the pathogenicity of another respiratory virus of chickens, infectious bronchitis virus (IBV) (Pradhan et al., 1984 ; Dhinakar Raj & Jones, 1996a). In the present work involving precocious oviducts, a comparison of APV pathogenicity for chickens was made with that for turkeys, the species where its effects on the reproduction tract is known (Jones et al., 1988; O'Loan & Allan, 1990).

PART I: *IN VITRO* STUDIES ON PATHOGENICITY OF APV FOR OVIDUCT ORGAN CULTURES

In this section, a comparison of pathogenicity was made between OOC prepared from precocious oviducts of young chicks and poults. APV pathogenicity for OOC prepared from normal oviducts of adult female chickens was studied. The pathogenicity in OOC was assessed by (i) measurement of ciliary activity, (ii) immunofluorescence (IF) staining and (iii) virus titres.

7.2. MATERIALS AND METHODS

7.2.1. Birds

SPF chicken eggs and turkey poults free of maternal antibodies (MA) to APV were obtained from separate commercial sources. The eggs were hatched in this laboratory. The birds were maintained in complete isolation with food and water *ad libitum*.

7.2.2. Viruses

APV strain #8544 of turkey origin (Wilding et al., 1986) described in Chapter 3 and strain CP-1 of chicken origin were used. The strain CP-1 was previously isolated from broilers affected with mild respiratory disease with corneal ulceration (Jones et al., 1991). It has been maintained in this laboratory and underwent 8 passages in chicken embryo tracheal organ cultures (TOC). Before use, both strains were titrated in TOC as described in Chapter 3.

7.2.3. Precocious oviducts

Precocious oviducts were induced by oestrogen treatment of unsexed birds following the method of Pradhan et al. (1984) with modifications. Briefly, one-day old chicks or poults were inoculated with 1mg of oestradiol benzoate (Intervet, Cambridge, UK) subcutaneously on 3 occasions with 3 day interval between injections. Two or three days after the final injection (i.e. day 11 or 12 of age), birds were euthanased and oviducts were aseptically collected from females and used as required.

The precocious oviduct (Fig. 7a & 7b) was differentiated into 2 regions and for the purpose of this study, they were treated separately. The anterior elongated tubular part corresponded to magnum and isthmus in mature birds and the posterior pouch-like part corresponded to the uterus. Unlike in the mature oviduct, there was no gross demarcation that could differentiate between regions of magnum and isthmus in the precocious oviduct. In the following text, OOCs prepared from these two regions of the precocious oviduct are referred to as OOC (m) for the part corresponding to magnum and isthmus, and OOC (u) for the part corresponding to uterus.

7.2.4. Normal oviducts from adult chickens

Pieces of magnum and uterus were aseptically removed from each of three SPF female chickens of 22 months of age and used as required. The details of the source of these chickens follow in Part III of Materials and Methods of this Chapter.

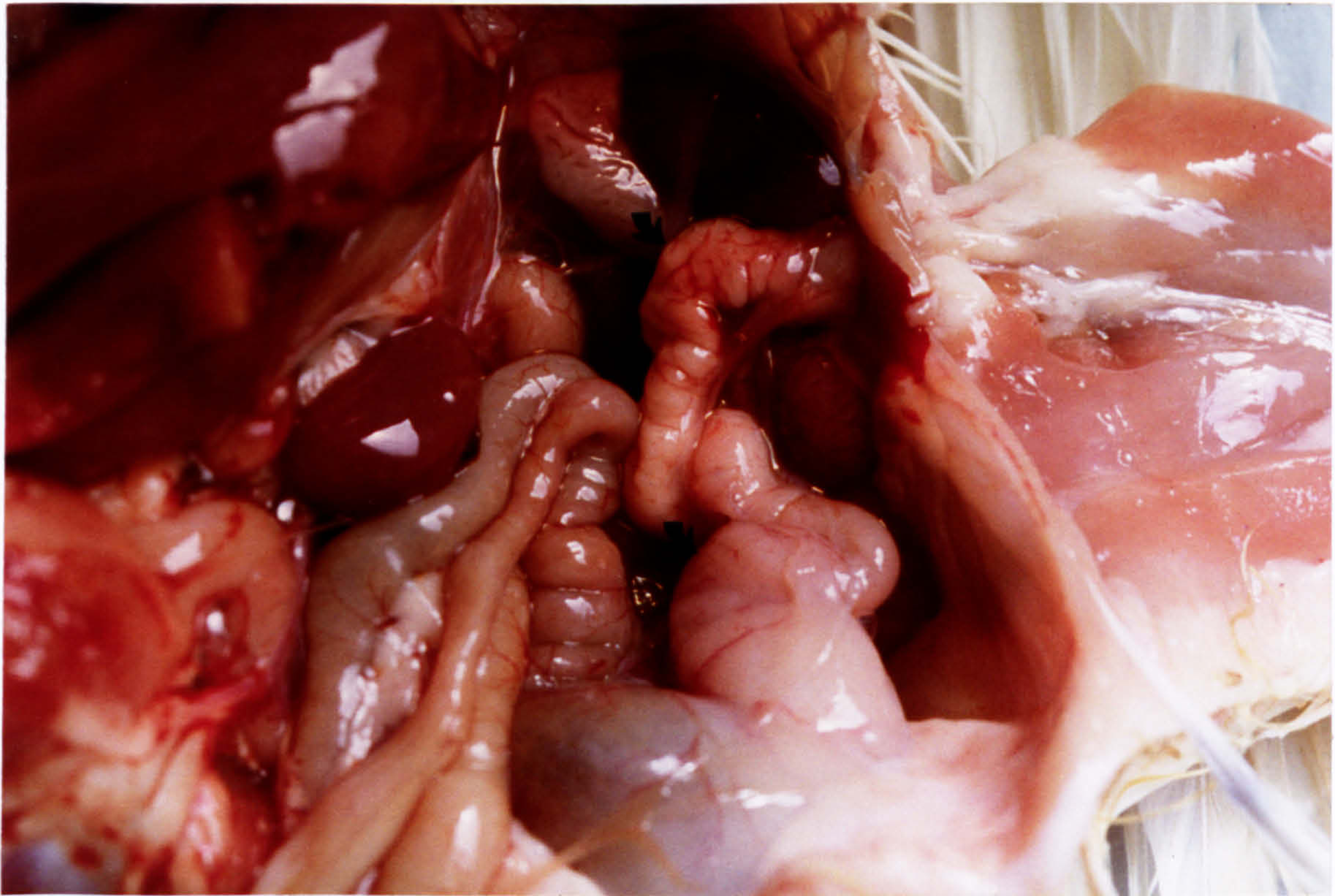

Fig. 7a. Precociously-induced oviduct in an oestrogen-treated 12-day old chick. Arrow on the top indicates magnum-isthmus and the bottom arrow indicates uterus.

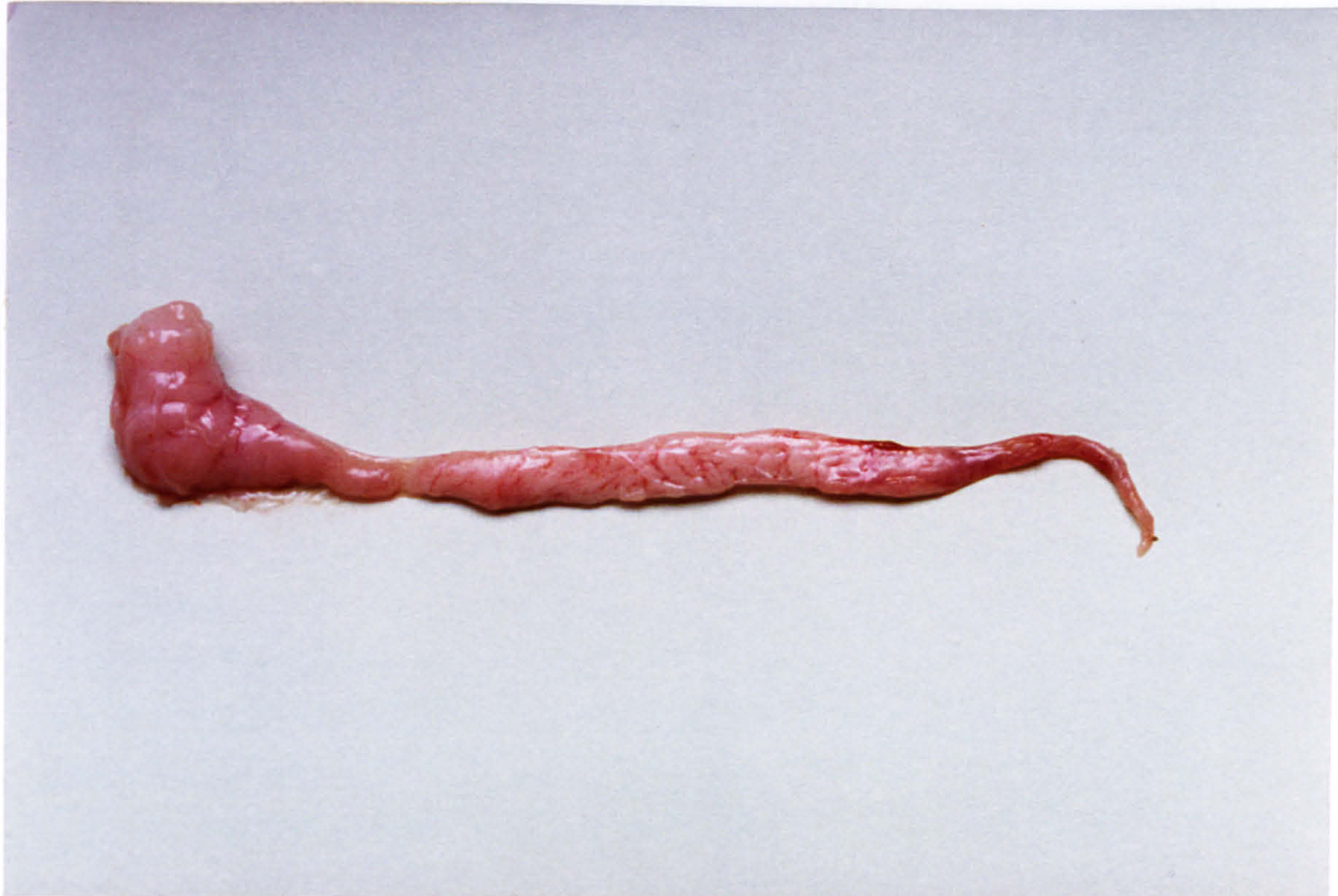

Fig. 7b. Differentiation of a precocious oviduct into 2 regions, i.e. the elongated tubular part (right) corresponding to magnum and isthmus in the mature bird and the rounded pouch-like part (left) corresponding to uterus in the mature bird.

7.2.5. Oviduct organ culture

OOC were prepared using the method described for TOC (Chapter 3) with some modifications. Briefly, 600 µm thick and 5 mm long sections of the oviducts were cut using a tissue chopper (Mickle Laboratory Ltd., Surrey, UK) and each section was placed in a sterile tube containing 0.7 ml of OOC medium (see Appendix). The tubes were set rotating in a roller drum at 8 revolutions/h at 37°C. After 24 h the sections were checked for ciliary beating and only those showing vigorous ciliary activity were used subsequently for studying ciliary damage following virus infection.

For virus titrations, 1000 µm thick and 5 mm long sections and for IF staining, approximately 2 mm thick and 7 mm long sections of oviducts were used similarly. After infection, cultures were left stationary for 1 h at 37°C. Following adsorption, the inoculum was removed and fresh medium was added after the tissue had been washed.

7.2.6. Experimental design

Infected OOC were examined in the following ways: (i) measurement of ciliary activity, (ii) IF staining and (iii) virus titre curve. For precocious oviducts, two identical trials were performed and for the normal mature oviduct, only one.

Measurement of ciliary activity

OOCs were examined for ciliary activity 24 hours after preparation and infected with either of the two APV strains at an input dose of 3.5 $\log_{10}$ CD_{50}/0.1ml. End-points were calculated as per the method of Power and Jordan (1976). Briefly, all OOCs were examined once daily and the day on which complete loss of ciliary activity occurred was recorded for each section, for computing the time taken for 50% (10 of the 20) of sections to lose the ciliary activity.

Immunofluorescence of OOC

Oviducal tissues were infected with each strain at the input dose mentioned above. The infected tissues were collected on days 1, 3, 5, 7 and 9 post infection (pi), snap-frozen and processed for IF staining as described in Chapter 3. Three sections of each tissue were examined. The fluorescence of epithelial cells was scored on 0 to 3 scale using the method of Dhinakar Raj and Jones (1996a). Briefly, 0 indicated no fluorescence, 1 a few fluorescing cells, 2 discrete areas of fluorescence and 3 extensive fluorescence.

Virus titres in OOC

Oviducal tissues were infected with each strain of APV at input dose as stated above and supernatants were collected daily from days 1 to 9 pi. Ten tubes of OOCs were

used for each strain. The pooled harvest of 10 tubes on days 1, 3, 5, 7 and 9 pi was titrated in TOC (Chapter 3).

7.2.7. Statistical analysis

The results of ciliary activity, IF scores and virus titres were compared for significant differences ($p<0.05$) using student's t-test.

7.3. RESULTS

7.3.1. Precocious oviducts

Following oestrogen treatment, the birds remained apparently normal and active. At necropsy, the mean length of the precocious oviducts (n=10) was 6.3 cm for chicks and 9.5 cm for poults.

7.3.2. Loss of ciliary activity

Precocious oviducts

Figs. 7.1 and 7.2 show the time taken in days for ciliostasis in 50% of OOC sections infected with the two APV strains. Both strains showed similar pathogenicity for OOCs prepared from either chickens or turkeys as indicated by their ciliostatic end-points. Between the two species, the ciliostatic end-point was similar for OOC(u), but significantly longer for OOC (m) of chicken origin. In fact, approximately 10-15% of sections of chicken OOC (m) failed to show complete ciliostasis. Within species, in chickens, the time taken to attain the ciliostatic end-point for OOC (m) was longer than for OOC (u) and in turkeys, time taken by OOC (m) was shorter than in OOC (u).

Adult oviducts

The measurement of ciliary damage to OOCs prepared from adult chickens could not be performed due to the difficulty in maintaining the cultures beyond 5 days, in contrast to OOC from young birds, where the cultures were able to be maintained in a healthy state with intact ciliary activity for at least 14 days.

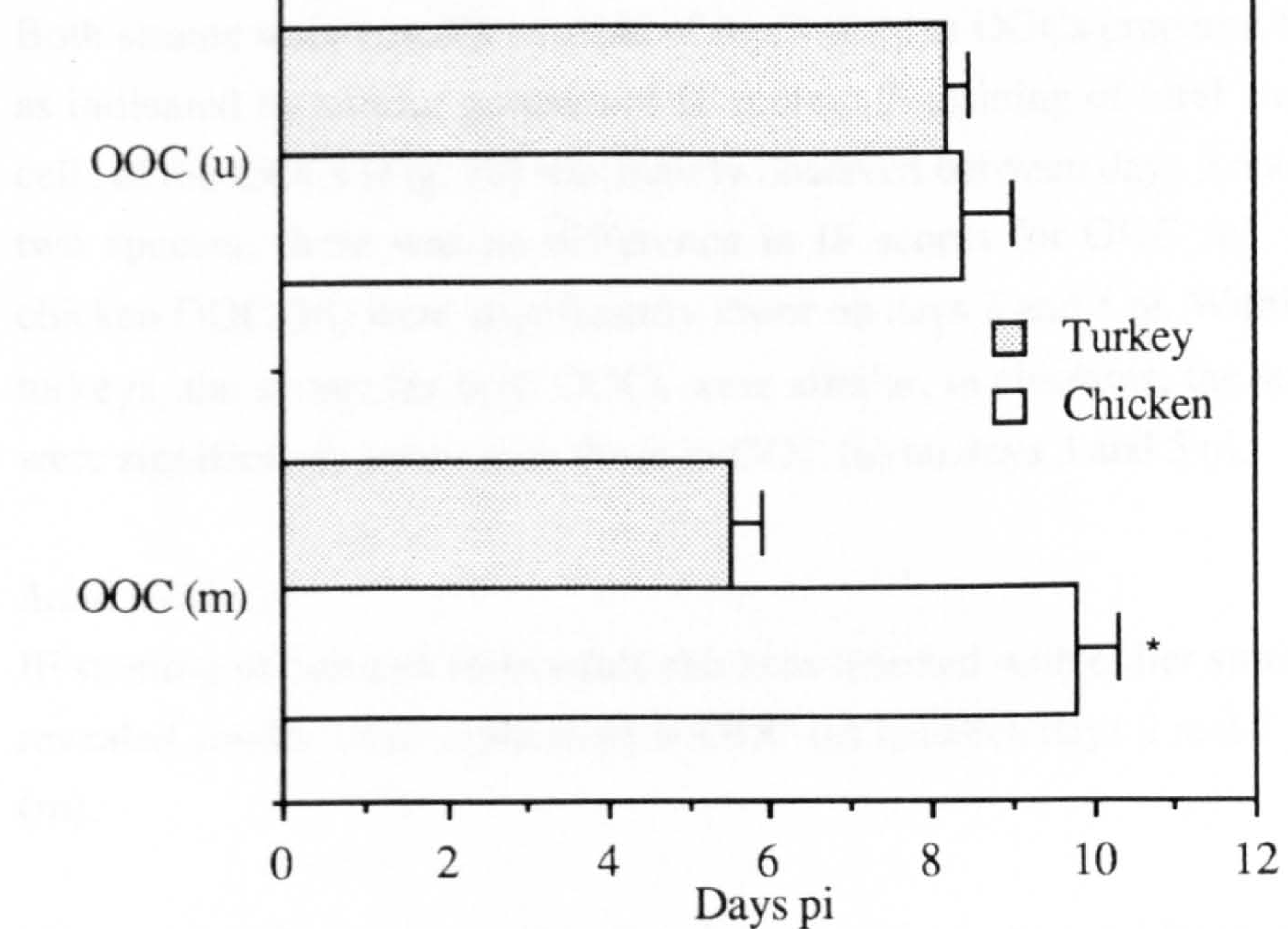

Fig. 7.1. Time taken (mean ± sd) for ciliostasis in 50% of OOC sections prepared from precocious oviducts infected with strain 8544. * : Significant difference ($p<0.05$) between OOC (m) of two species.

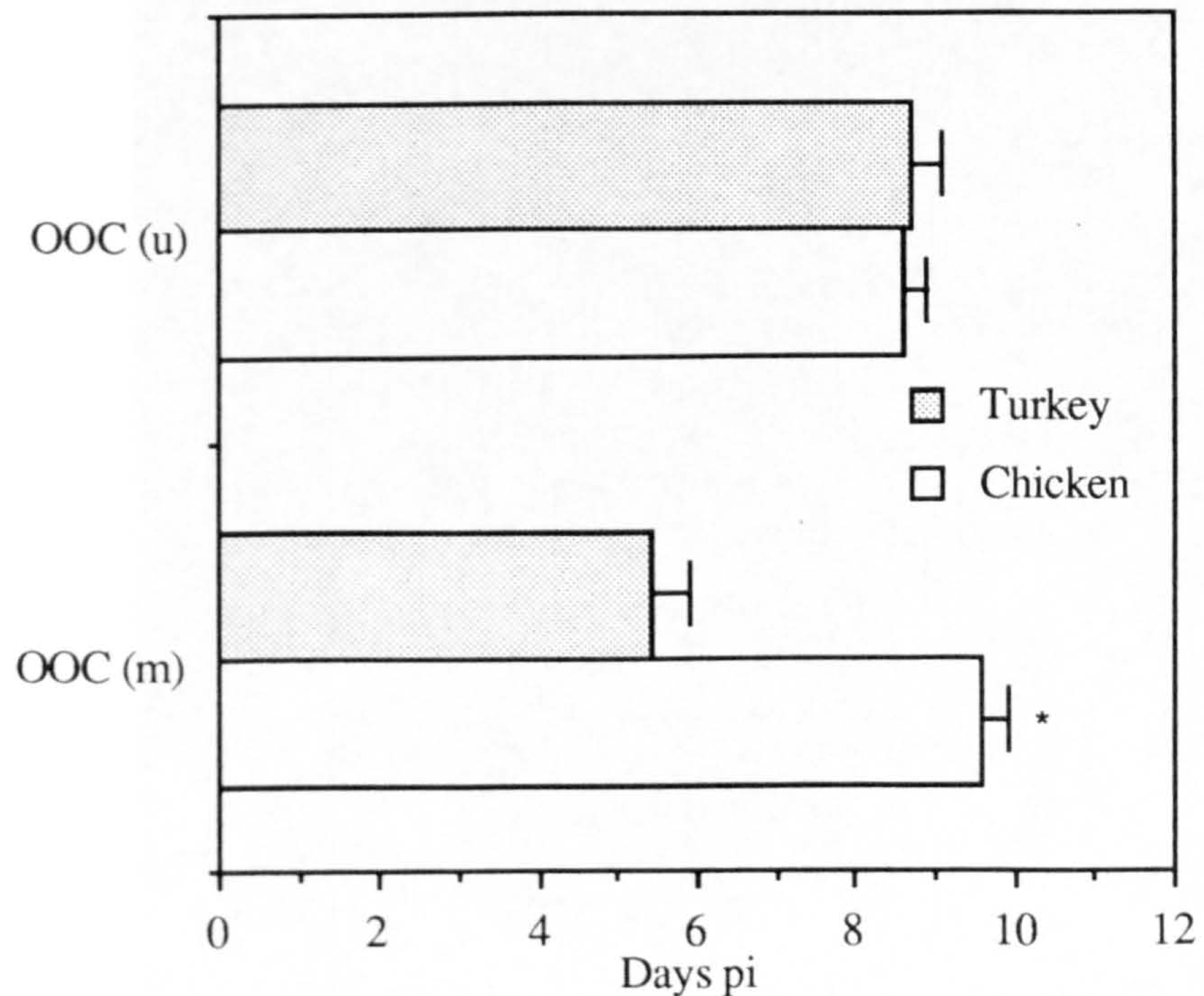

Fig. 7.2. Time taken (mean ± sd) for ciliostasis in 50% of OOC sections prepared from precocious oviducts infected with strain CP-1. * : Significant difference ($p<0.05$) between OOC (m) of two species.

7.3.3. IF staining of OOC

Precocious oviducts

Figs. 7.3 and 7.4 show the mean IF scores in OOCs infected with the two APV strains. Both strains were equally capable of replicating in OOCs prepared from either species as indicated by similar patterns of IF scores. IF staining of viral antigens in epithelial cells of the OOCs (Fig. 7c) was mainly observed between days 3 and 9 pi. Between the two species, there was no difference in IF scores for OOC (u), but the scores for chicken OOC (m) were significantly lower on days 3 and 5 pi. Within species, while in turkeys, the scores for both OOCs were similar, in chickens, the scores for OOC (m) were significantly lower than those in OOC (u) on days 3 and 5 pi.

Adult oviducts

IF staining of cultures from adult chickens infected with either strain (Figs. 7.5 & 7.6) revealed similar virus replication in OOC (u) between days 3 and 7 pi, but not in OOC (m).

No specific IF was detected in uninfected cultures obtained from either precocious or adult oviducts.

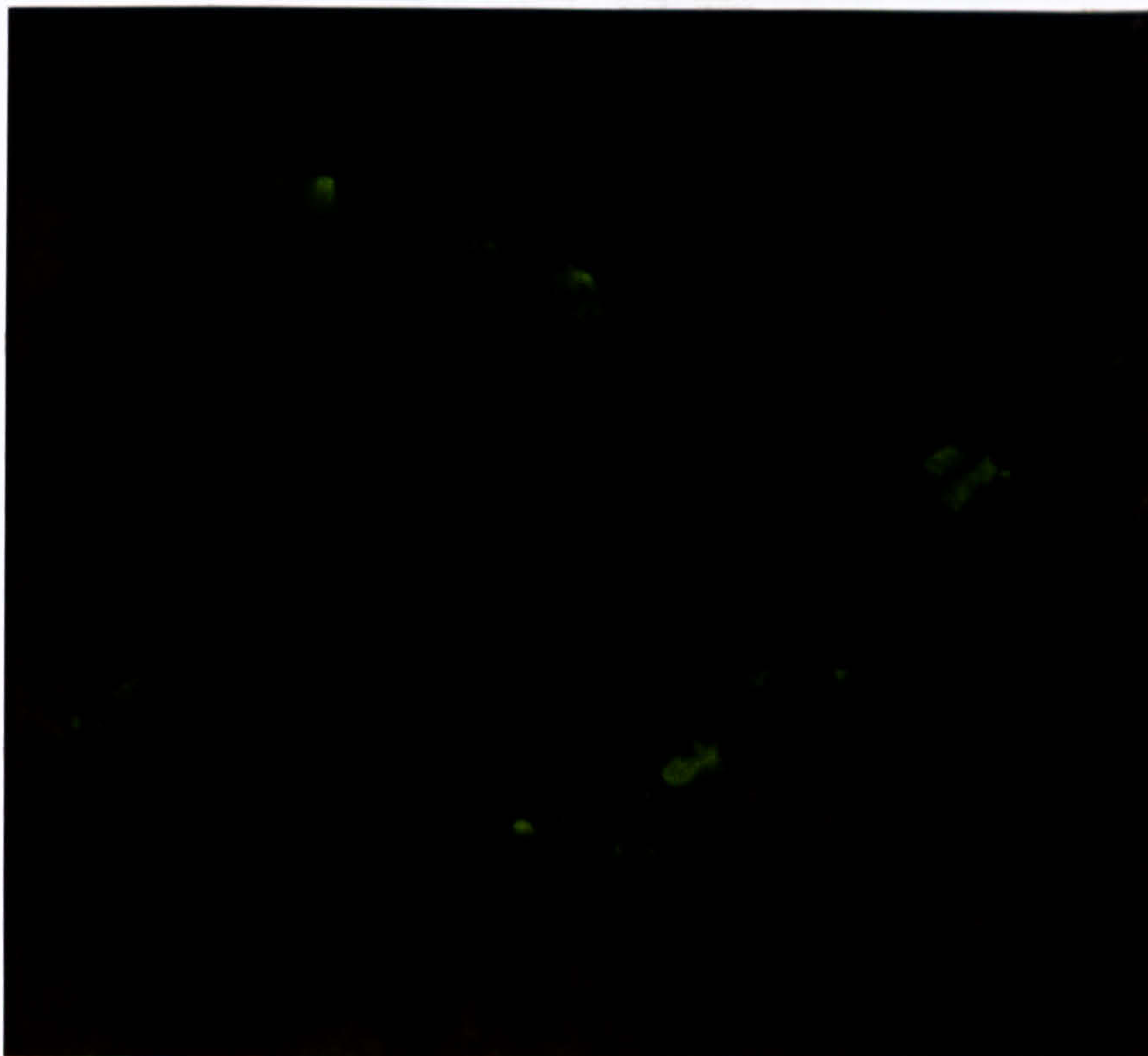

Fig. 7c. IF staining of a section of OOC showing APV antigens in epithelial cells. Magnification x312.5.

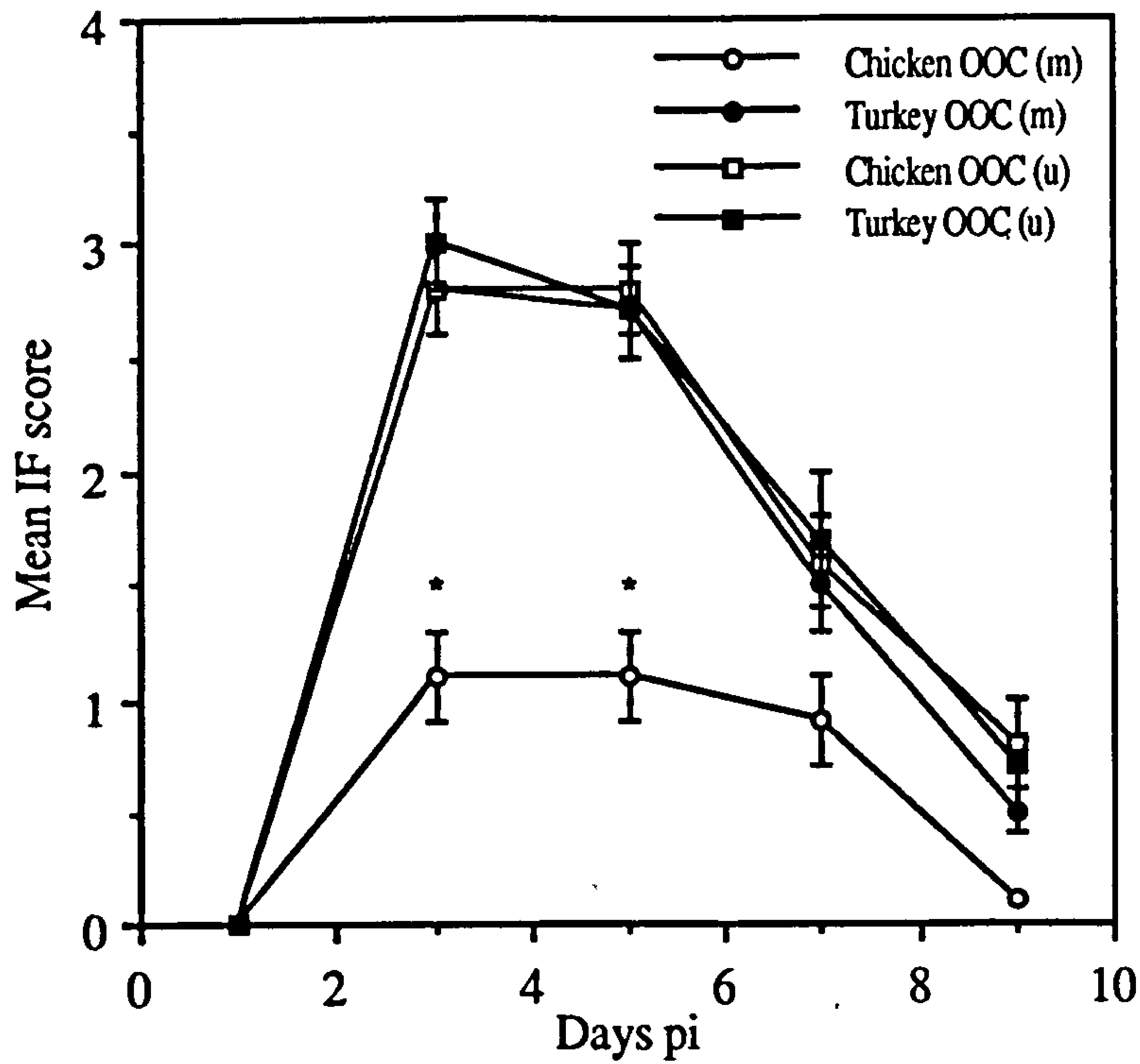

Fig. 7.3. IF scores (mean ± sd) in OOC prepared from precocious oviducts infected with strain 8544. *: Significantly lower ($p<0.05$) scores for chicken OOC (m).

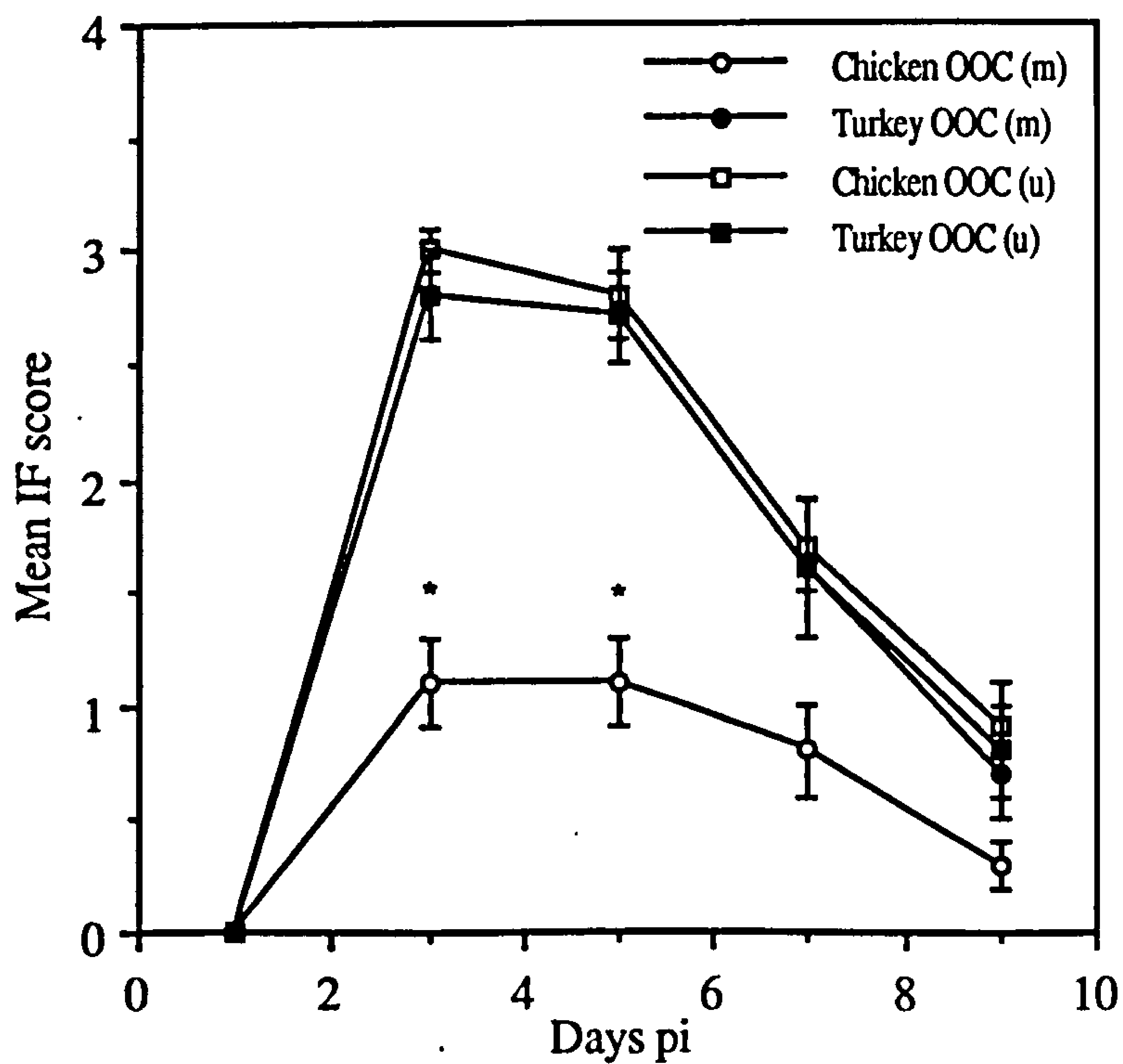

Fig. 7.4. IF scores (mean ± sd) in OOC prepared from precocious oviducts infected with strain CP-1. *: Significantly lower ($p<0.05$) scores for chicken OOC (m).

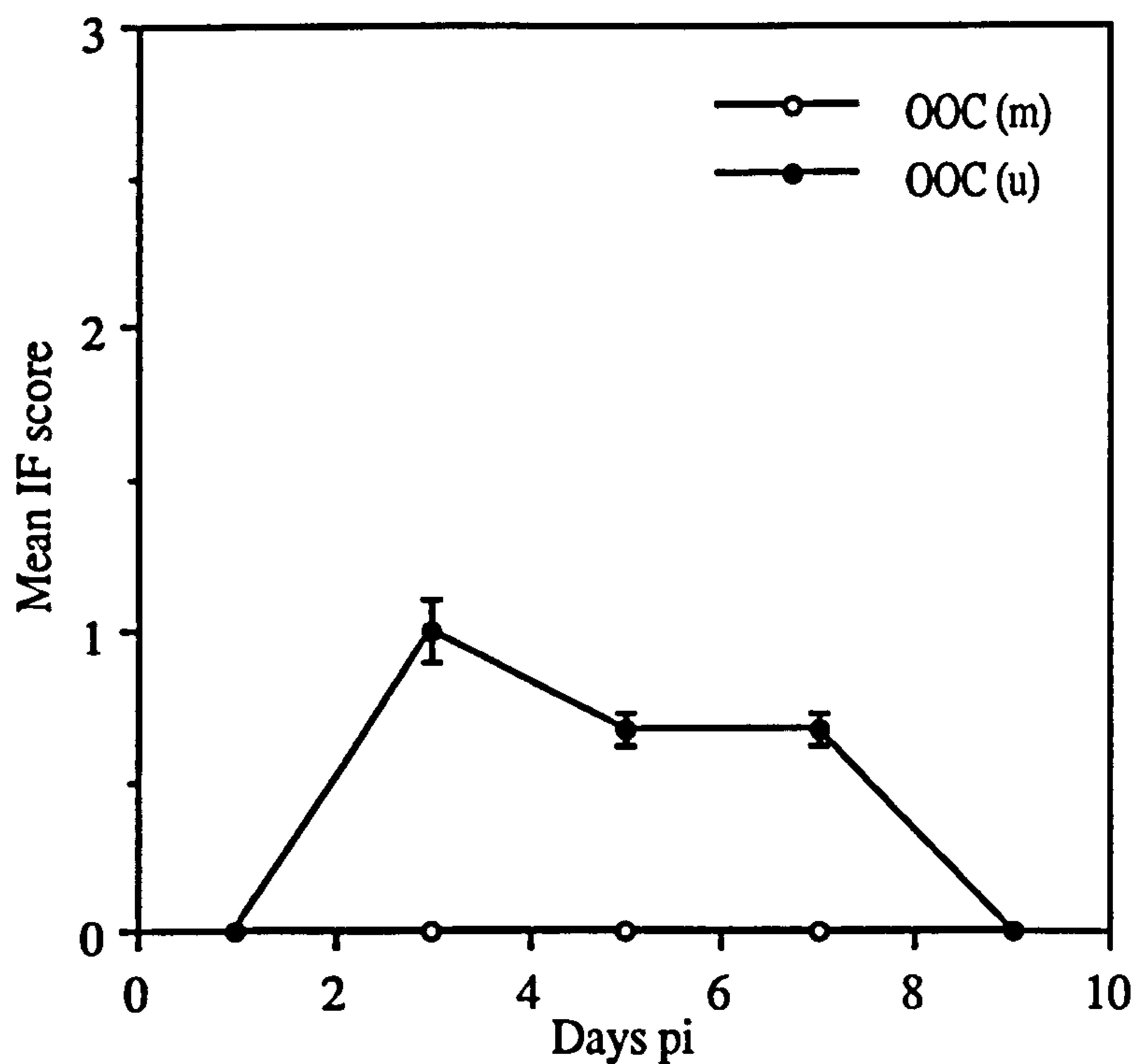

Fig. 7.5. IF scores (mean ± sd) in OOC prepared from normal oviducts of adult chickens and infected with strain 8544.

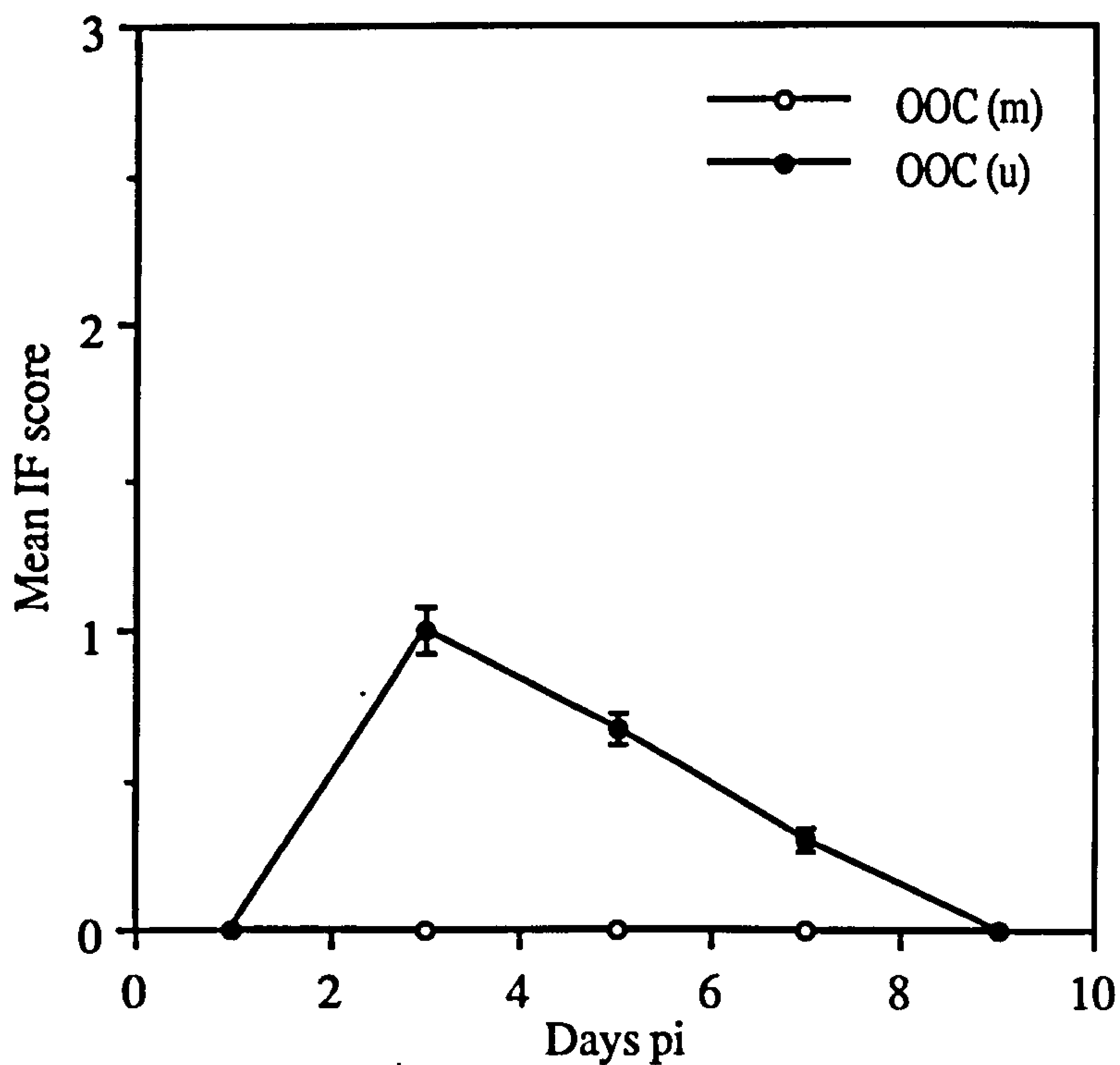

Fig. 7.6. IF scores (mean ± sd) in OOC prepared from normal oviducts of adult chickens and infected with strain CP-1.

7.3.4. Virus titres in OOC

Precocious oviducts

Figs. 7.7 and 7.8 depict the mean virus titres in OOCs infected with the two APV strains. Both were equally capable of growing in OOCs prepared from either chickens or turkeys as indicated by similar patterns of virus titres. Between the two species, there was no difference in virus titres in OOC (u), but the titres in chicken OOC (m) were significantly lower on days 3, 5 and 7 pi. Within species, while in turkeys the titres were similar in both OOCs, in chickens, the titres in OOC (m) remained significantly lower than those in OOC (u) on days 3, 5 and 7 pi.

Adult oviducts

Virus titres in OOCs prepared from adult chickens infected with the two APV strain (Figs. 7.9 & 7.10) were strikingly similar. Titres in OOC (u) persisted longer and were markedly higher than in OOC (m), where virus was detected on day 3 only.

The supernatants from uninfected OOC prepared from either precocious or adults oviducts did not result in any ciliary damage when assayed in TOC.

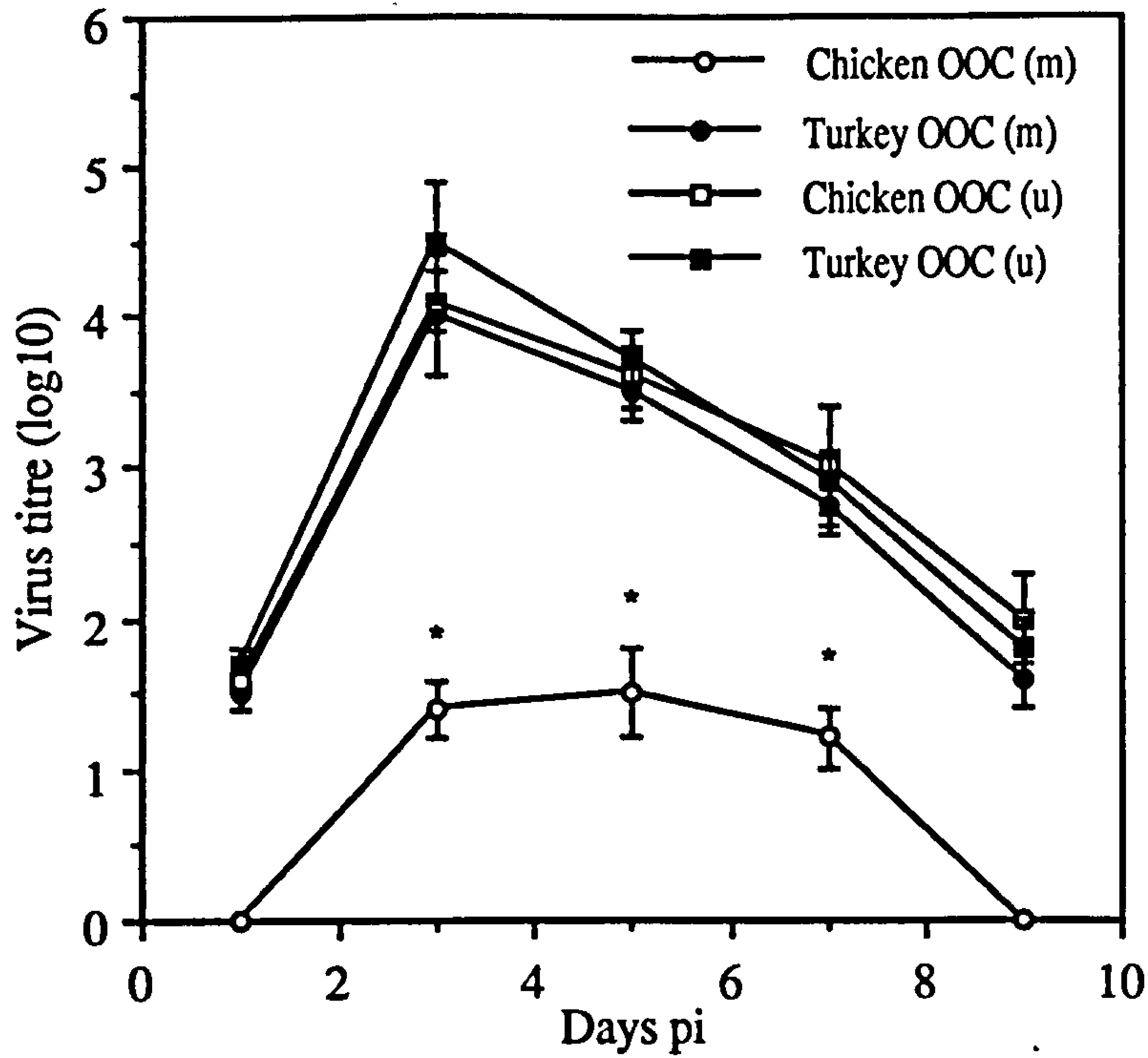

Fig. 7.7. Virus titres (mean ± sd) in OOC prepared from precocious oviducts infected with strain 8544. *: Significantly lower ($p<0.05$) titres for chicken OOC (m).

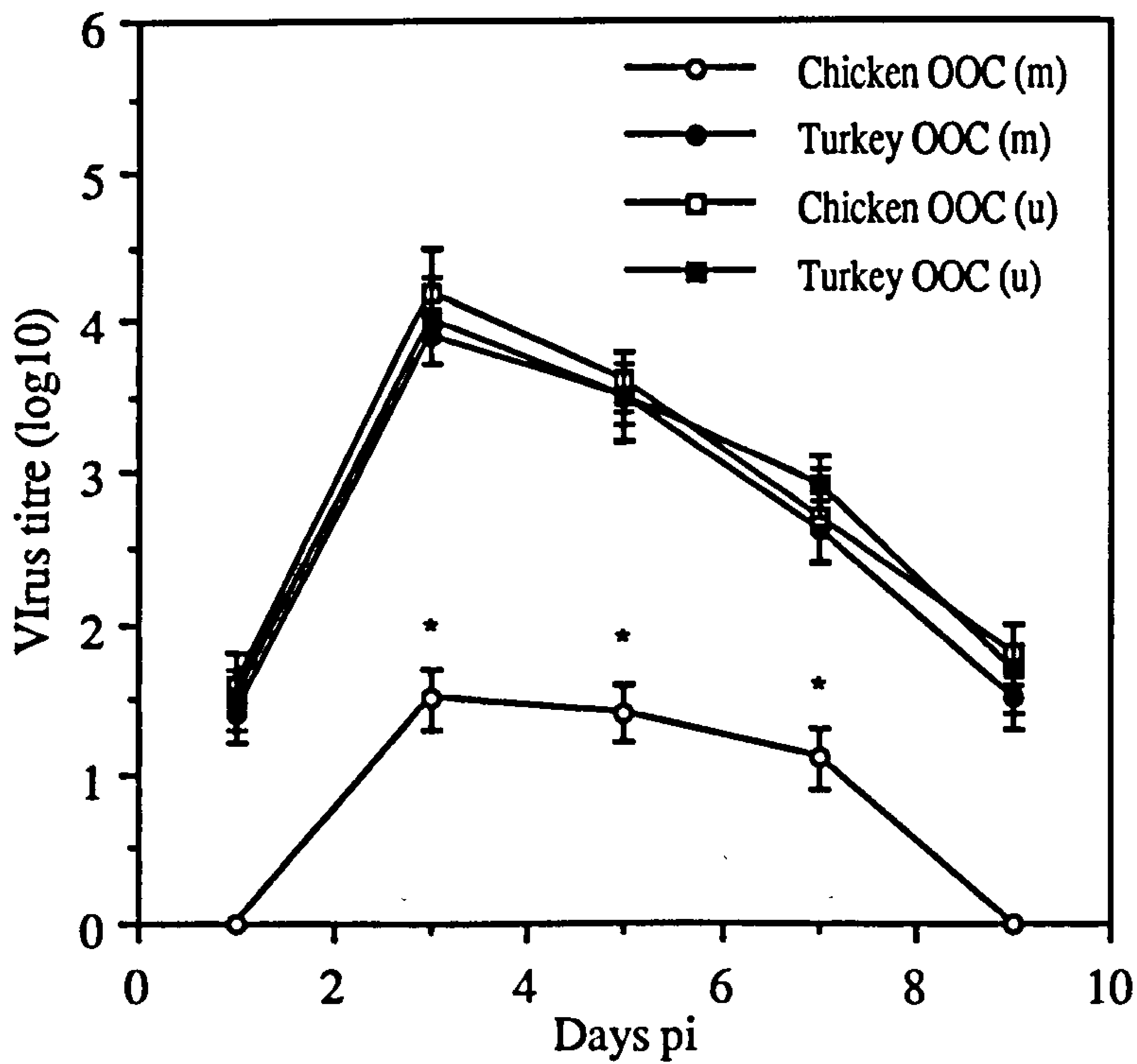

Fig. 7.8. Virus titres (mean ± sd) in OOC prepared from precocious oviducts infected with strain CP-1. *: Significantly lower ($p<0.05$) titres for chicken OOC (m).

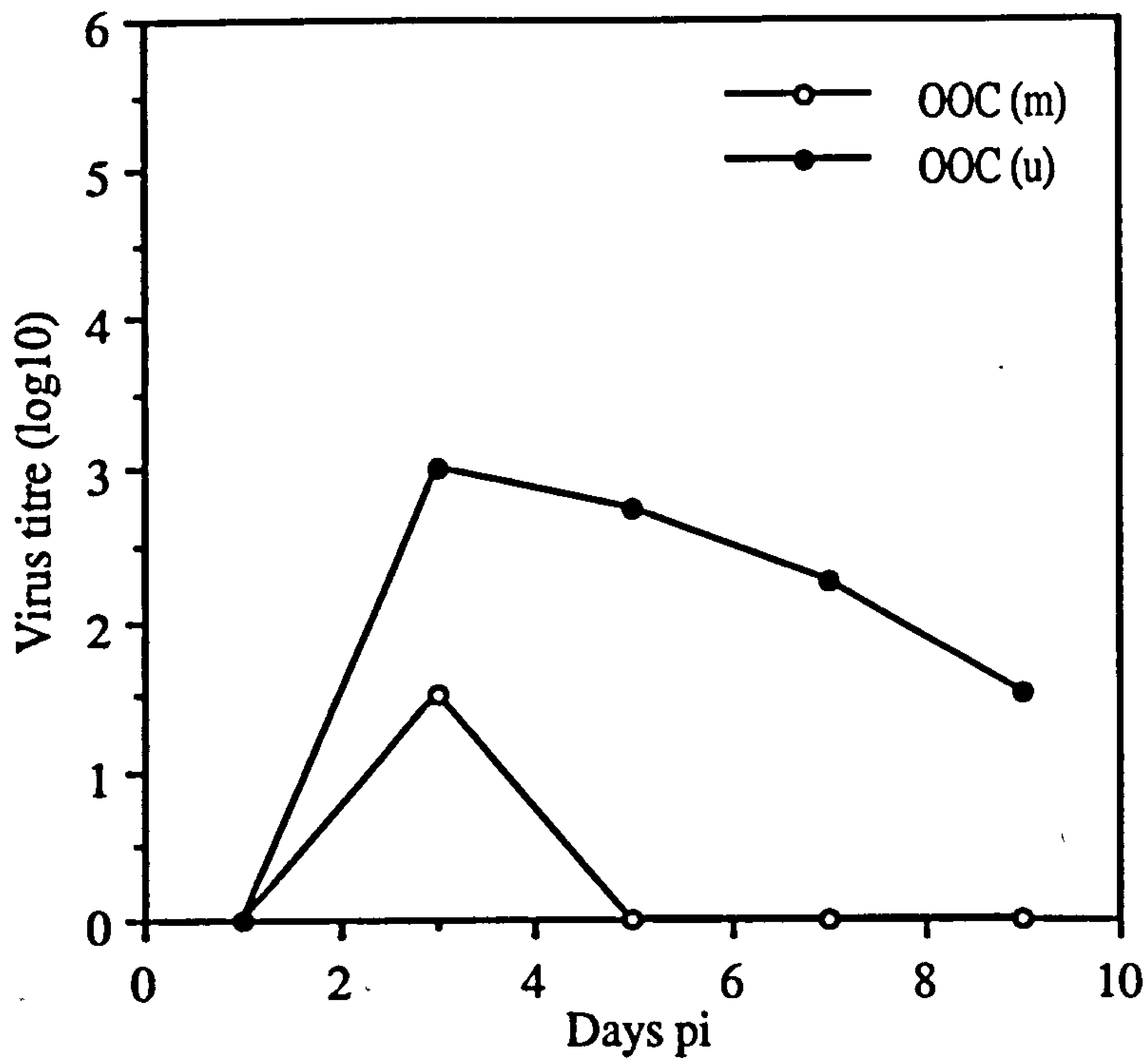

Fig. 7.9. Virus titres in OOC prepared from normal oviducts of adult chickens and infected with strain 8544.

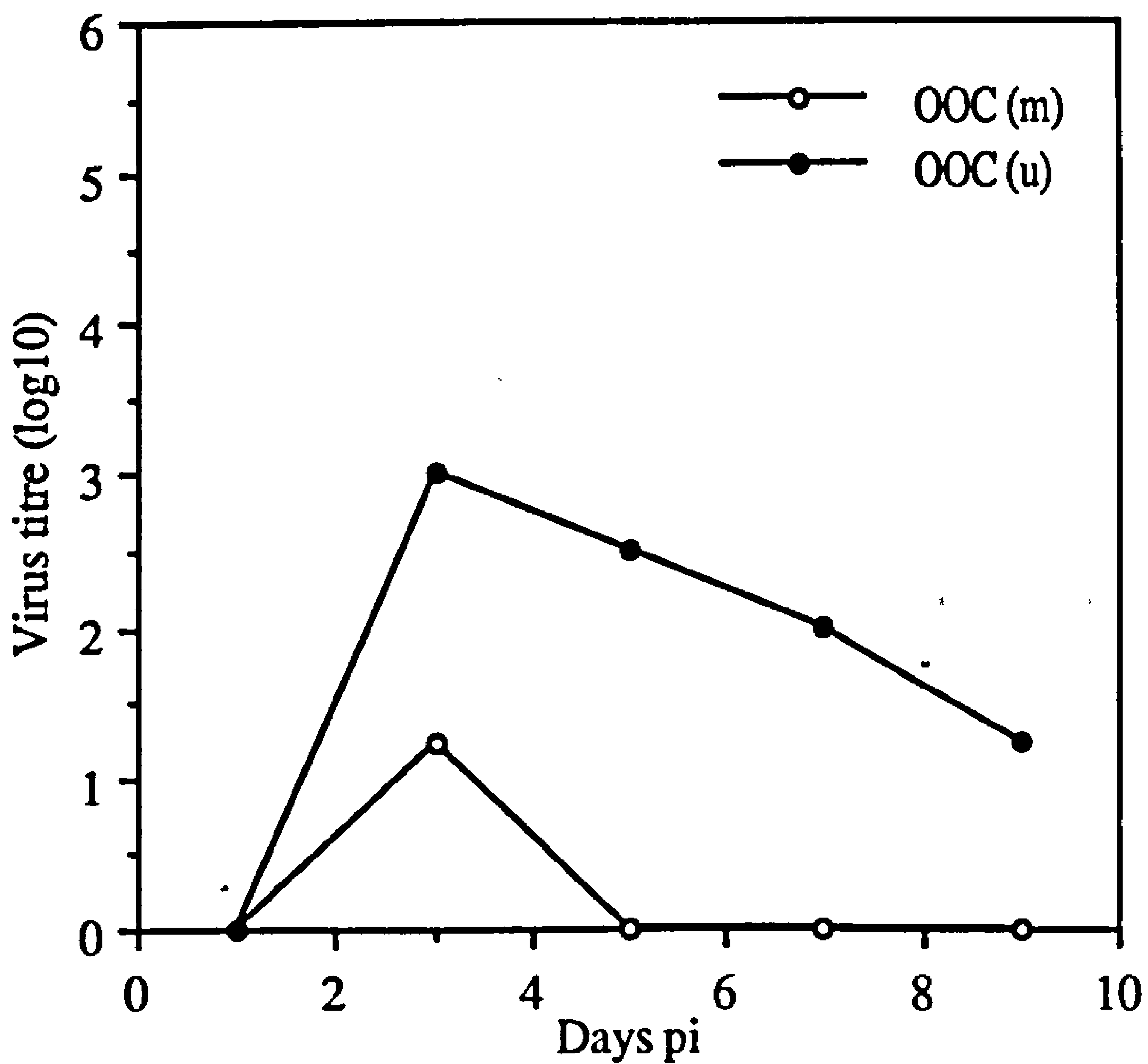

Fig. 7.10. Virus titres in OOC prepared from normal oviducts of adult chickens and infected with strain CP-1.

PART II: *IN VIVO* STUDIES ON THE PATHOGENICITY OF APV FOR THE PRECOCIOUS OVIDUCT

7.4. MATERIALS AND METHODS

7.4.1. Experimental birds

SPF chicks and poults free of MA to APV were obtained and raised as described in Materials and Methods of Part I of this Chapter.

7.4.2. Precocious oviducts

One day old chicks or poults were treated by oestrogen for induction of precocious oviducts (see section 7.2.3).

7.4.3. Virus

APV strain #8544 (Wilding et al., 1986) described in Chapter 3 was used. Each bird was inoculated with 3.5 $\log_{10}$ CD_{50}/0.1 ml of virus oculonasally at 5 days of age.

7.4.4. Experimental design

Two identical experiments were performed, one each in chicks and poults. Unsexed birds were divided into two groups. Both groups were given oestrogen treatment for 14 days starting from day one of age and one group was infected with APV on day 5 of age (i.e. 4 days after start of oestrogen treatment). Following infection, 10 birds in each group were examined daily for clinical signs. On days 3, 5, 7 and 9 pi, birds were euthanased and each time from 4 females in each group, pieces of precocious oviduct portions (magnum-isthmus and uterus) and tracheas were collected for assessment of ciliary activity, virus isolations and IF staining.

In addition, the effect of oestrogen treatment on *in vivo* replication of APV in the trachea was evaluated. For this purpose, a group of birds without oestrogen treatment was infected similarly. Pieces of tracheas from five birds each in this group and the infected group with oestrogen treatment were collected on day 5 pi for virus titration.

7.4.5. Clinical signs

The severity of clinical signs was scored on 0 to 3 scale by the method of Jones et al. (1992) as described in Chapter 3.

7.4.6. Ciliary activity

The damage to tissues was assessed by measuring loss of ciliary activity as per the method of Cook et al. (1989a) with slight modifications. Briefly, tracheas and oviduct

portions after collection were sectioned immediately and placed in tubes containing pre-warmed TOC medium (see Appendix). Ten 600 μm thick sections of each tissue were used and loss of ciliary activity was scored on a scale from 0 (100% ciliary activity) to 4 (total cessation of activity). Student's t-test was used to examine significant differences ($p<0.05$) between the infected and uninfected group.

7.4.7. Virus isolation

Aseptically collected tissues from birds were processed and used for virus isolations in TOC as described in Chapter 3. A minimum of three passages was given and the presence of virus assessed by ciliostasis. Ciliostatic virus was identified by IF staining of unfixed TOC using hyperimmune serum against APV strain #8544 (Chapter 3).

7.4.8. Immunofluorescence

Pieces of tracheas and oviduct portions after collection, were snap frozen in liquid nitrogen, stored at -70°C and processed for examining for the presence of viral antigens by IF staining (Chapter 3).

7.4.9. Virus titrations

Pieces of tracheas collected from oestrogen-treated and untreated but infected birds, were processed and titrated for virus in TOC as described in Chapter 3. The results were compared for significant differences ($p<0.05$) using student's t-test.

7.5. RESULTS

7.5.1. Clinical signs

Fig. 7.11 shows the mean clinical scores following APV infection of oestrogen-treated chicks or poults. As previously described, the respiratory disease in poults was more severe than in chicks as indicated by significantly higher scores on day 3 to 9 pi (end of experiment). No clinical signs were recorded in uninfected but oestrogen-treated birds (data not shown).

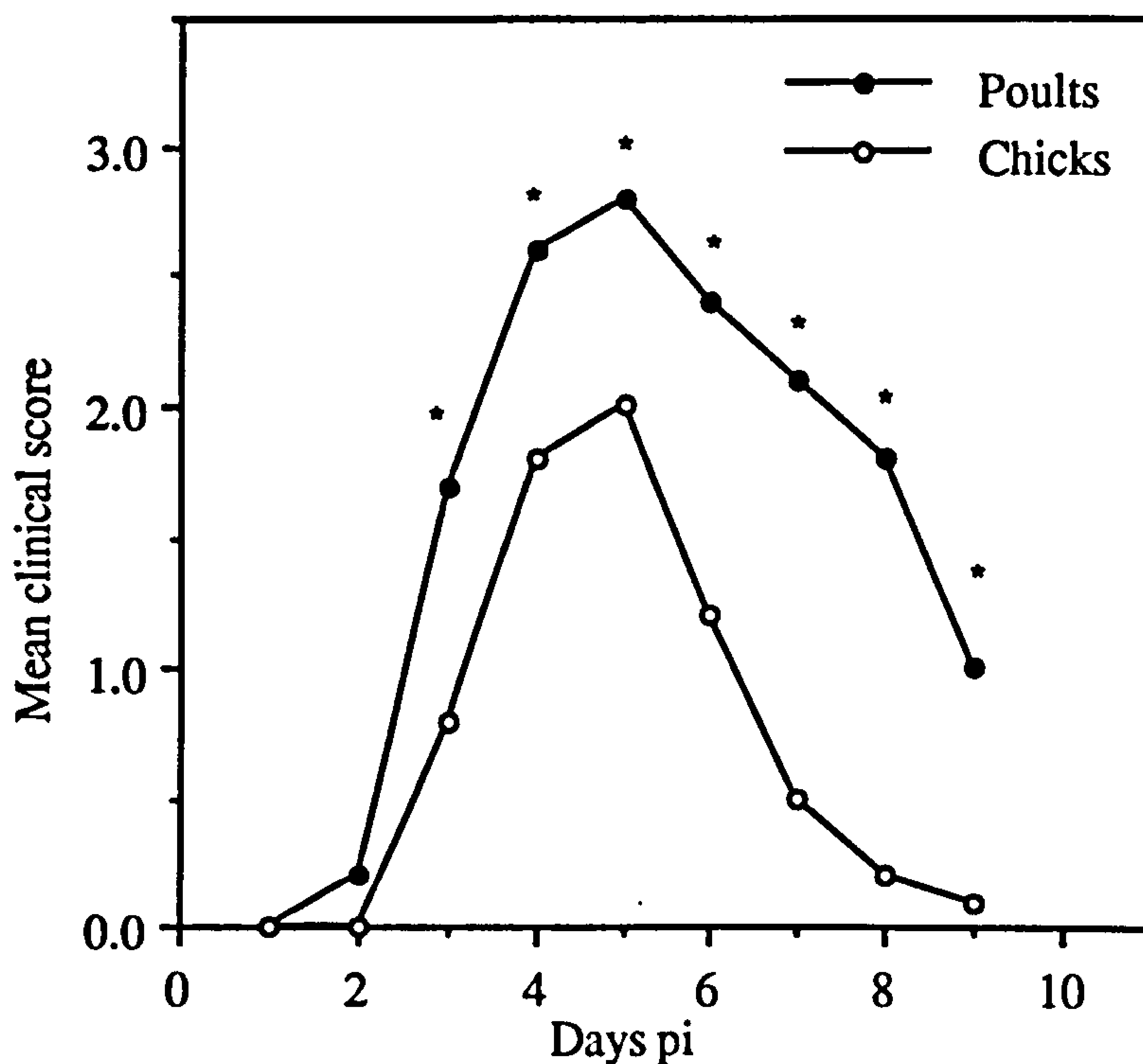

Fig. 7.11. Mean clinical scores of oestrogen-treated chicks and poults following APV infection. *: Significantly higher ($p<0.05$) scores of poults compared to chicks using chi-square test.

7.5.2. Loss of ciliary activity

The measurement of ciliary activity reflected significant damage to the tracheal tissue in chicks from days 3 to 7 pi and in poults from days 3 to 9 pi (Table 7.1). However, there was no damage inflicted on the oviducal tissues of either chicks or poults as indicated by similar mean ciliary scores between infected and uninfected birds.

Table 7.1. Mean ciliary scores in tissue explants after APV infection

		Mean ciliary scores					
		Chicks			Poults		
Days pi	Group	T@	M	U	T	M	U
3	Uninfected	4[a]	8*	9	6[a]	8	9
	Infected	15[b]	9	9	25[b]	8	10
5	Uninfected	3[a]	9	8	7[a]	8	10
	Infected	18[b]	10	9	29[b]	9	9
7	Uninfected	5[a]	8	9	6[a]	7	9
	Infected	16[b]	10	8	28[b]	8	8
9	Uninfected	5	8	9	5[a]	10	9
	Infected	9	9	9	19[b]	9	9

@: Tissues T=Trachea, M=Magnum-isthmus and U=Uterus.

*: Mean score of 4 tissues examined each time, for scoring method see section 7.4.6.

Values with different superscripts differ significantly between groups ($p<0.05$).

7.5.3. Virus isolation and immunofluorescence

APV replication in tracheas of both chicks and poults was detectable by virus isolation and IF staining (Table 7.2). However, neither method detected APV in the oviducal tissues of infected chicks or poults (Table 7.2). Also no virus was detected in any tissues of uninfected controls (not shown).

Table 7.2. Detection of APV in tissues of infected chicks or poults by virus isolation and IF staining

Tissue	Days post-infection							
	Chicks				Poults			
	3	5	7	9	3	5	7	9
Virus isolation								
Trachea	3*	4	0	0	4	4	1	0
Magnum/uterus	0	0	0	0	0	0	0	0
IF staining								
Trachea	3	3	0	0	3	4	0	0
Magnum/uterus	0	0	0	0	0	0	0	0

*: No. positive of 4 samples tested each time.

7.5.4. Virus titres in tracheas of oestrogen-treated and untreated birds

There was no significant difference in the amount of virus recovered on day 5 pi from tracheas of oestrogen-treated and untreated chicks or poults (Fig. 7.12).

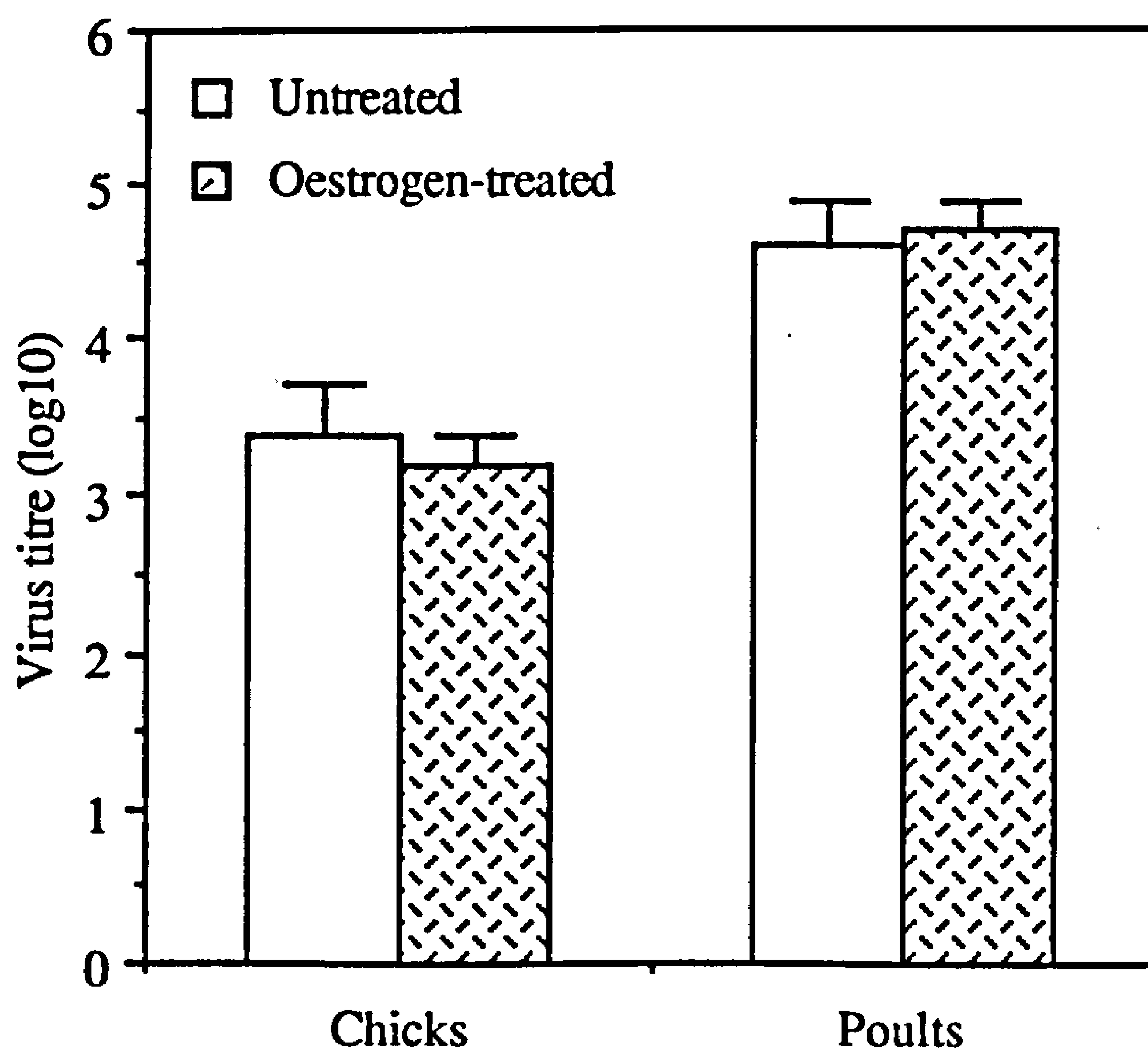

Fig. 7.12. Virus titres (mean ± sd) in tracheas of oestrogen-treated and untreated chicks or poults on day 5 pi. Titres expressed as CD_{50} log_{10}/g tissue. No significant difference between the two groups.

PART III: *IN VIVO* STUDIES ON APV PATHOGENESIS IN ADULT FEMALE CHICKENS

7.6. MATERIALS AND METHODS

7.6.1. Chickens

SPF WLH female chickens at 22 months of age were used for this study. These chickens had been raised in this laboratory under isolation conditions as a source of fertile eggs until they were used for the experimentation. Before the start of the experiment, the status of each bird was confirmed by collecting tracheal swabs for virus isolation. The hens were found free of evidence of APV based on negative virus isolation results.

7.6.2. Virus

APV strain #8544 (Wilding et al., 1986) described in Chapter 3 was used.

7.6.3. Experimental design

Thirty-seven hens were divided into 3 groups, comprising one uninfected, the second, oculonasally (O/N) infected and the third, intravenously (IV) infected. According to the group, each bird was inoculated either O/N or IV with 4.3 $\log_{10}$ CD_{50} of virus in 200 µl volumes. All birds were daily assessed for the presence of clinical signs. Three birds from each infected group and one from the uninfected group were euthanased on days 3, 5, 7, 10, and 14 pi, and pieces of harderian gland (HG), turbinates, trachea, lungs, ovary, magnum and uterus were collected for virus isolations. Samples of trachea, magnum and uterus were snap-frozen in liquid nitrogen for use in IF staining. Serum samples were collected from five birds in each group on days 7 pi and from three birds each on day 14 pi for measuring virus-specific antibodies by ELISA.

7.6.4. Clinical signs, virus isolation and immunofluorescence

The methods for assessing clinical disease, virus isolation and IF staining were essentially the same as described in Materials and Methods of Part II of this Chapter, except that for IF staining in this study, a pool of 8 monoclonal antibodies (Mabs) directed against the G protein of APV was used instead of hyperimmune serum. These Mabs were kindly provided by Dr. D Cavanagh of the Institute for Animal Health, Compton, UK.

7.6.5. Serology

Sera were titrated for specific antibodies to APV using an ELISA (Naylor et al., 1992) as described in Chapter 3. The results of ELISA were compared for significant differences ($p<0.05$) using the student's t-test.

7.7. RESULTS

7.7.1. Clinical signs

Very mild clinical signs in the form of nasal exudate on beak squeezing were recorded between days 4 and 7 pi in the group of birds infected by oculonasal route (Fig. 7.13), but none were seen in the birds infected IV or in the uninfected controls.

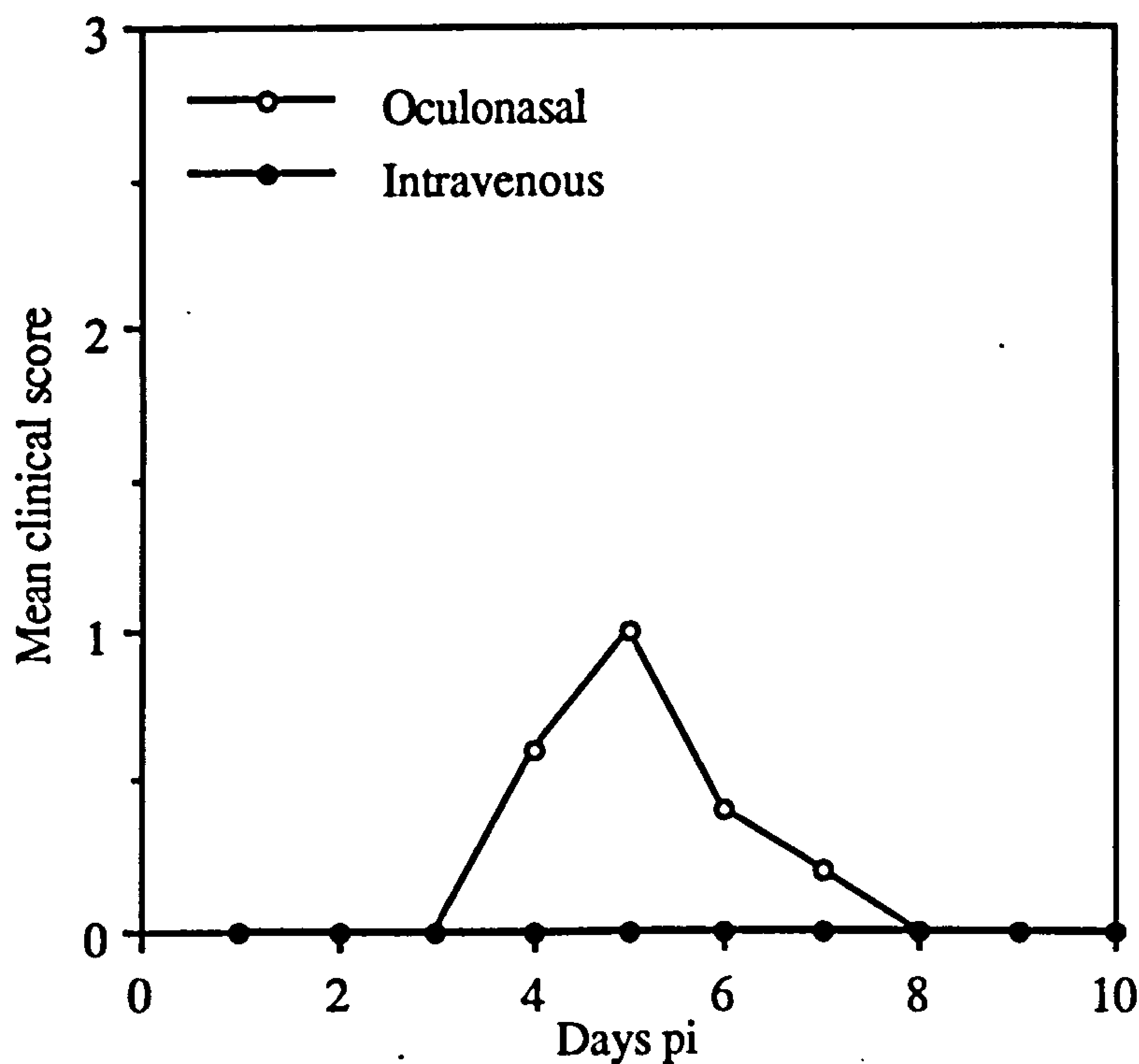

Fig. 7.13. Mean clinical scores of adult female chickens following APV infection.

7.7.2. Virus isolation and immunofluorescence

APV isolations were made from HG, turbinates and tracheas on days 3 and 5 pi from hens infected by the O/N route (Table 7.3). In those infected IV, APV was isolated from tracheas only on days 3 and 5 pi. No isolations were made from lungs and tissues of the reproductive tract of either group (Table 7.3). Also no virus was isolated from any tissue from uninfected controls (not shown).

By IF, APV was demonstrated on days 3 and 5 pi in tracheas of hens infected by either route (Table 7.3). None of the infected birds on any sampling occasion revealed the APV-specific antigens in the epithelium of the magnum or uterus (Table 7.3). No viral antigens were detected in tissues of uninfected birds (not shown).

Table 7.3. Detection of APV in tissues of hens by virus isolation and IF

Infection route	Virus isolation only				Virus isolation & IF		
	HG	Turbinates	Lungs	Ovary	Trachea	Magnum	Uterus
O/N							
3*	2[a]	2	0	0	1	0	0
5	2	3	0	0	1	0	0
7-14	0	0	0	0	0	0	0
IV							
3	0	0	0	0	2	0	0
5	0	0	0	0	2	0	0
7-14	0	0	0	0	0	0	0

*: Days pi; a: No. positive of 3 samples tested each time.

7.7.3. Serology

In both infected groups, the antibody titres were higher than that in uninfected controls with significant differences on day 14 pi (Fig. 7.14). The apparent significant amounts (> 6.1 $\log_2$) of antibody in controls birds was probably due to the lipaemic nature of the sera causing elevated absorbance readings in ELISA.

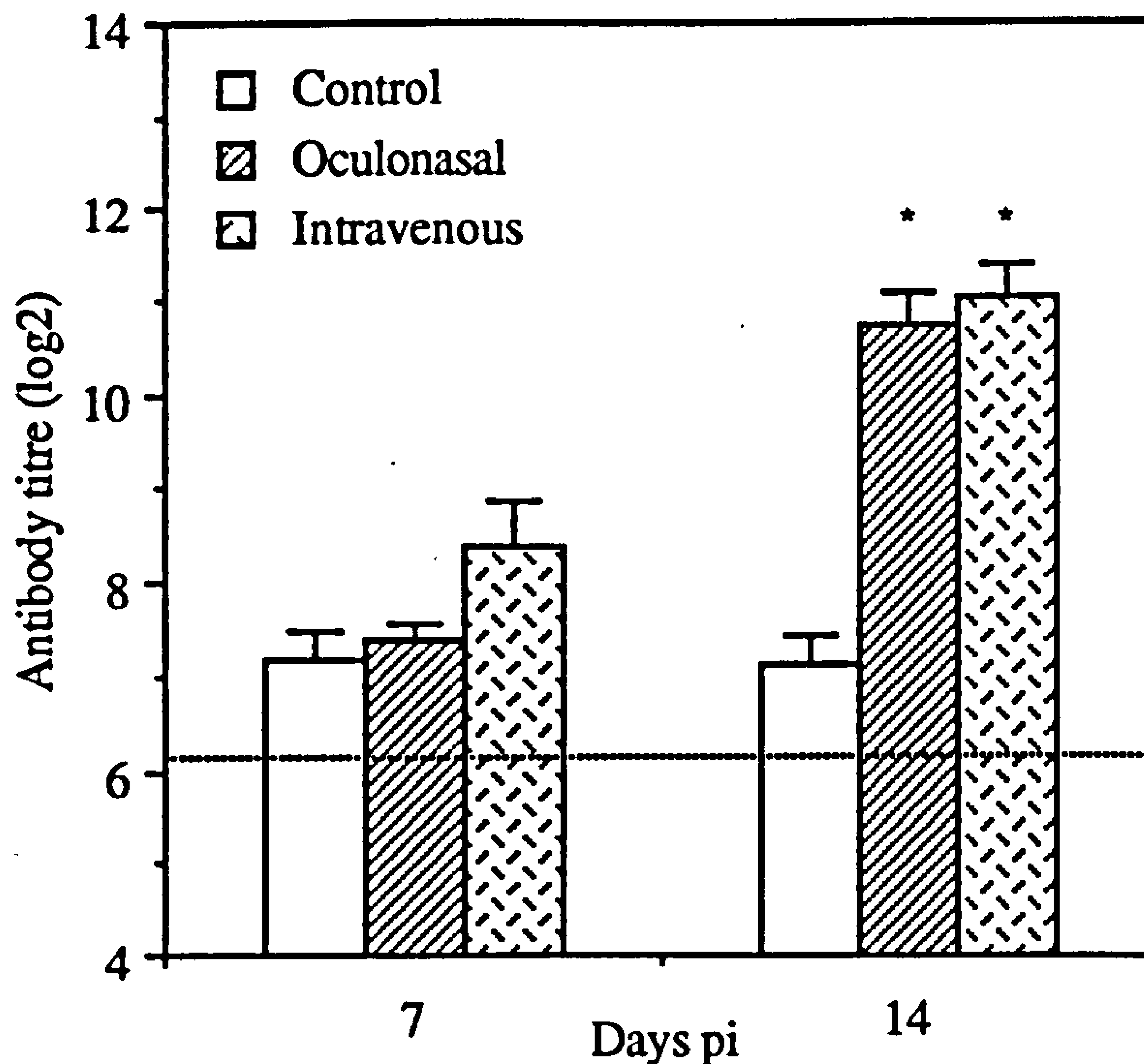

Fig. 7.14. Antibody titres (mean ± sd) in adult female chickens following APV infection. *: Significantly higher ($p<0.05$) titres compared to uninfected controls.

7.8. DISCUSSION

Due to the practical constraints in procurement of adult chickens or raising the birds until sexual maturity, the pathogenicity of APV for the chicken reproductive tract was initially evaluated using young chicks with precocious oviducts.

The results of *in vitro* experiments on OOCs obtained from precocious oviducts showed the innate susceptibility of the chicken reproductive tract to APV. The ciliary damage, positive IF staining and virus titres in OOCs, all indicated the pathogenic ability of APV for the chicken oviduct. There was no overall difference between the chicken and turkey APV strains in their pathogenicity for OOCs obtained from either species. However, on comparing OOCs of chicken origin with those of the turkey, some differences in susceptibility of OOC prepared from magnum and isthmus referred to as OOC (m) was apparent. The pathogenicity in terms of ciliary damage, IF scores and virus titres of both APV strains for the chicken OOC (m) was significantly lower than that for turkey OOC (m). The pathogenicity of APV for the chicken OOC (m) was also lower compared to the chicken OOC (u).

This difference in pathogenicity for the chicken OOC (m) was further validated by the similar results in OOC prepared from the magnum (not isthmus) and uterus of adult female chickens. APV showed markedly poor growth in OOC prepared from magnum compared to OOC from uterus. The reason for this difference in pathogenicity is not clear. One possibility could be difference in susceptibility of epithelial cells of the magnum and the uterus for APV. Another factor might be that some secretions from magnum eg. ovomucin interfered with the virus growth, however, the tissues were thoroughly washed before use while preparing OOC.

The oviduct of the chicken synthesizes those components which surround the ovum in the hard-shelled egg; the magnum producing the albumin, the isthmus the keratinous shell membranes and the uterus the calcareous shell, pigment and cuticle (Aitken, 1971). Thus, the *in vitro* finding that the chicken magnum (and immature isthmus) is apparently less susceptible to APV than the uterus would suggest that in the adult hen, if APV does indeed replicate in the oviduct *in vivo*, the virus might be more likely to affect shell quality of eggs than components produced by magnum or isthmus.

The impetus from successful *in vitro* experiments, showing pathogenic potential of APV for the chicken oviduct lead to the *in vivo* studies. The difficulties associated with raising or procurement of laying hens as mentioned earlier, made us decide to initially use an *in vivo* precocious oviduct model. This model has been used previously to study the cross-protection of various IBV strains at the oviduct level by *in vitro* challenge of OOC prepared from precocious oviducts of immunised chicks (Dhinakar Raj & Jones, 1996b). Ganapathy (1997) also used the same model for studying a mixed *Mycoplasma gallisepticum* and IBV infection in chicks. They found an increase in invasiveness of both agents for the precocious oviduct accompanied by an increase in lesions including ciliary damage of the oviducts.

In the present study, although both the chicken and turkey strains showed similar *in vitro* pathogenicity, the turkey strain was selected for use in *in vivo* studies due to its known *in vivo* replication in the turkey reproductive tract (Jones et al., 1988). In order to maximise the chances of APV adapting to the oviduct epithelium, the chicks were first treated with oestrogen, and when the size of oviduct increased notably by 5 days, the virus infection was given and the treatment continued every 4 days throughout the sampling. For comparison, an identical study was also conducted in turkey poults, the species in which replication of APV in mature oviduct is known (Jones et al., 1988; O'Loan & Allan, 1990).

Although APV infection resulted in clinical disease and replicated in the tracheas of both species, the results of ciliary damage, virus isolation in TOC and IF staining failed to reveal any evidence of APV replication in precocious oviducts of either chickens or surprisingly, turkeys. The suitability of the virus detection methods could be questioned. However, TOC is the preferred system for primary isolation of APV from tissues of infected birds (McDougall & Cook, 1986; Buys et al., 1989a). Also, the sensitivity of IF for detecting APV in the trachea (Jones et al., 1986) and tissues of the reproductive tract (Jones et al., 1988) have been demonstrated previously.

The question of whether oestrogen could influence APV replication needed to be addressed. Oestrogen treatment of chicks or poults did not significantly alter the virus titres in their tracheas compared to titres in untreated birds. However, the effect of oestrogen treatment on APV replication in oviducts could not be evaluated in the same way, since appropriate oviducts could not be obtained from untreated birds at a very young age. Recently, in this laboratory, C.E. Savage (personal communication) found that a vaccine strain of APV was difficult to grow in rapidly dividing vero-cells which otherwise multiply in confluent monolayers. In the present work, the failure to detect APV replication in precocious oviducts even of poults might have been due to oestrogen-induced rapid multiplication of oviducal epithelial cells. In the *in vitro* study with precocious oviducts, the tissue had stopped multiplying, and virus was able to grow.

The adult female chickens used for the *in vivo* work were formerly part of an SPF laying flock maintained in this department. The flock had been kept for longer than normal and egg production was in decline at 22 months of age. Two routes of virus infection were chosen. The O/N route simulated a natural means of infection, but other birds were inoculated IV. The latter was intended to induce viraemia without prior respiratory replication. Also, it would guarantee high levels of virus in the blood stream for distribution to all sites.

Despite these two approaches, APV replication in the ovary or oviduct could not be demonstrated by virus isolation and/ or IF, even though respiratory infection and seroconversion did occur in both groups. In a recent study conducted elsewhere (J.K.A. Cook, personal communication), the pathogenicity of a chicken strain of APV for oviducts of hens at peak of lay was evaluated in similar manner. Following natural infection, APV was not detected in the oviduct, however, IV inoculation of this strain revealed APV replication in the epithelium of the oviduct as demonstrated by immunoperoxidase staining.

In the present study, the hens were markedly older and atrophy of reproductive tract in about 20% of birds was seen at necropsy. Thus age difference might be one explanation for our failure to demonstrate the virus in oviducts, even after infection via IV route. However, the results of *in vitro* experiments of this study with tissues from hens of the same group had shown the mature oviducts to be innately susceptible to APV infection.

The difference in findings between the results of J.K.A. Cook and those presented here could also be related to virus strain differences. Although, the in *vitro* work in the present study, showed similar pathogenicity of both the chicken and turkey strain. In a previous study, Cook et al. (1993a) reported some differences in biological behaviour of a chicken and a turkey strain. It was found that these strains caused more marked disease in their homologous species. Perhaps the use of the chicken strain would have resulted in virus replication in the chicken oviduct.

In conclusion, from the results of this and the Cook study, it seems that while the epithelium of the chicken oviduct is innately susceptible to APV infection with strains of both the chicken and turkey origin, it may remain unaffected after infection via a natural route. This brings into question whether APV infection in commercial hens causes suspected falls in egg production simply through the stress of the respiratory disease rather than virus itself multiplying in the reproductive tract. What prevents the APV multiplying in the reproductive tract of chickens following infection via natural route is unknown.

CHAPTER 8

DUAL INFECTION OF TRACHEAL ORGAN CULTURES AND CHICKS WITH AVIAN PNEUMOVIRUS AND INFECTIOUS BRONCHITIS VIRUS

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CHAPTER 8

DUAL INFECTION OF TRACHEAL ORGAN CULTURES AND CHICKS WITH AVIAN PNEUMOVIRUS AND INFECTIOUS BRONCHITIS VIRUS

8.1. INTRODUCTION

Infectious bronchitis virus (IBV) is the cause of a highly infectious and contagious respiratory disease of chickens. Besides causing disease of the respiratory tract, the virus may also affect the oviduct, kidneys and other tissues (Jordan, 1996). Intercurrent infections with certain bacteria and mycoplasma predispose chickens to more severe and prolonged respiratory disease (Jordan, 1996).

The interaction of IBV with other respiratory viruses of chickens has been variable. Aerosol vaccination with IBV increased the mortalities due to infectious laryngotracheitis in chickens (Pattison et al., 1971). IBV strains have been shown to interfere with Newcastle disease virus (NDV) growth in cell culture (Beard, 1967), chicken embryos (Raggi et al., 1963) and chickens (Raggi & Lee, 1964). When administered simultaneously, live IBV vaccines have been reported to interfere with live NDV vaccines by resulting in less protection of chickens against Newcastle disease challenge (Raggi & Lee, 1964; Thornton & Muskett, 1975).

Avian pneumovirus causes mild respiratory disease in chickens and sometimes it is associated with swollen head syndrome (SHS) (Alexander, 1997). There have been examples of the occurrence of both APV and IBV together in respiratory infections of chickens. Gough et al.(1994) isolated a pneumovirus from SHS-affected broiler chickens which had seroconverted to a IBV variant strain 793/B. Likewise, Capua et al. (1994) recorded a severe natural outbreak of a IBV strain 624/I in chickens, which had antibodies to APV.

In a recent longitudinal study of commercial broiler flocks affected with respiratory disease despite the use of both IBV Massachusetts (Mass) type and APV vaccines, the polymerase chain reaction (PCR) detected IBV (Mass) or IBV variants throughout the broiler's life, with APV appearing in the last days before slaughter (D. Cavanagh & others, personal communication). However, no experimental work on the interaction of APV and IBV has been reported.

The present study was therefore undertaken to investigate the effects of these two agents in dual infections of tracheal organ cultures (TOC) *in vitro* and of chicks *in vivo*.

PART I: DUAL INFECTION OF TOC

8.2. MATERIALS AND METHODS

8.2.1. Viruses

IBV strain Mass (M-41) (Ambali & Jones, 1991) was used. This strain has been maintained in this laboratory for several years and underwent numerous passages in TOC. Before use, it was passaged once in TOC, followed by a passage in SPF embryonated chicken eggs, then 48 hours post infection (pi), allantoic fluid was harvested and titrated in TOC as described in Chapter 3.

APV strain #8544 (Wilding et al., 1986) described in Chapter 3 was used. Before use in this study, it was also titrated in TOC.

8.2.2. Tracheal organ culture

TOC prepared from 19-day old embryonated SPF chicken eggs (Chapter 3) were used for studying dual infection with APV and IBV.

8.2.3. Experimental design

The effect of dual infection of APV and IBV in TOC was evaluated by (i) measurement of ciliary activity and (ii) immunofluorescence (IF) staining to detect the viral antigens.

Measurement of ciliary activity

For studying single or dual infections, TOCs were divided into ten groups (i) 1 uninfected controls, (ii) 2 single infections and (iii) 7 dual infections. Dual infections were done simultaneously or APV was given prior to IBV at 24, 48 or 72 h intervals, or IBV was given prior to APV also at 24, 48 or 72 h intervals. Two experimental trials were performed, one with equal input doses i.e. 3.5 $\log_{10}$ CD_{50} each of IBV and APV, and the second with a lower IBV dose i.e. 1.5 $\log_{10}$ and 3.5 $\log_{10}$ of APV. A volume of 100 µl of each virus was used for infecting the TOCs. In single infections, for equalising the input dose, 100 µl of TOC medium was added. The uninfected controls were mock-infected with 200 µl of TOC medium.

End-points were calculated as per the method of Power & Jordan (1976). Briefly, following infection, all TOCs were examined daily and the day on which complete loss of ciliary activity occurred was recorded for each TOC section, for computing the time taken for 50% (10 of the 20) of sections to lose the ciliary activity.

Immunofluorescence staining

The protocol for studying single or dual infections by IF staining was similar to that described above for measurement of ciliary activity. IF staining was performed on unfixed TOC rings for demonstration of the viral antigens following the method of Bhattarcharjee et al. (1994) described in Chapter 3. APV-specific antigens were detected using hyperimmune serum against APV strain 8544 and for detecting IBV-specific antigens, hyperimmune serum against IBV strain M-41 was used. IF staining was performed daily for 7 days pi and each time five TOCs were used for each infection group. The fluorescence of epithelial cells was scored on 0 to 3 scale by the method of Dhinakar Raj & Jones (1996a) (see Chapter 7).

8.3. RESULTS

8.3.1. Loss of ciliary activity

Figs. 8.1 to 8.3 show the time taken in days for ciliostasis in 50% of TOC sections infected with APV (constant dose) and/ or IBV (two doses). There was no ciliostasis in the mock-infected TOC (not shown). The ciliostatic end-point for IBV alone was considerably shorter than for single APV infection even with a 100 times lower IBV dose (Fig. 8.1). Dual infection of TOC either simultaneously (Fig. 8.1) or where IBV preceded APV (Fig. 8.2), both at equal input doses or with a lower IBV dose, resulted in the end-points at similar times compared to the respective IBV controls. This suggested that there was no effect of APV on IBV growth in TOC, and that IBV was the predominant virus.

When APV infection preceded that of IBV the results varied with regard to inoculation dose (Fig. 8.3). At equal doses, the time taken for dual infections to reach 50% ciliostasis was shorter than that for the APV control, even when IBV was inoculated 72 h following APV infection. This indicated that IBV was still able to grow in TOC, despite prior APV infection. At the lower IBV dose, the time taken for dual infections was shorter than the APV control if the interval between infections was 24 h, similar if it was 48 h and slightly longer if the gap was 72 h. This showed that with APV 100 times in excess, it could interfere with the growth of IBV if its infection preceded that of IBV by a sufficient time interval.

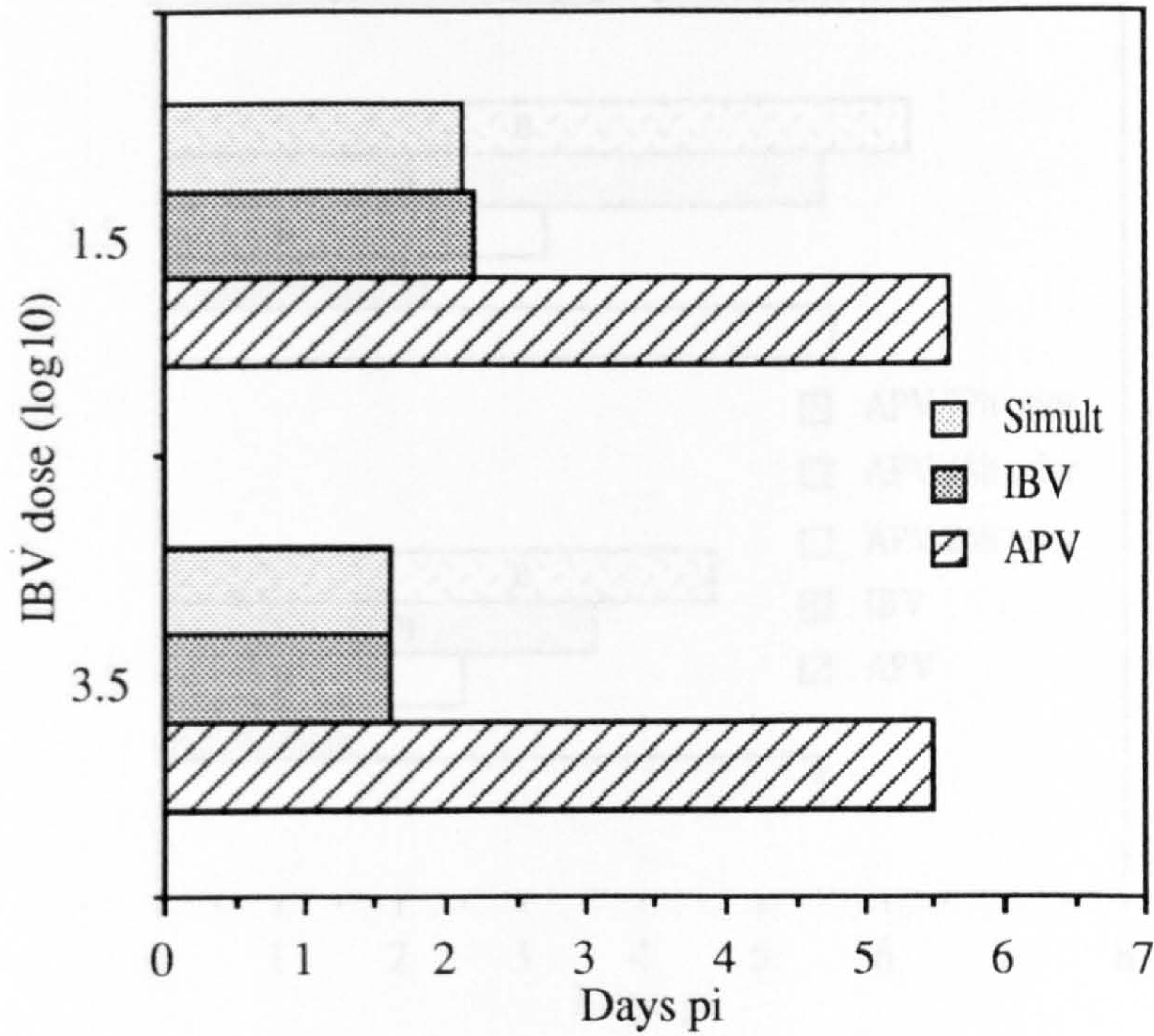

Fig. 8.1. Time taken for ciliostasis in 50% of TOC sections after single infection with APV or IBV or dual infection simultaneously.

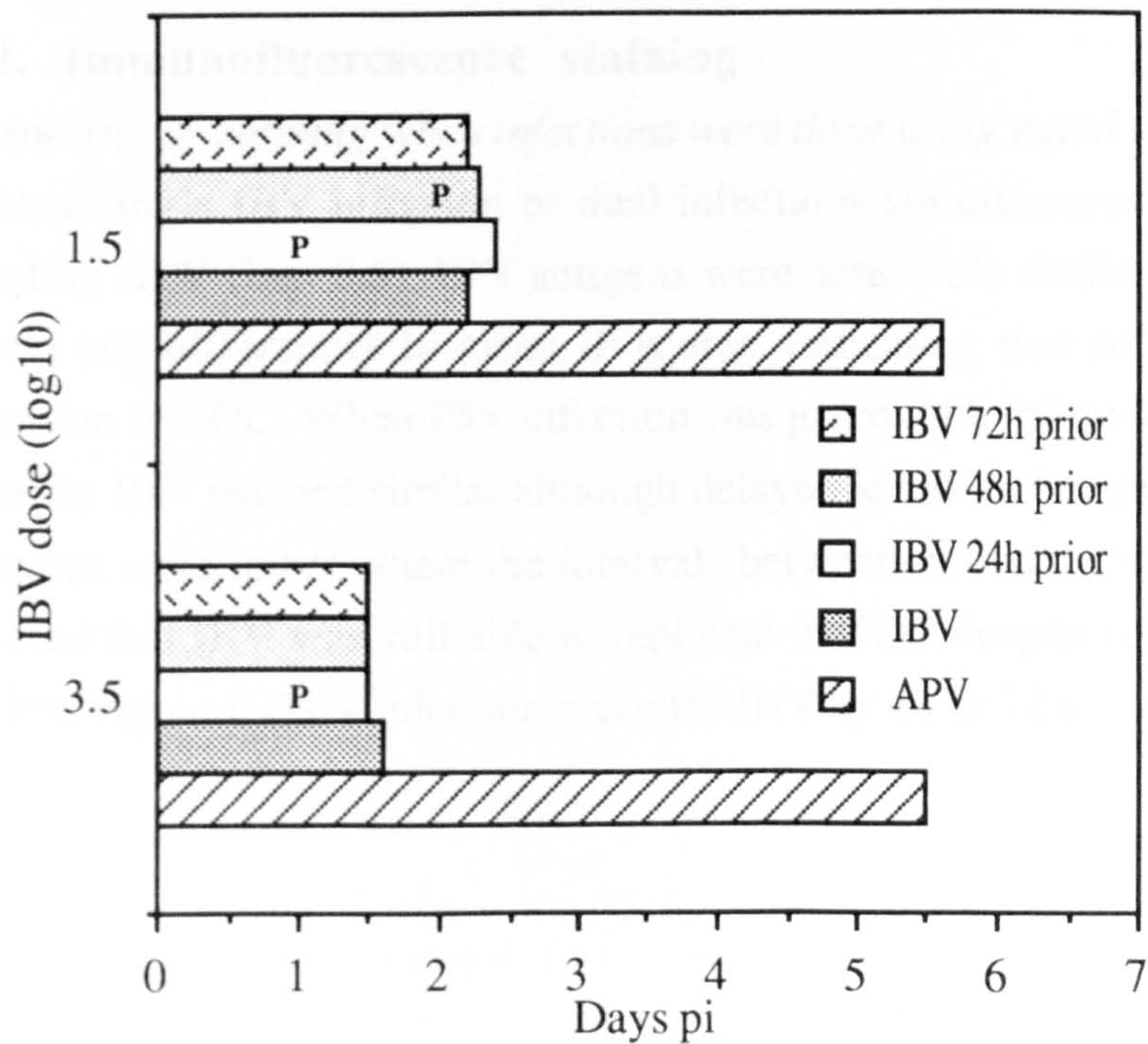

Fig. 8.2. Time taken for ciliostasis in 50% of TOC sections after single infection with APV or IBV or dual infection with IBV preceding APV. **P**: time of APV inoculation.

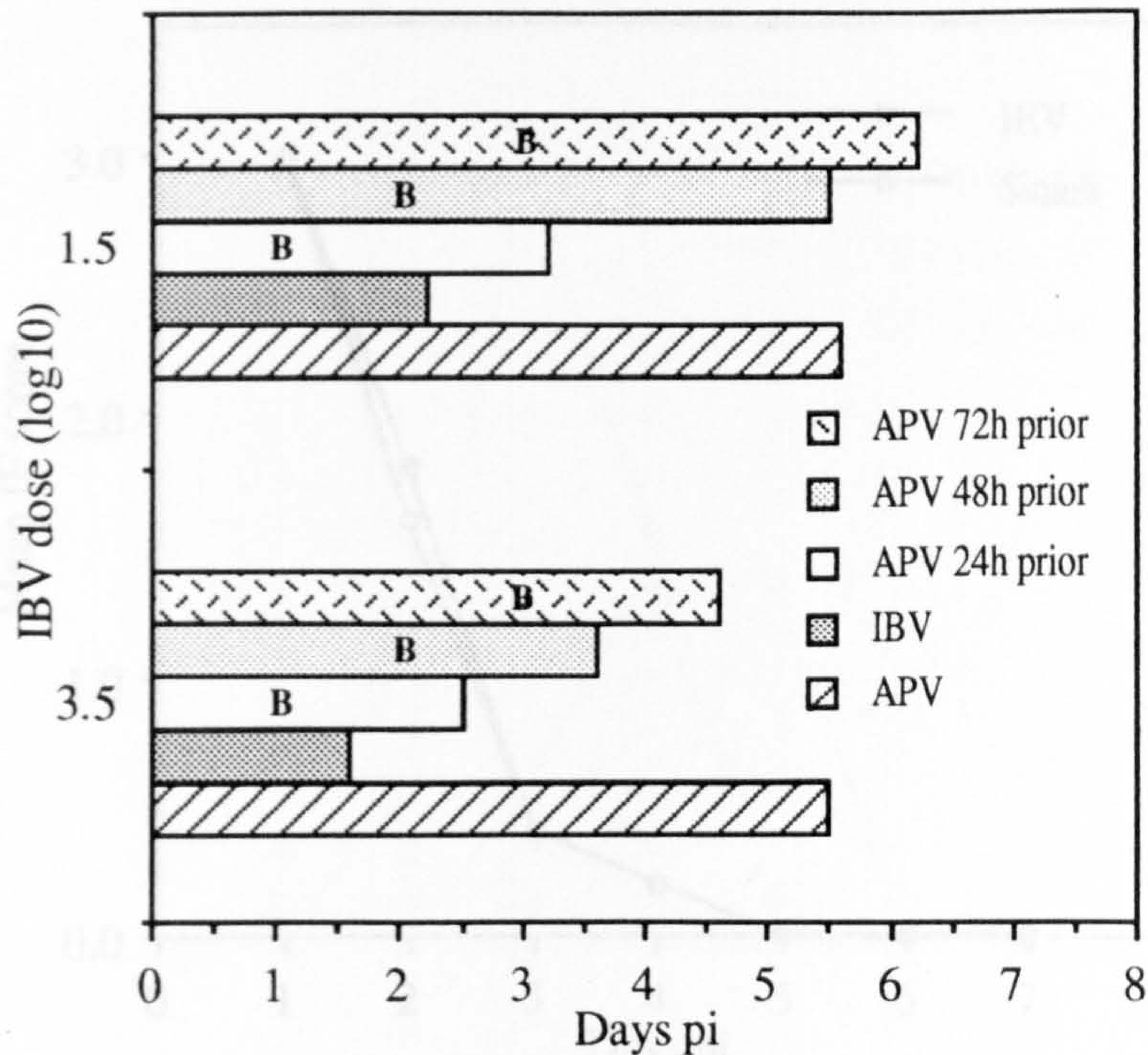

Fig. 8.3. Time taken for ciliostasis in 50% of TOC sections after single infection with APV or IBV or dual infection with APV preceding IBV. **B**: time of APV inoculation.

8.3.2. Immunofluorescence staining

IBV-specific IF staining when infections were done using equal doses of either virus

In either single IBV infection or dual infections simultaneously (Fig. 8.4) and IBV preceding APV (Fig. 8.5), IBV antigens were detectable similarly from days 1 to 4 pi without any differences in mean IF scores, indicating that APV did not affect IBV replication in TOC. When IBV infection was given 24 h following APV (Fig. 8.6), the scores for IBV reached similar although delayed levels as for single IBV infection, but the scores were lower where the intervals between infections were 48 and 72 h. This indicated that IBV was still able to replicate in TOC despite prior APV infection but with less vigour if APV infection preceded IBV by 48 or 72 h.

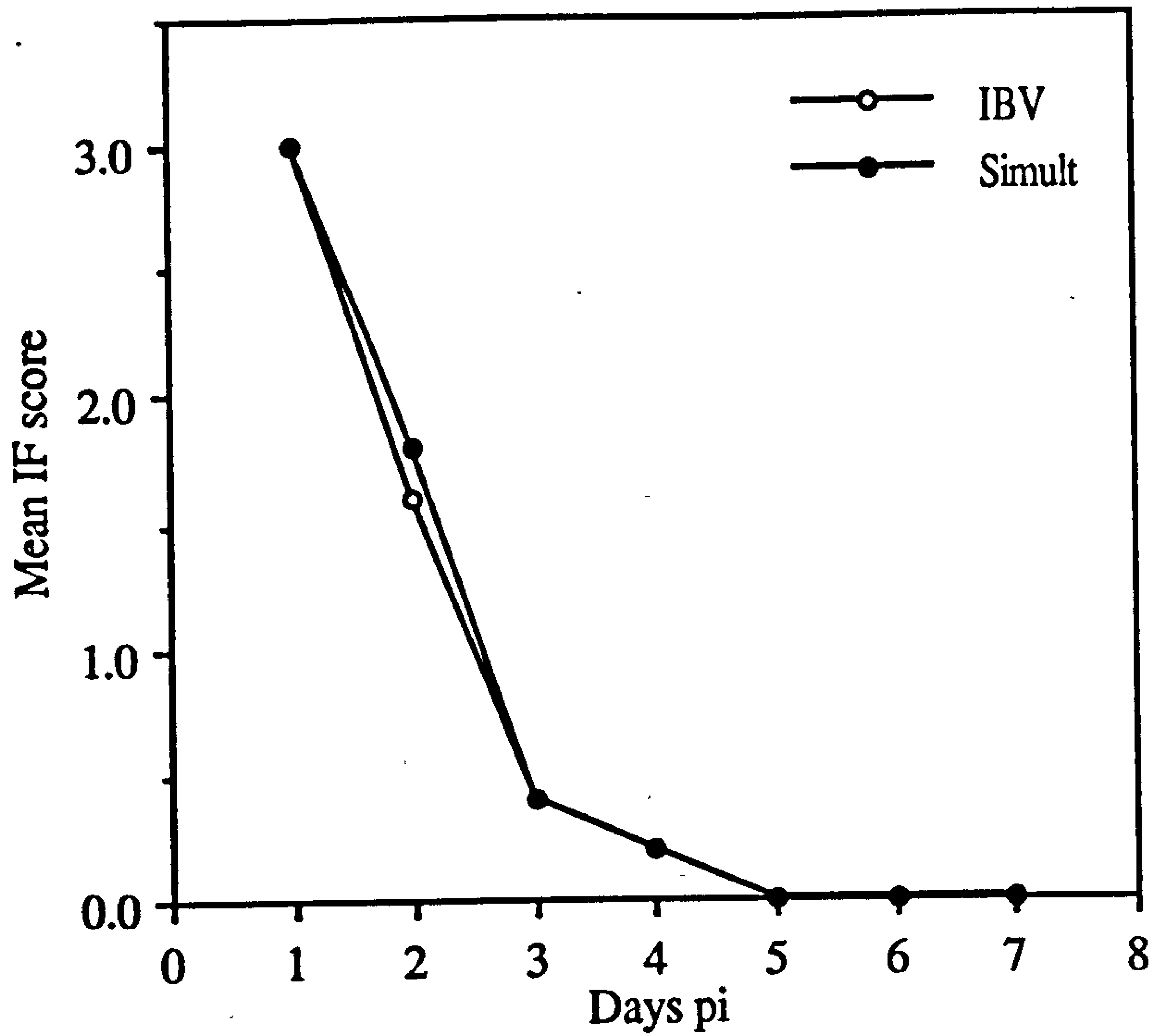

Fig. 8.4. IBV IF scores of TOC following single infection with IBV or dual infection with APV and IBV simultaneously at equal doses.

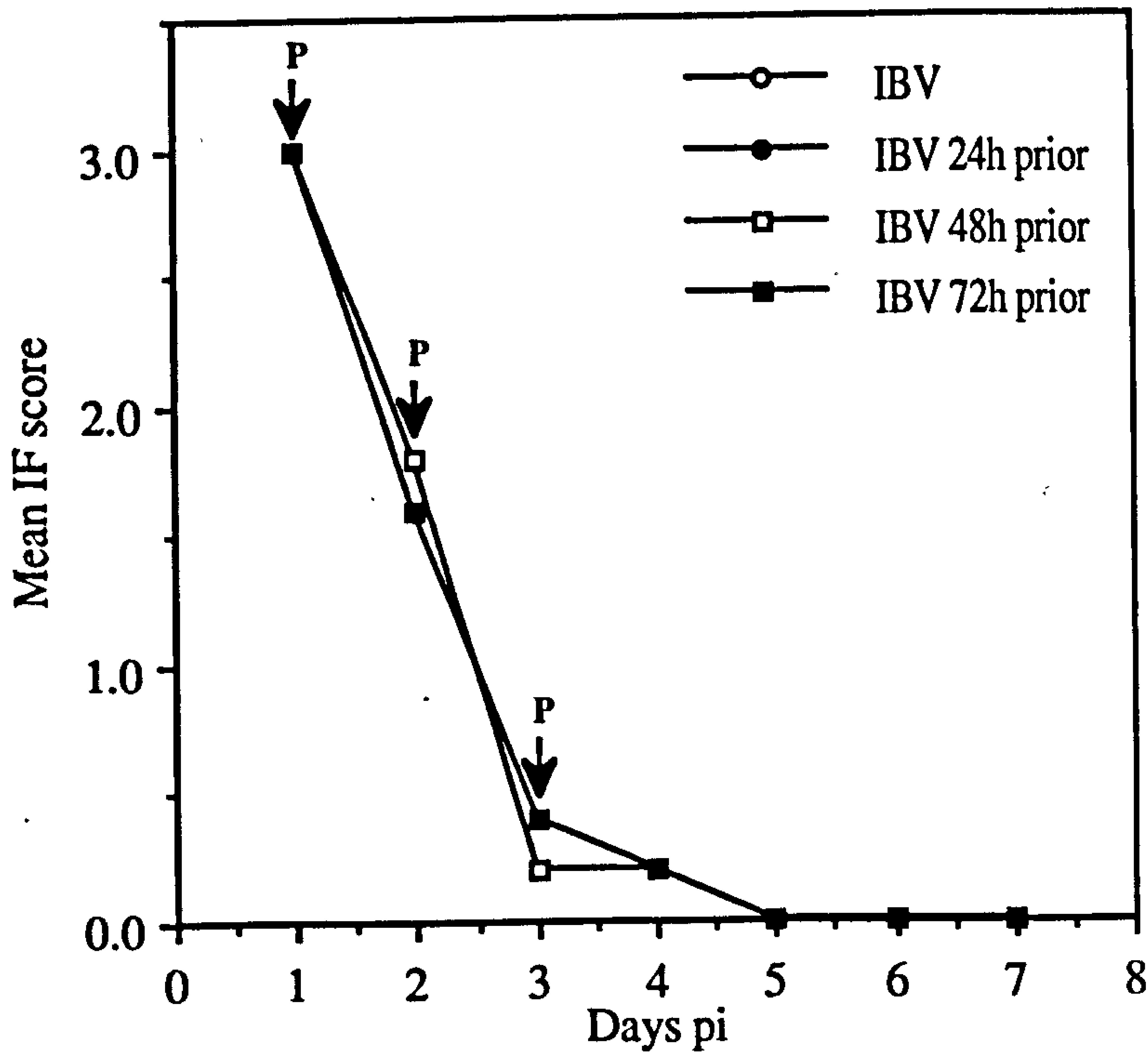

Fig. 8.5. IBV IF scores of TOC following single infection with IBV or dual infection with IBV preceding APV at equal doses. P: time of APV inoculation.

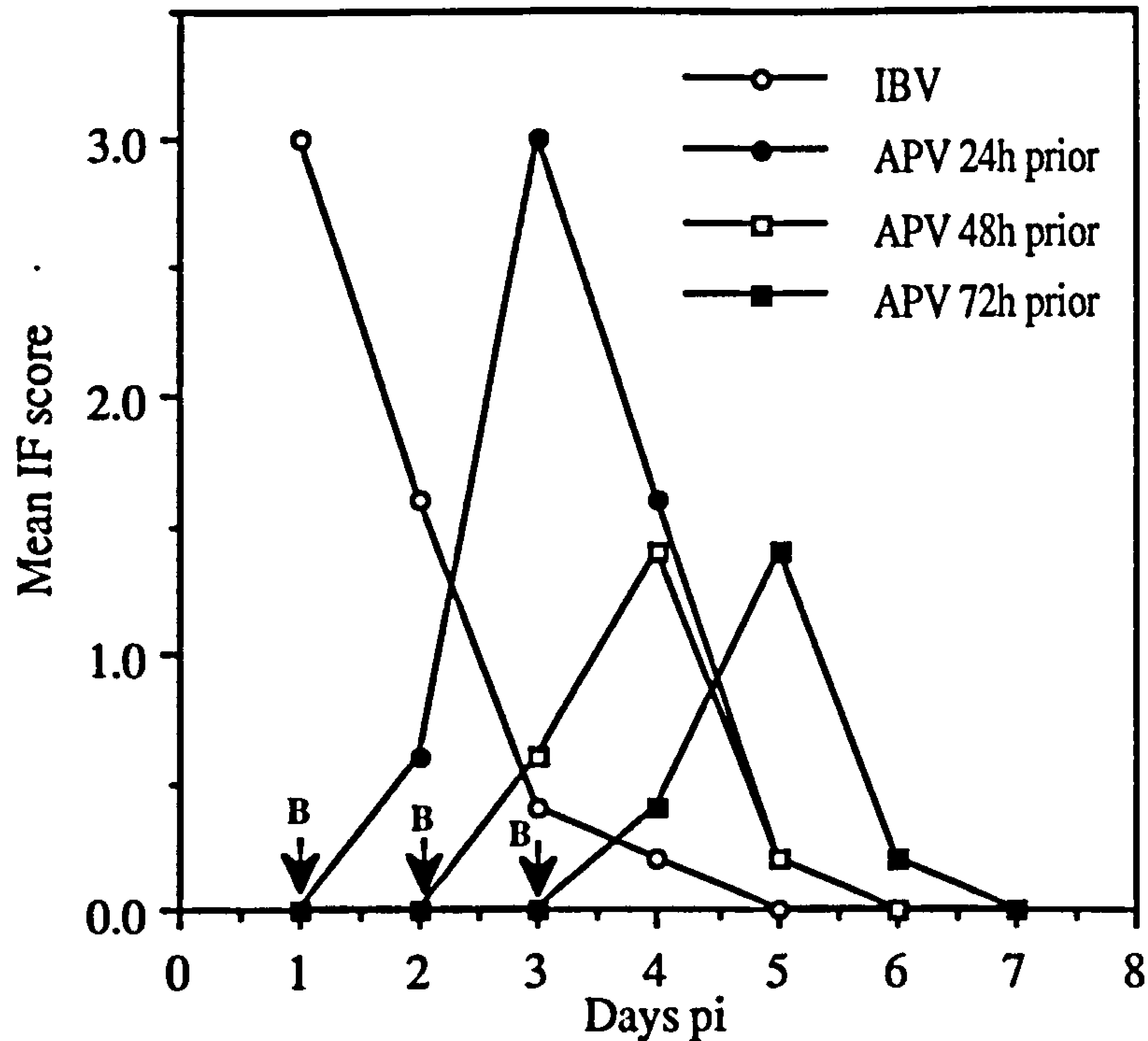

Fig. 8.6. IBV IF scores of TOC following single infection with IBV or dual infection with APV preceding IBV at equal doses. **B**: time of IBV inoculation.

APV-specific IF staining when infections were done using equal doses of either virus
After single infection (Fig. 8.7 or 8.8 or 8.9), APV was detected from days 1 to 7 pi, but in dual infections either simultaneously (Fig. 8.7) or with IBV preceding APV (Fig. 8.8), it was detected for one day only, indicating that IBV interfered with the APV replication. Where APV infection was given prior to IBV (Fig. 8.9), again IBV interfered with APV replication, even when APV infection preceded IBV by 72 h, as shown by the rapid reduction in the mean IF scores for these groups compared to APV control.

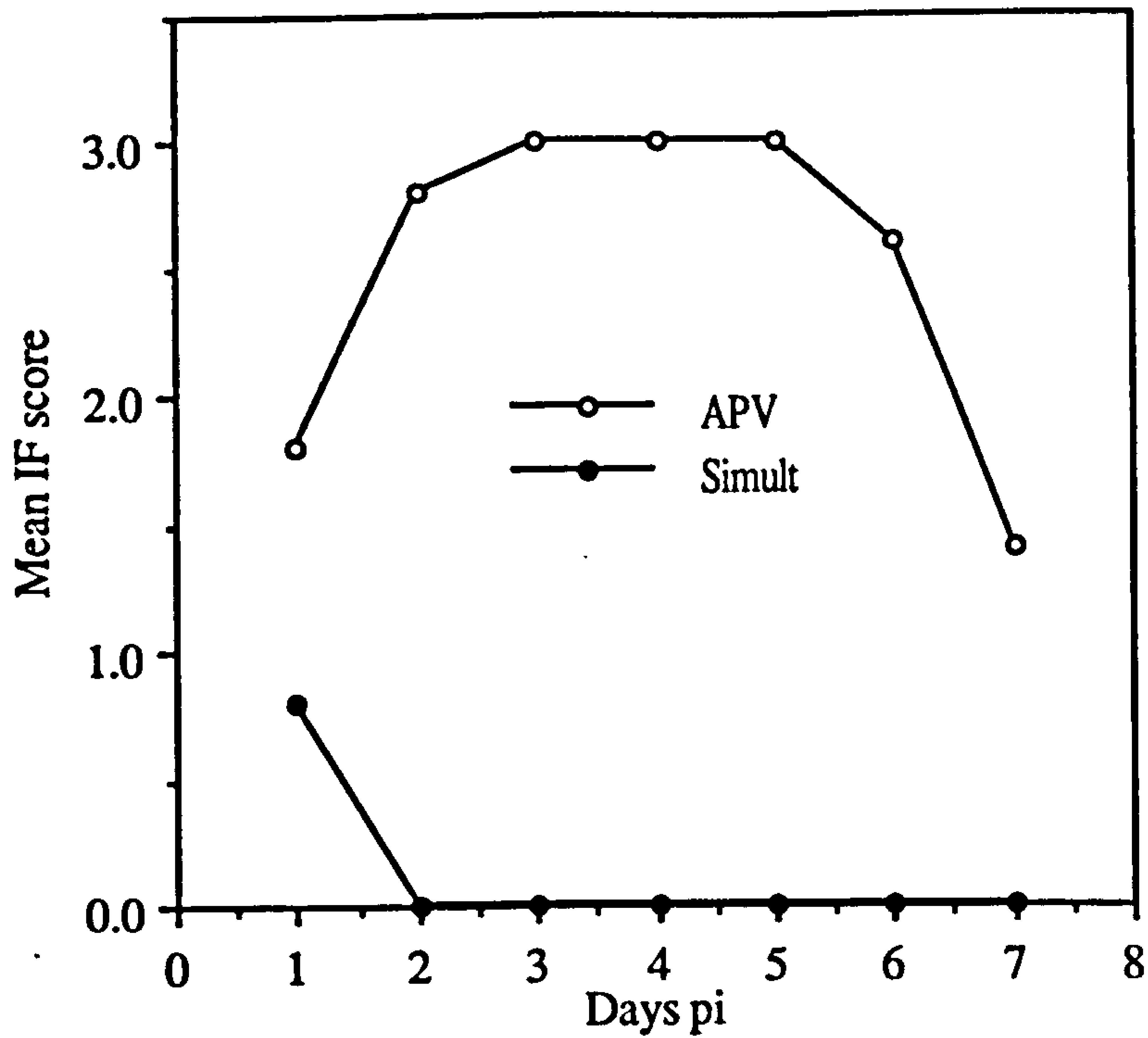

Fig. 8.7. APV IF scores of TOC following single infection with APV or dual infection with APV and IBV simultaneously at equal doses.

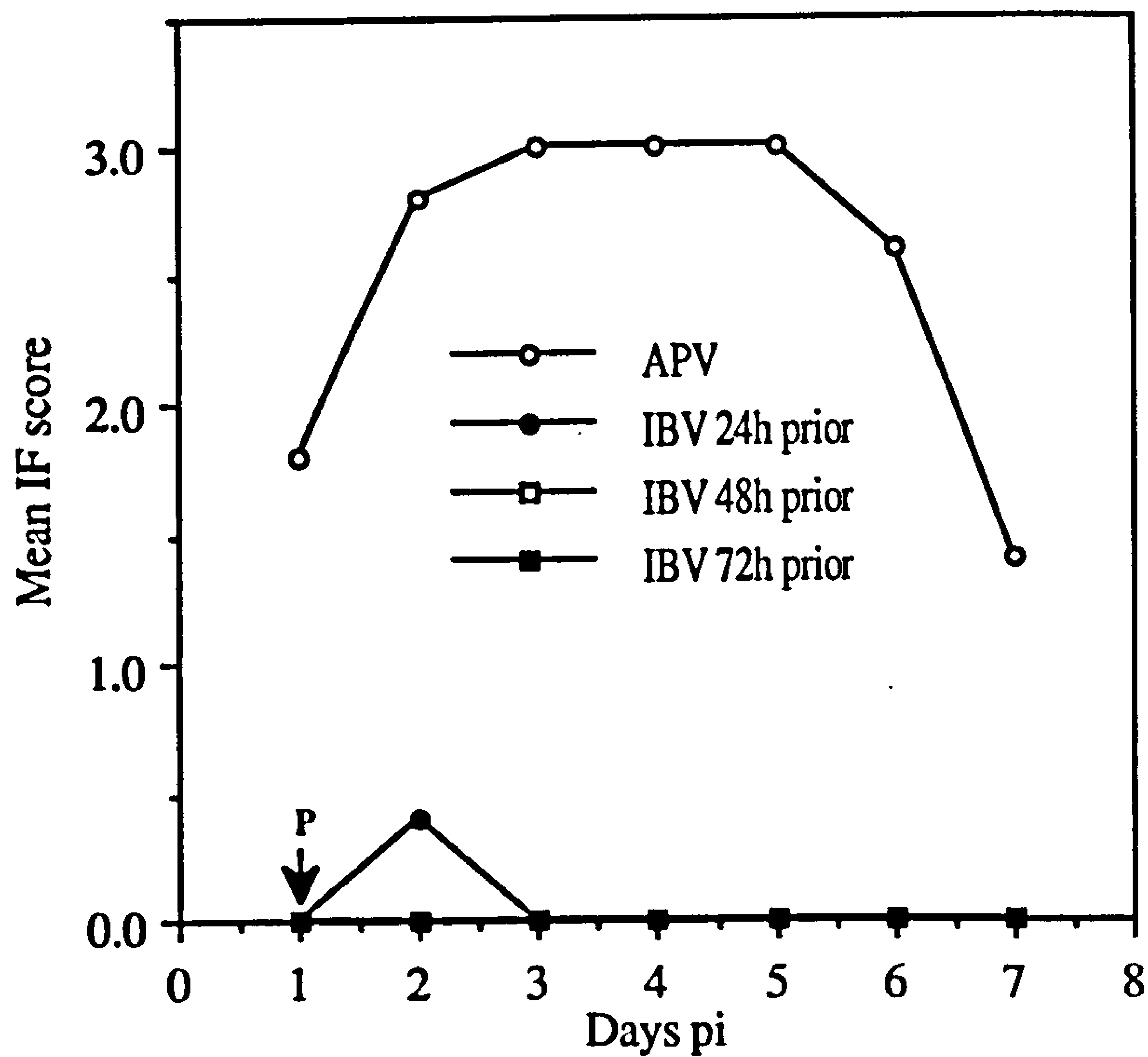

Fig. 8.8. APV IF scores of TOC following single infection with APV or dual infection with IBV preceding APV at equal doses. P: time of APV inoculation.

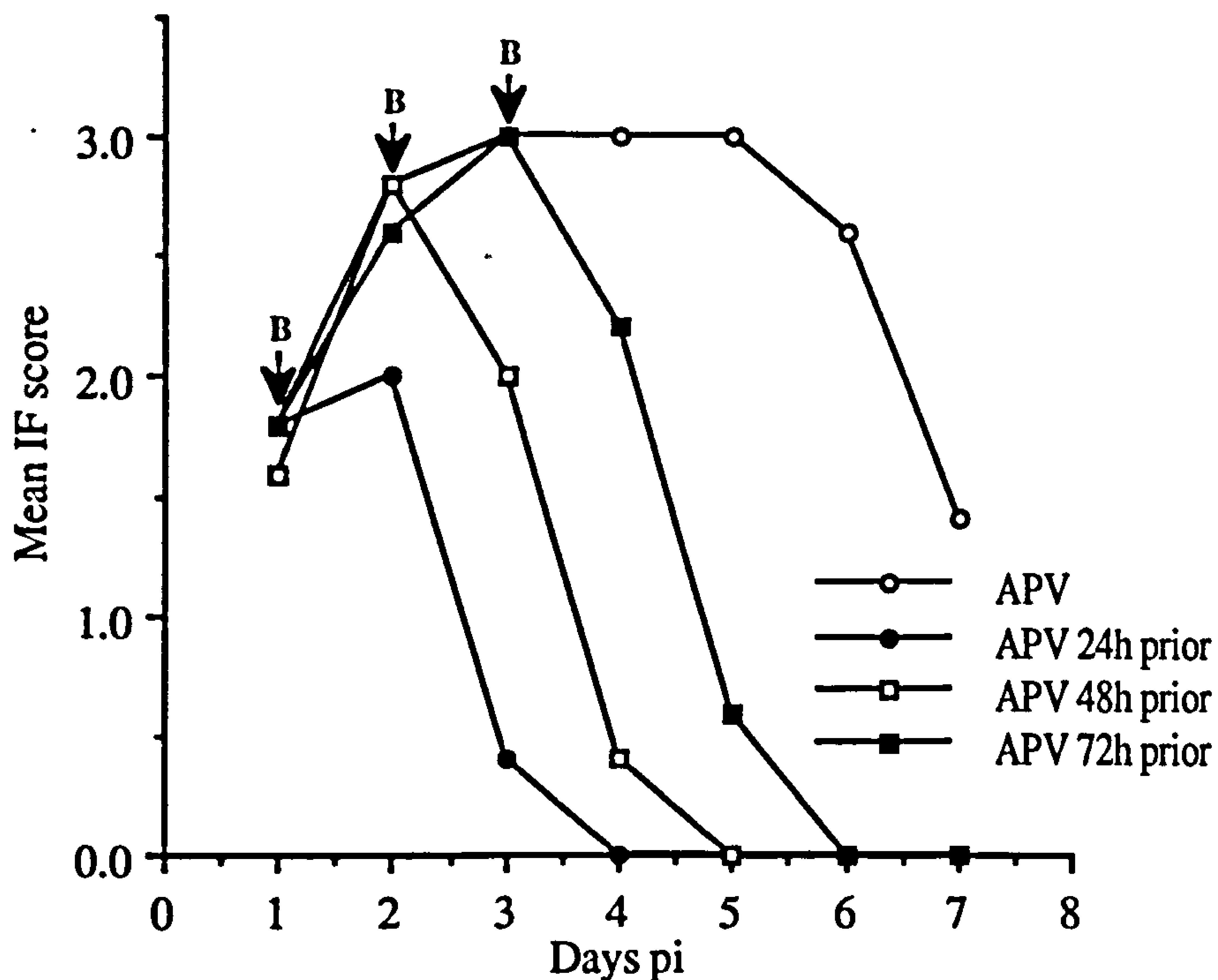

Fig. 8.9. APV IF scores of TOC following single infection with APV or dual infection with APV preceding IBV at equal doses. B: time of IBV inoculation.

IBV-specific IF staining when infections were done using a lower IBV dose

In the single infection (Fig. 8.10 or 8.11 or 8.12), IBV was detectable for longer than with the higher dose, i.e. up to day 6 pi due to the slower damage to tracheal epithelial cells. Following dual infections either simultaneously (Fig. 8.10) or when IBV preceded APV (Fig. 8.11), the mean IF scores and the times for which IBV was detected remained similar to that for IBV alone, indicating that APV, despite being in excess, did not affect IBV replication. Where APV infection preceded IBV (Fig. 8.12), it caused interference as shown by lower scores and shorter periods of IBV detection compared to the IBV control.

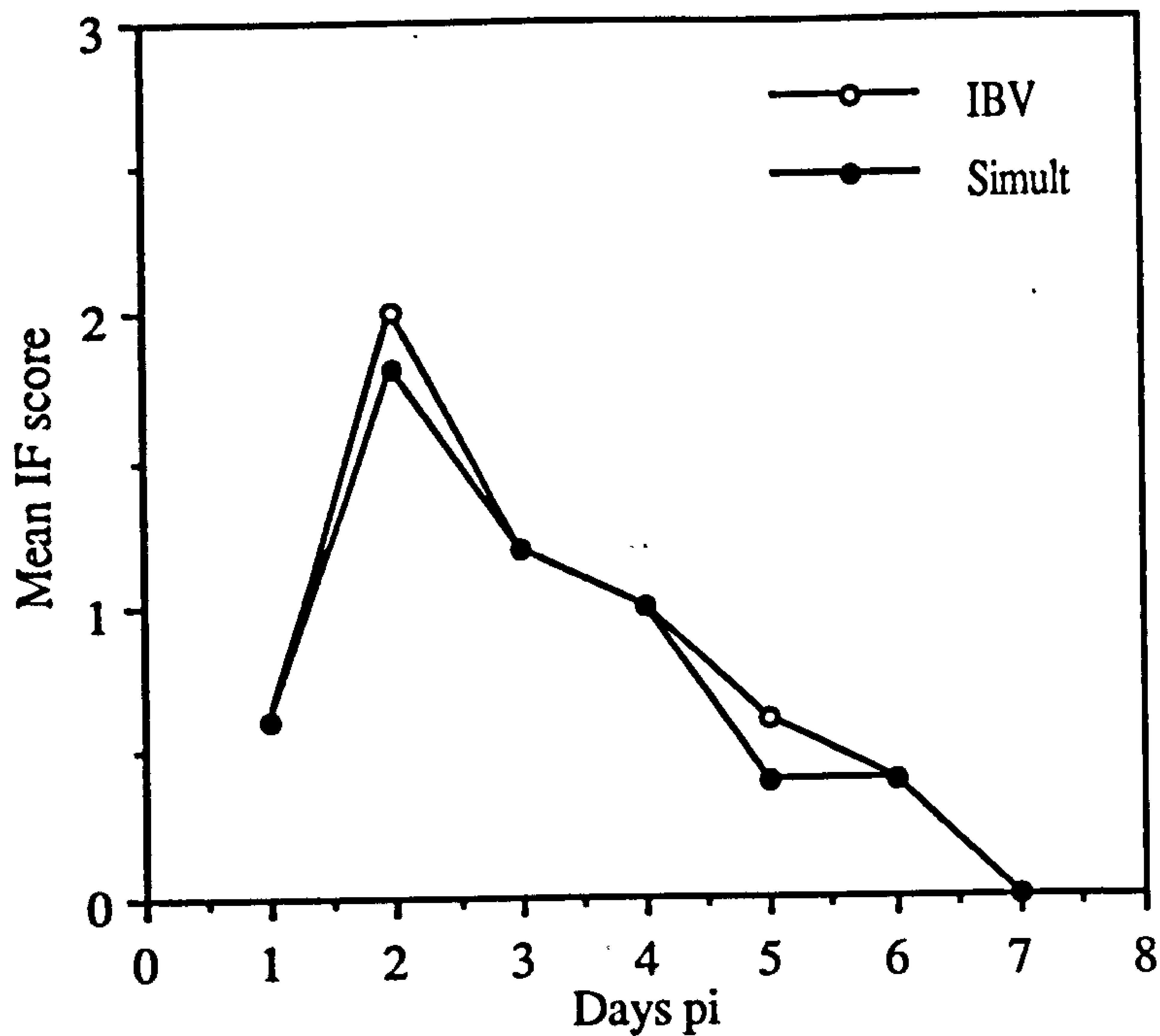

Fig. 8.10. IBV IF scores of TOC following single infection with IBV or dual infection with APV and IBV simultaneously at the lower IBV dose.

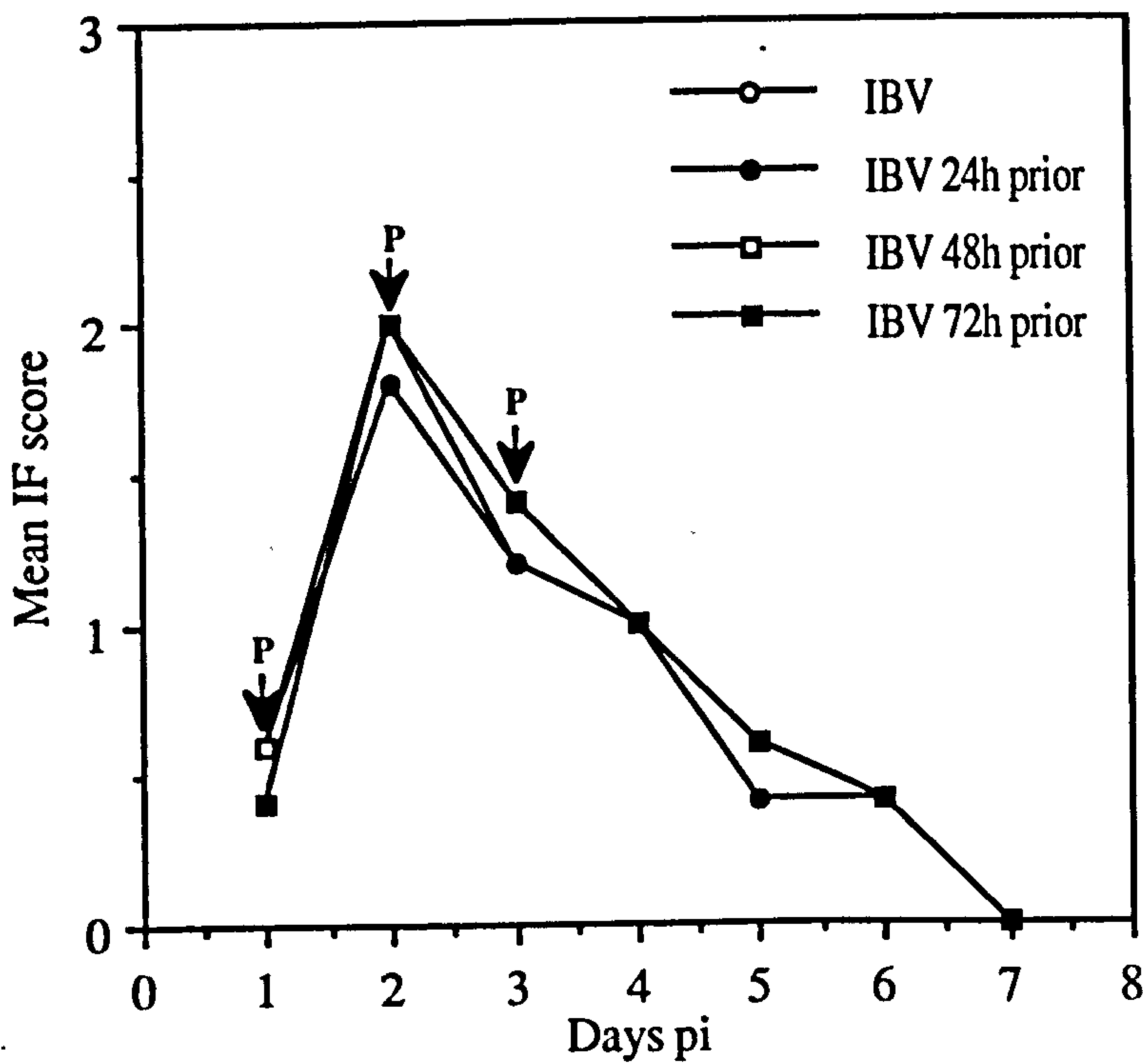

Fig. 8.11. IBV IF scores of TOC following single infection with IBV or dual infection with IBV preceding APV at the lower IBV dose. P: time of APV inoculation.

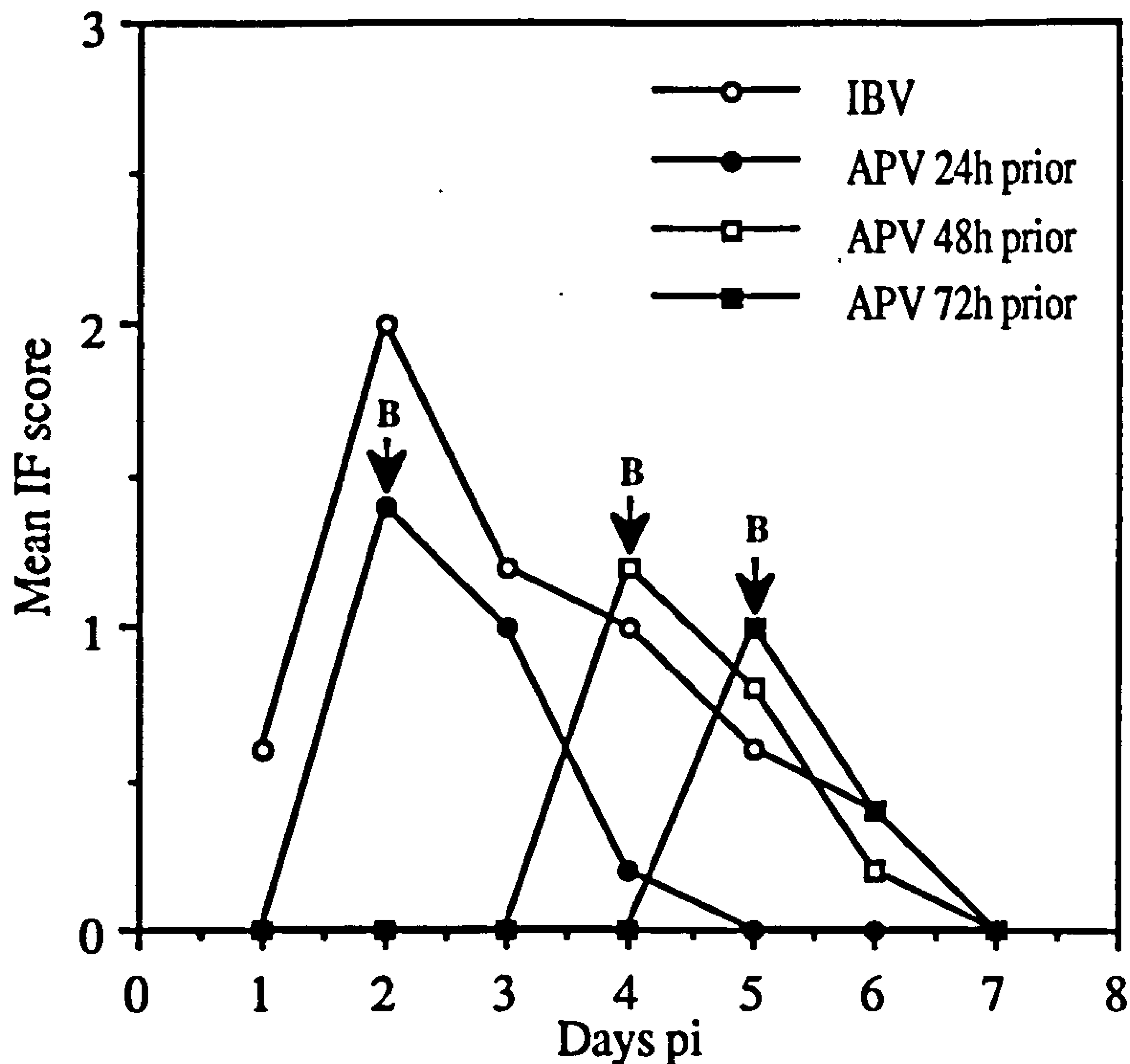

Fig. 8.12. IBV IF scores of TOC following single infection with IBV or dual infection with APV preceding IBV at the lower IBV dose. B: time of IBV inoculation.

APV-specific IF staining when infections were done using a lower IBV dose

In all dual infections (Fig. 8.13 to 8.15), APV antigens were detectable for considerably longer than in dual infections with equal doses, suggesting that APV had more chance to replicate when IBV was at the lower dose. However, the mean IF scores for APV were generally lower in all the dual infected groups compared to APV alone, probably due to interference by IBV even at the lower dose. IBV interference with APV replication was maximal when IBV infection occurred prior to APV (Fig. 8.14) and it was dependant on the time interval. The longer the time lag of APV inoculation following IBV, the greater was IBV-related interference.

No APV- or IBV-specific IF was observed in uninfected controls. Also no APV-specific antigens were detected in IBV controls groups and no IBV-specific antigens were detectable in APV controls (not shown).

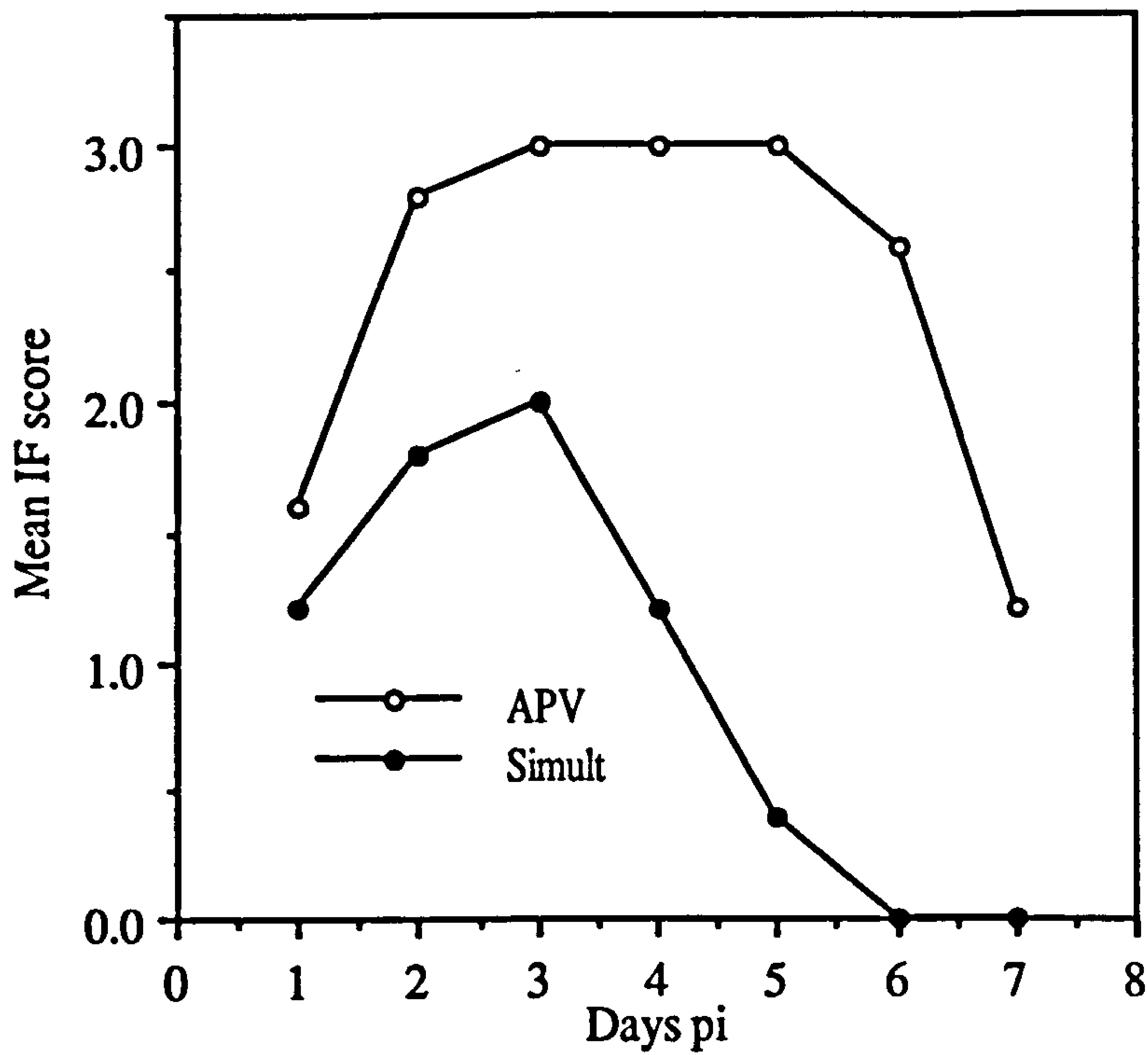

Fig. 8.13. APV IF scores of TOC following single infection with APV or dual infection with IBV and APV simultaneously at the lower IBV dose.

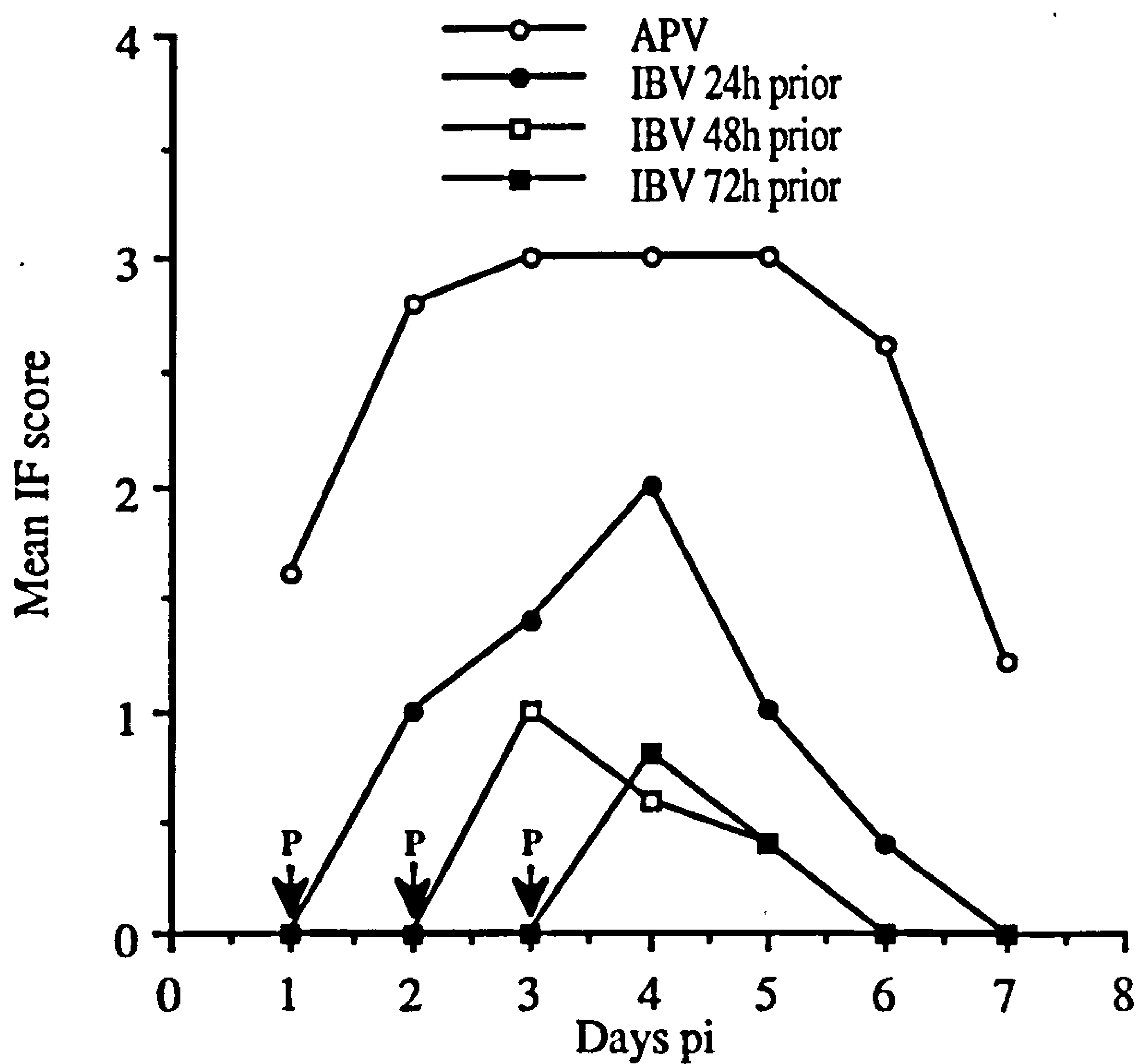

Fig. 8.14. APV IF scores of TOC following single infection with APV or dual infection with IBV preceding APV at the lower IBV dose. P: time of APV inoculation.

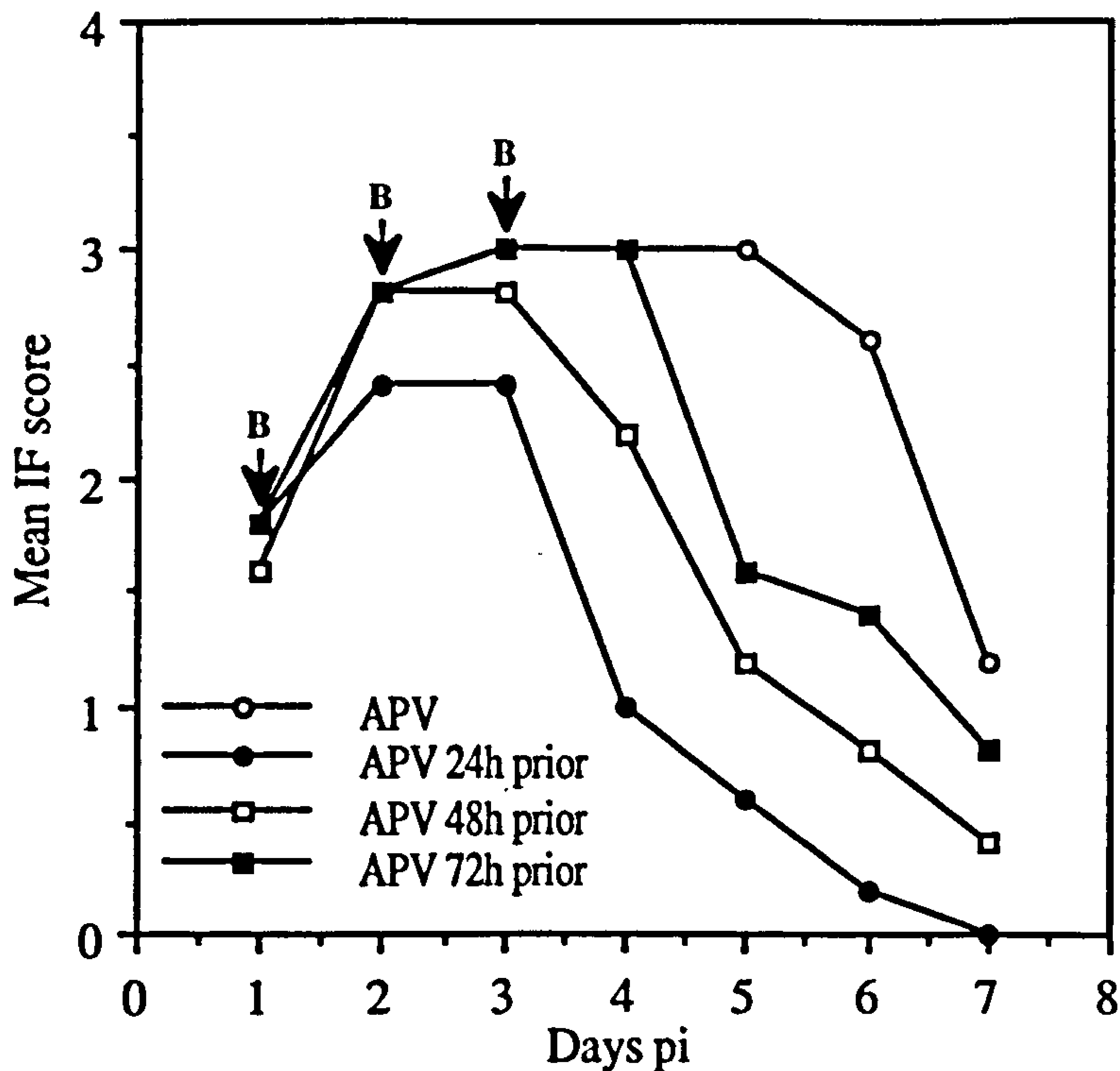

Fig. 8.15. APV IF scores of TOC following single infection with APV or dual infection with APV preceding IBV at the lower IBV dose. B: time of IBV inoculation.

PART II: DUAL INFECTION OF CHICKS

8.4. MATERIALS AND METHODS

8.4.1. Chicks

SPF chicken eggs from a commercial source were hatched in this laboratory and the chicks were maintained in complete isolation with food and water *ad libitum.*

8.4.2. Viruses

IBV strain M-41 and APV strain 8544 described in Materials and Methods of Part I of this chapter were used.

8.4.3. Experimental design

Two hundred and forty birds were divided into 6 groups (Table 8.1) comprising uninfected controls, APV control, IBV control and three dual infected groups viz.: APV infection 48 h prior to IBV, simultaneous infection of APV and IBV, and IBV infection 48 h prior to APV. Birds were infected oculonasally with either IBV, APV or both, at a dose rate of 3.5 log CD_{50}/0.1ml of either virus as shown in Table 8.1. For single

infections, the uninfected eye-nostril (EN) was inoculated with 0.1 ml of TOC medium (see Appendix). The uninfected controls were similarly inoculated with the medium.

Table 8.1. Experimental design

Group	Inoculum			
	5 day-old		7 day-old	
	Left EN*	Right EN	Left EN	Right EN
Uninfected control	–	–	–	–
APV control	–	–	–	APV
IBV control	–	–	IBV	–
APV prior to IBV	–	APV	IBV	–
Simultaneous	–	–	IBV	APV
IBV prior to APV	IBV	–	–	APV

*: eye and nostril.

Following infection, 10-15 birds in each group were examined daily for clinical signs. Five birds each in each group were identified for repeated tracheal swabbing for virus isolation and PCR-based detection. This was done on days 1, 3, 5, 7, 9, 12, 15, 19 and 21 pi (post infection = post infection at 7 day-old). On day 7 pi, seven birds from each infected group and 3 from uninfected controls were euthanased and necropsied. Pieces of lower trachea, lungs and kidneys were collected for virus isolation, and they were swabbed for PCR examination. Pieces of tracheas from 4 birds in each infected group and 2 from uninfected controls on days 1, 3, 5, 7, 9, 12, 15 and 21 pi were collected for IF staining and on days 3, 7, 12, 15 and 21 pi also for histopathology. Sera were collected from 6 birds in each group on days 7, 14 and 21 pi for measuring antibodies by enzyme-linked immunosorbent assays (ELISA) and serum neutralisation tests (SNT).

8.4.4. Clinical signs

The severity of clinical signs was scored on 0 to 3 scale following the method of Jones et al. (1992) as described in Chapter 3.

8.4.5. Virus isolation and titrations

Virus isolation

Tracheal swabs or tissues after collection, were processed as described in Chapter 3 and subsequently used for APV and IBV isolations in TOC. Since both viruses cause ciliostasis in TOC and if present together in the inoculum e.g. in the samples from dually infected birds, they can interfere with growth and detection of each other as shown by results of present work on dual infection of TOC. This difficulty was overcome by (i) early detection of both agents by IF staining before the ciliostatic effect and (ii) neutralising one virus by treating with its antiserum and identifying the other.

The early detection of viruses was based on an in *vitro* pilot study (not shown) where TOC were infected with the mixtures of APV and IBV, and IF staining on unfixed TOC was performed to detect viral antigens (for methods, see section 8.2.3). It was possible to detect both APV and IBV between 24 to 36 h following infection of TOC. Therefore, detection of both viruses in TOC infected with samples from this experiment was performed at 30 h following their inoculation in TOC.

The differentiation of viruses after neutralisation was based on another pilot experiment (not shown). A dilution of homologous antiserum against APV or IBV was adjusted to neutralise 4.0 $\log_{10}$ of the relevant virus in the inoculum containing a mixture of the two viruses and thus allowing heterologous virus to grow in TOC, which was then identified by IF staining. For processing of samples of this experiment, neutralisation of either virus was therefore performed using the pre-determined dilution of the respective antiserum. Equal volumes of both sample and antiserum were mixed and allowed to react for 30 min at room temperature. The resultant mixture was used for inoculating the TOC. Any sample with a ciliostatic component was passaged once further and identified by IF staining (see section 8.2.3).

Detection of viruses before the ciliostatic effect and after neutralisation was also compared with the routine method of isolation described in Chapter 3. Briefly, each sample was passaged three times in TOC and the ciliostatic virus was identified by IF staining using hyperimmune serum to APV and IBV.

Virus titrations

All the samples positive in virus isolation were pooled on each sampling occasion within the experimental group and titrated in TOC as described in Chapter 3. Since both APV and IBV in the samples from dually infected birds can contribute to the amount of virus measured by TOC, the titres are expressed as total ciliostatic virus titres instead of

APV or IBV titres. This was used as parameter to assess the severity of disease following dual infections.

8.4.6. Immunofluorescence

Pieces of tracheas after collection, were snap frozen in liquid nitrogen, stored at -70°C and processed for detection of virus-specific antigens by IF staining as described in Chapter 3. APV-specific antigens were identified by using antiserum to APV strain 8544 (Chapter 3) and for detection of IBV-specific antigens, hyperimmune serum against IBV strain M-41 was used. This was raised in 4-week old SPF chickens by inoculating oculonasally with 2 doses (3.5 $\log_{10}$ CD_{50} each) of strain M-41, 21 days apart and serum collected 2 weeks following the second inoculation of the virus.

8.4.7. Reverse transcription-PCR (Nested RT-PCR)

Tracheal swabs after collection from birds were allowed to dry at ambient temperature. Five swabs of each sampling occasion were pooled within the experimental group for extraction of RNA (Jing et al., 1993) and RT-PCRs were performed as described by Naylor et al. (1997a). For APV PCR, oligonucleotides from the G protein gene were selected and for IBV, oligonucleotides from S1 protein gene sequences were used. Two RT-PCR 1 reactions, one for APV using oligonucleotides G1+ and G6- and the other for IBV using oligonucleotides XCE1+ and XCE2-, were performed in 35 cycles. This gave DNA products of approximately 450 bp. These were used as a template in second, nested PCRs (35 cycles) using oligonucleotides G8+A and G5- for APV and oligonucleotides MCE1+ and XCE3- for IBV. This yielded DNA products of approximately 300 bp. These were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining.

8.4.8. Histopathology

Pieces of tracheas collected in 10% formalin were processed by conventional methods and the sections stained with haemotoxylin and eosin.

8.4.9. Serology

The antibody responses to both IBV and APV were determined using ELISA and SNT. ELISA for IBV was performed using a commercial IBV antibody test kit (Biochek C.V., Gouda, Holland) and the test conducted strictly as per manufacturer's instructions. Neutralising antibodies to IBV strain M-41 (100 CD_{50}) were determined in TOC following the method described by Darbyshire et al. (1979). The methods used for APV ELISA (Naylor et al., 1992) and SNT (Cook et al., 1988) are described in Chapter 3. Serum samples were tested individually by ELISAs and for SNTs, pooled samples were used.

8.4.10. Statistical analysis

Mean clinical scores and the total numbers of virus isolations in different groups were compared by chi-square test ($p<0.05$). The ELISA antibody titres between experimental groups were compared by student's t-test ($p<0.05$).

8.5. RESULTS

8.5.1. Clinical signs

Figs. 8.16 to 8.22 show the mean clinical scores of chicks following single or dual infections. After single infections, signs were more severe and prolonged with IBV than APV infection (Fig. 8.16). The mean scores for IBV-infected chicks remained higher than those given APV with significant differences between days 7 to 9 pi. The scores for both virus infections peaked at the same time, i.e. between days 4 to 6 pi. However the nasal exudate was profuse and more turbid in IBV-infected chicks.

There were no significant differences in the mean scores or durations of signs between IBV controls and the three dually infected groups, except during the intervals where infection by one virus preceded the other (Figs. 8.17 to 8.19).

As with single IBV infection, in all three dually infected groups, signs were more prolonged and the mean scores were higher than those in APV controls (Figs. 8.20 to 8.22), with significant differences on several occasions, including the intervals where infection by one virus preceded the other.

No clinical signs were recorded in uninfected controls (not shown).

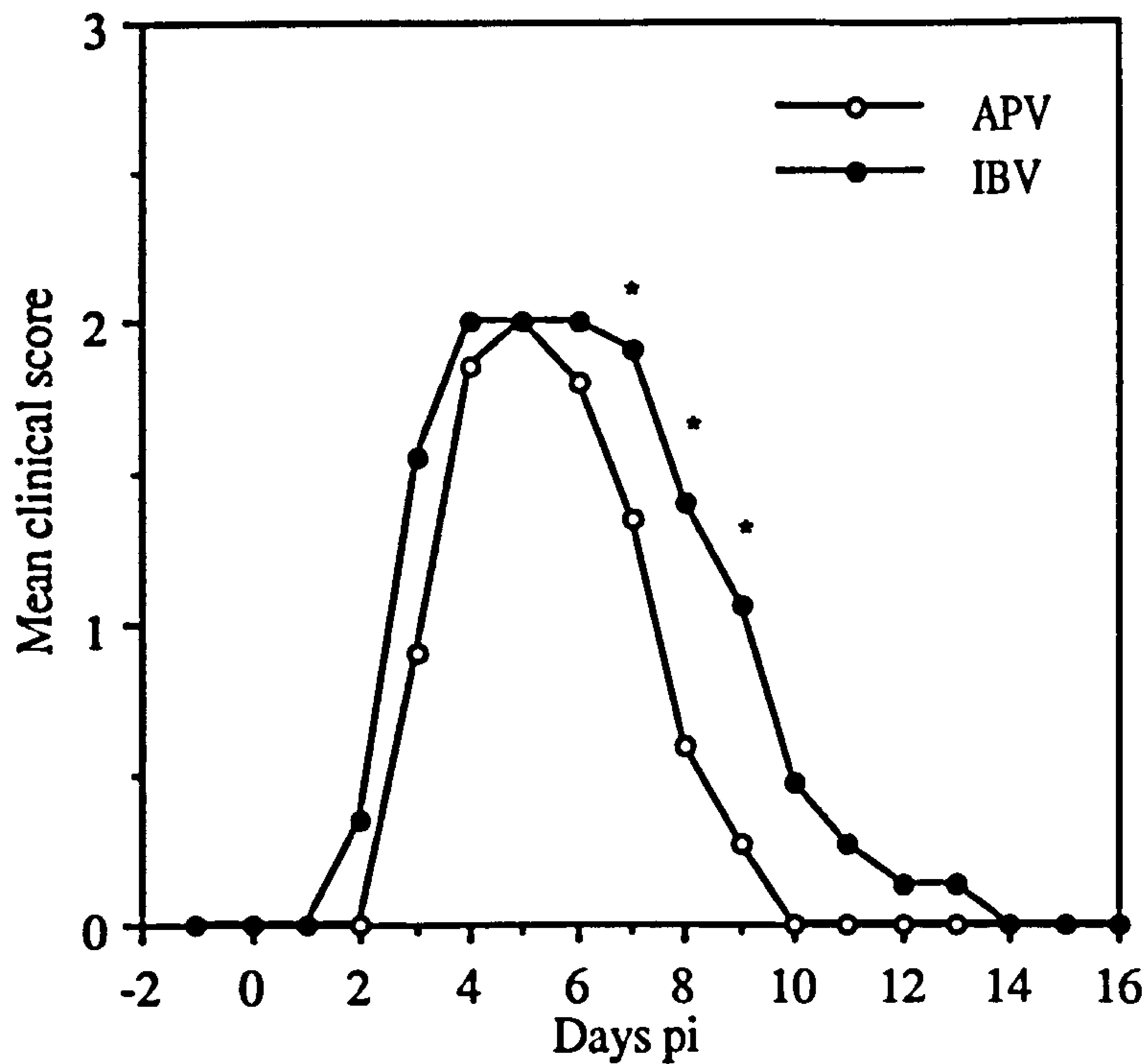

Fig. 8.16. Mean clinical scores of chicks following single infection with either APV or IBV. *: Significantly higher ($p<0.05$) scores in IBV infected chicks.

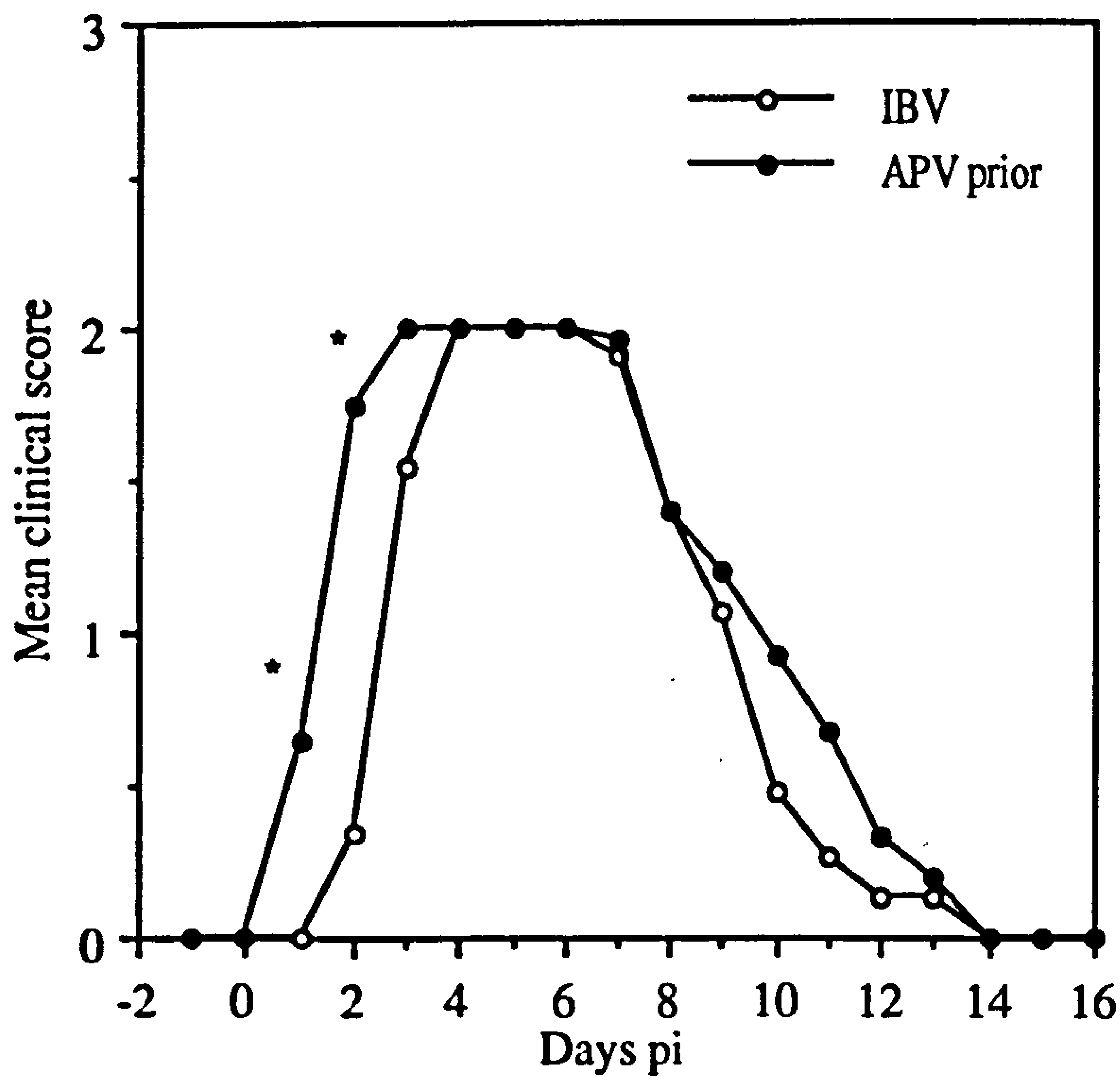

Fig. 8.17. Mean clinical scores of chicks following single infection with IBV or dual infection with APV preceding IBV. *: Significant differences ($p<0.05$) in scores.

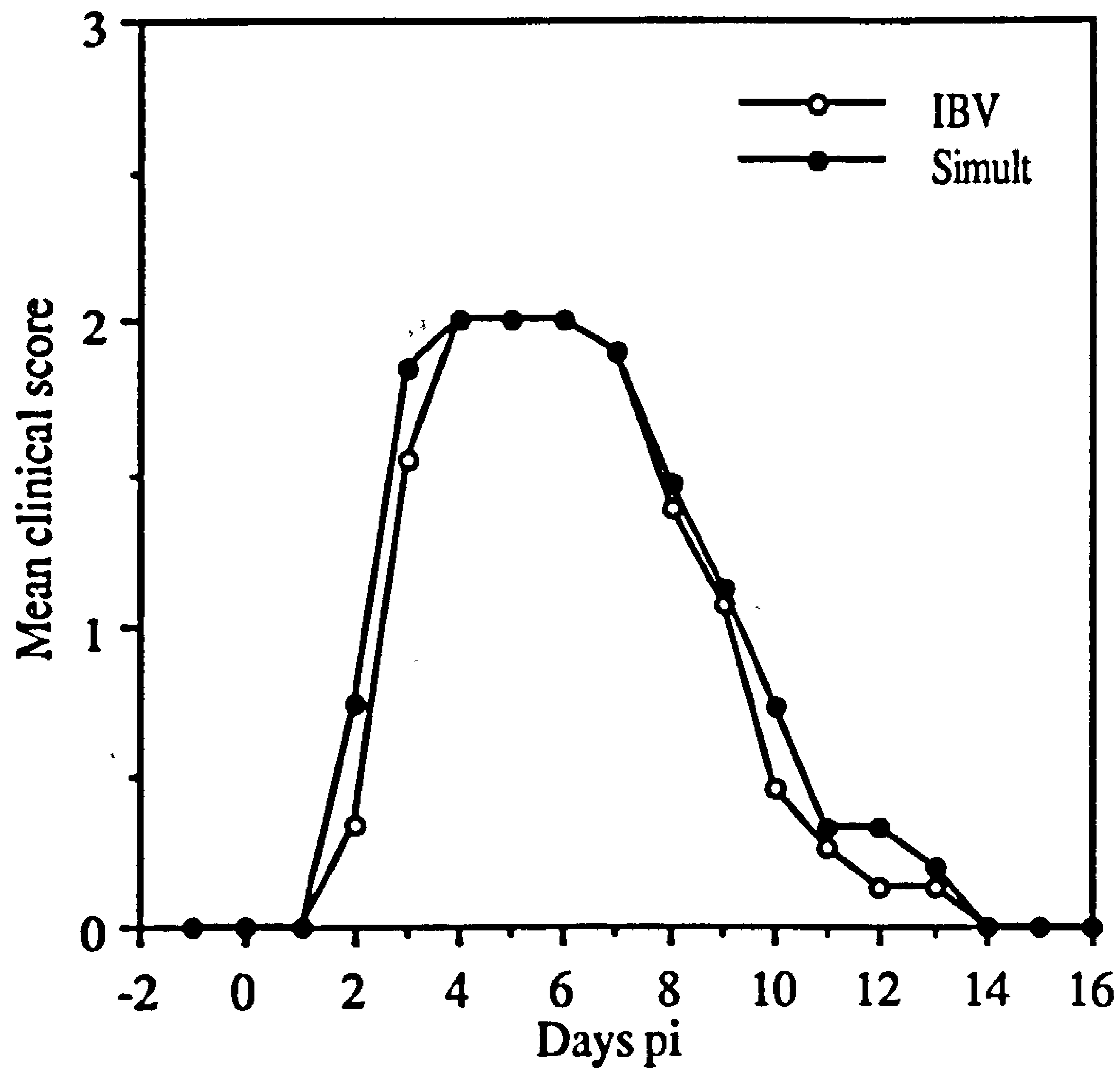

Fig. 8.18. Mean clinical scores of chicks following single infection with IBV or dual infection with APV and IBV simultaneously.

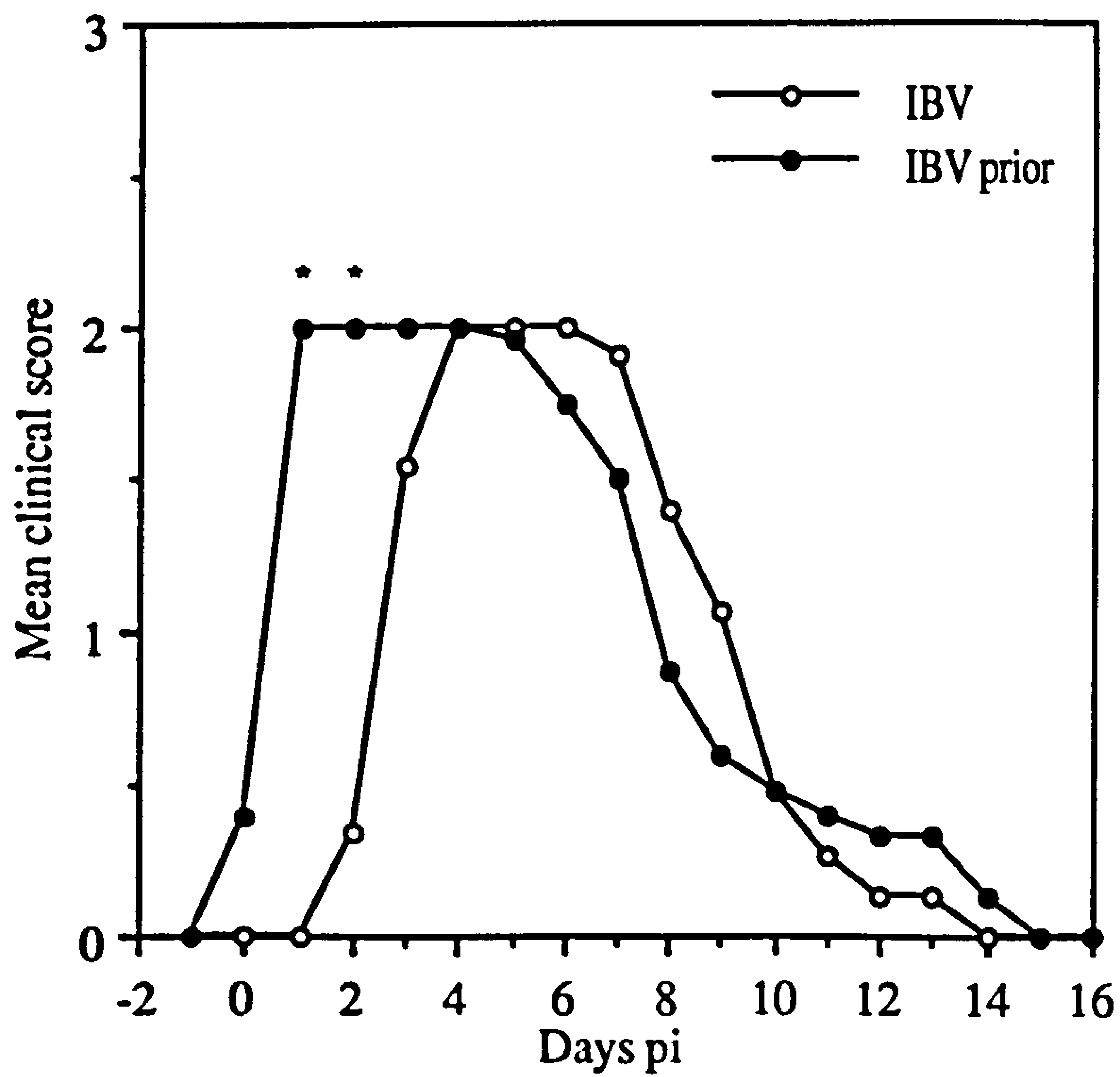

Fig. 8.19. Mean clinical scores of chicks following single infection with IBV and dual infection with IBV preceding APV. *: Significant differences ($p<0.05$) in scores.

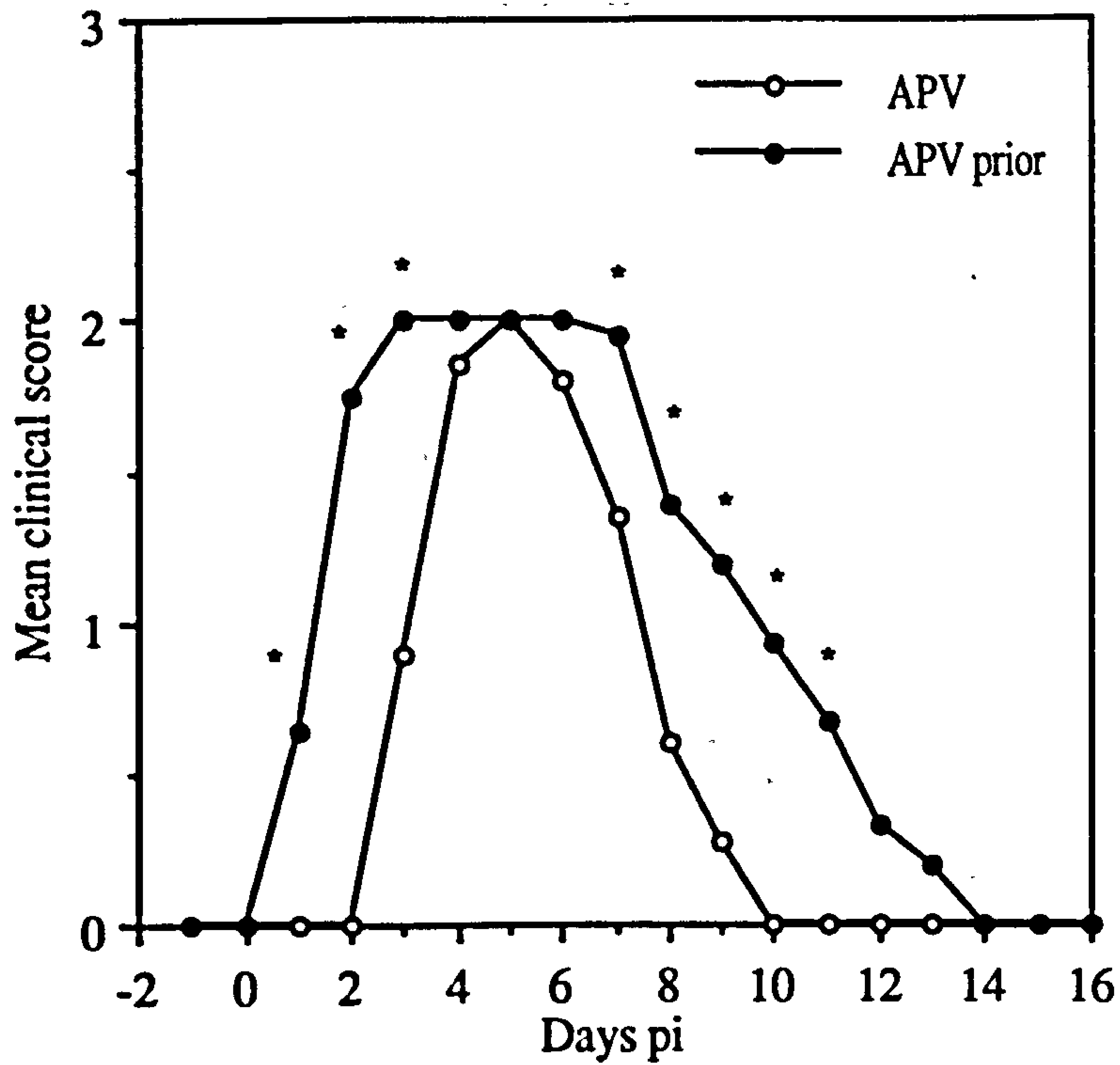

Fig. 8.20. Mean clinical scores of chicks following single infection with APV or dual infection with APV preceding IBV. *: Significant differences ($p<0.05$) in scores.

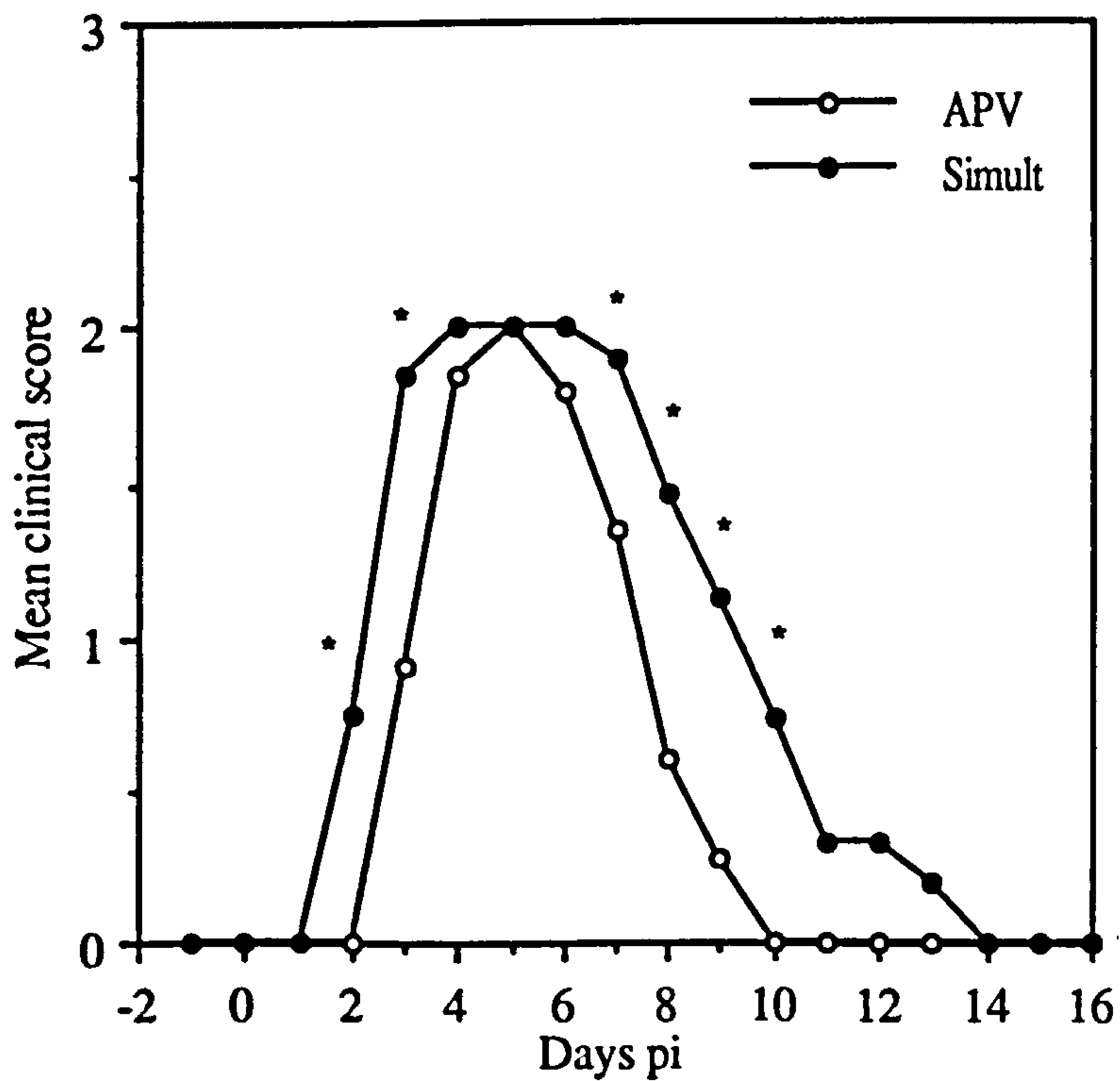

Fig. 8.21. Mean scores of chicks after single infection with APV or dual infection simultaneously with APV and IBV. *: Significant differences ($p<0.05$) in scores.

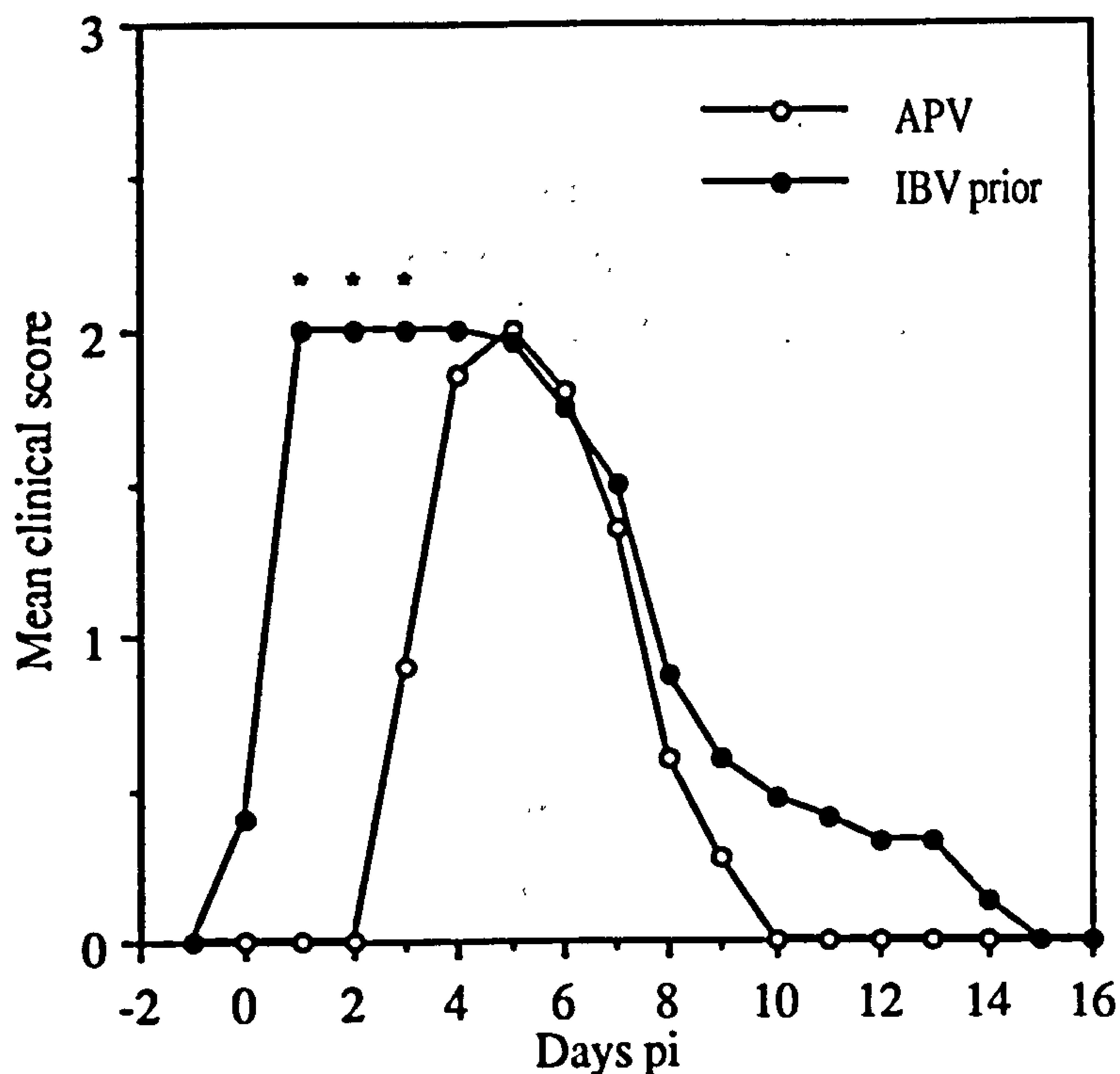

Fig. 8.22. Mean clinical scores of chicks following single infection with APV and dual infection with IBV preceding APV. *: Significant differences ($p<0.05$) in scores.

8.5.2. Virus isolations and titrations

Virus isolations

The results of APV and IBV isolations in tracheal swabs based on IF detection either before the ciliostatic effect (Table 8.2) or after neutralisation (Table 8.3) or by the routine method (Table 8.4) were all very similar. IBV was uniformly detected between days 1 to 9 pi in either IBV control or dual infections simultaneously and APV preceding IBV, but where IBV infection preceded APV, IBV was only detected until day 7 pi. There were no significant differences in total numbers of IBV isolations between the IBV control group and any of the dual infected groups. No IBV was detected in swabs from uninfected or APV controls.

APV was detected up to day 5 pi in the APV controls but not in dual infections either simultaneously or IBV preceding APV. Where APV was inoculated before IBV, APV was detected primarily on day 1 pi by all three methods of isolation and in one tracheal swab on day 3 pi only by IF staining before the ciliostatic effect. In this group, both APV and IBV were detectable on day 1 pi by all three methods and on day 3 pi only by

IF staining before ciliostatic effect. No APV was detected in swabs from uninfected or IBV controls.

In tissues examined on day 7 pi (Tables 8.5 & 8.6), IBV was detected in lower trachea, lungs and kidneys similarly in all the dual infected groups and the IBV controls by the three methods but not in the APV alone or uninfected controls. APV was not detected in these tissues in any of the groups including APV controls (not shown).

Table 8.2. APV and IBV isolations in tracheal swabs based on IF detection in TOC before the ciliostatic effect

Days pi	Controls				Dual infections					
	APV control		IBV control		APV prior		Simult		IBV prior	
	APV	IBV	APV	IBV	APV	IBV	APV	IBV	APV	IBV
1	0*	0	0	3	4	2	0	4	0	5
3	5	0	0	5	1	5	0	4	0	4
5	5	0	0	5	0	5	0	5	0	4
7	0	0	0	3	0	5	0	3	0	3
9	0	0	0	1	0	2	0	2	0	0
12-21	0	0	0	0	0	0	0	0	0	0
Totals										
APV	10^b	–	0^a	–	$5^{a,b}$	–	0^a	–	0^a	–
IBV	–	0^a	–	17^b	–	19^b	–	18^b	–	16^b

*: No. positive of 5 samples tested each time.

Values with different superscripts within virus type differ significantly ($p<0.05$).

Table 8.3. APV and IBV isolations in pooled tracheal swabs after neutralisation of heterologous virus

Days pi	Controls				Dual infections					
	APV control		IBV control		APV prior		Simult		IBV prior	
	APV	IBV	APV	IBV	APV	IBV	APV	IBV	APV	IBV
1	–	–	–	+	+	+	–	+	–	+
3	+	–	–	+	–	+	–	+	–	+
5	+	–	–	+	–	+	–	+	–	+
7	–	–	–	+	–	+	–	+	–	+
9	–	–	–	+	–	+	–	+	–	–
12-21	–	–	–	–	–	–	–	–	–	–

Table 8.4. APV and IBV isolations in tracheal swabs by the routine method

Days pi	Controls				Dual infections					
	APV control		IBV control		APV prior		Simult		IBV prior	
	APV	IBV	APV	IBV	APV	IBV	APV	IBV	APV	IBV
1	1*	0	0	3	4	1	0	5	0	5
3	5	0	0	5	0	5	0	5	0	5
5	5	0	0	5	0	5	0	5	0	4
7	0	0	0	3	0	5	0	3	0	3
9	0	0	0	1	0	2	0	2	0	0
12-21	0	0	0	0	0	0	0	0	0	0
Totals										
APV	11^{b}	–	0^{a}	–	4^{a}	–	0^{a}	–	0^{a}	–
IBV	–	0^{a}	–	17^{b}	–	18^{b}	–	20^{b}	–	17^{b}

*: No. positive of 5 samples tested each time.

Values with different superscripts within virus type differ significantly ($p<0.05$).

Table 8.5. IBV isolations in tissues on day 7 pi, based on either IF detection in TOC before ciliostatic effect or the routine method

Tissue	Controls		Dual infections		
	APV	IBV	APV prior	Simult	IBV prior
Trachea	0*	4	5	4	4
Lungs	0	3	3	3	3
Kidneys	0	1	2	2	2

*: No. positive of 7 samples examined. No statistical difference ($p<0.05$) among different groups.

Table 8.6. IBV isolations in pooled tissue samples on day 7 pi, after neutralisation

Tissue	Controls		Dual infections		
	APV	IBV	APV prior	Simult	IBV prior
Trachea	–	+	+	+	+
Lungs	–	+	+	+	+
Kidneys	–	+	+	+	+

Titration of total ciliostatic virus

In tracheal swabs, there were no marked differences in total ciliostatic virus titres between the IBV controls and all the dual infected groups (Fig. 8.23). The ciliostatic virus titres in the dual infected groups and single IBV infection were consistently higher than the APV control (Fig. 8.23)

In tissues examined on day 7 pi, virus titres in lower trachea, lungs and kidneys were similar between IBV controls and all the dual infected groups (Fig. 8.24).

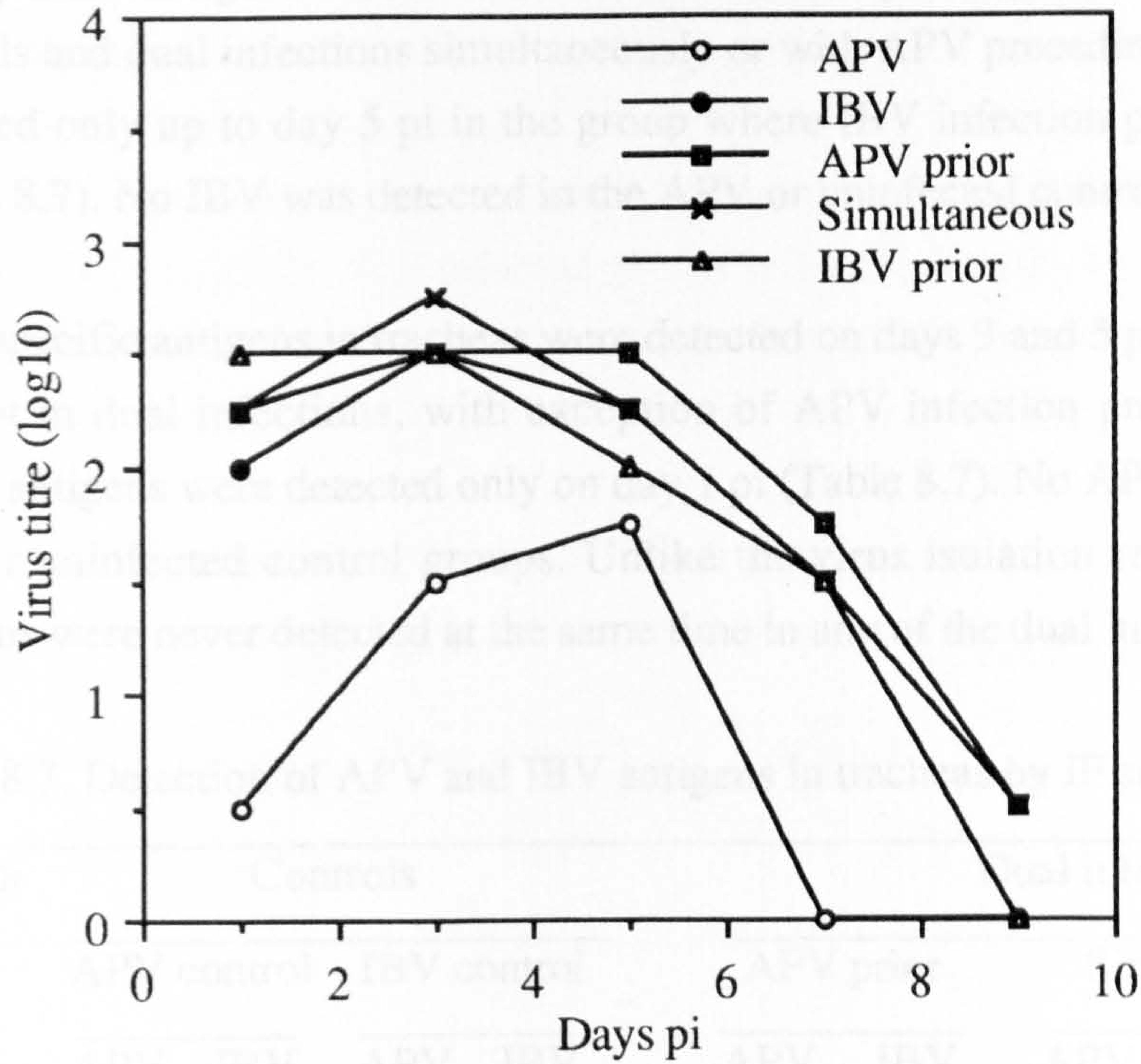

Fig. 8.23. Total ciliostatic virus titres in pooled tracheal swabs from chicks following single APV or IBV infection or various dual infections with APV and IBV.

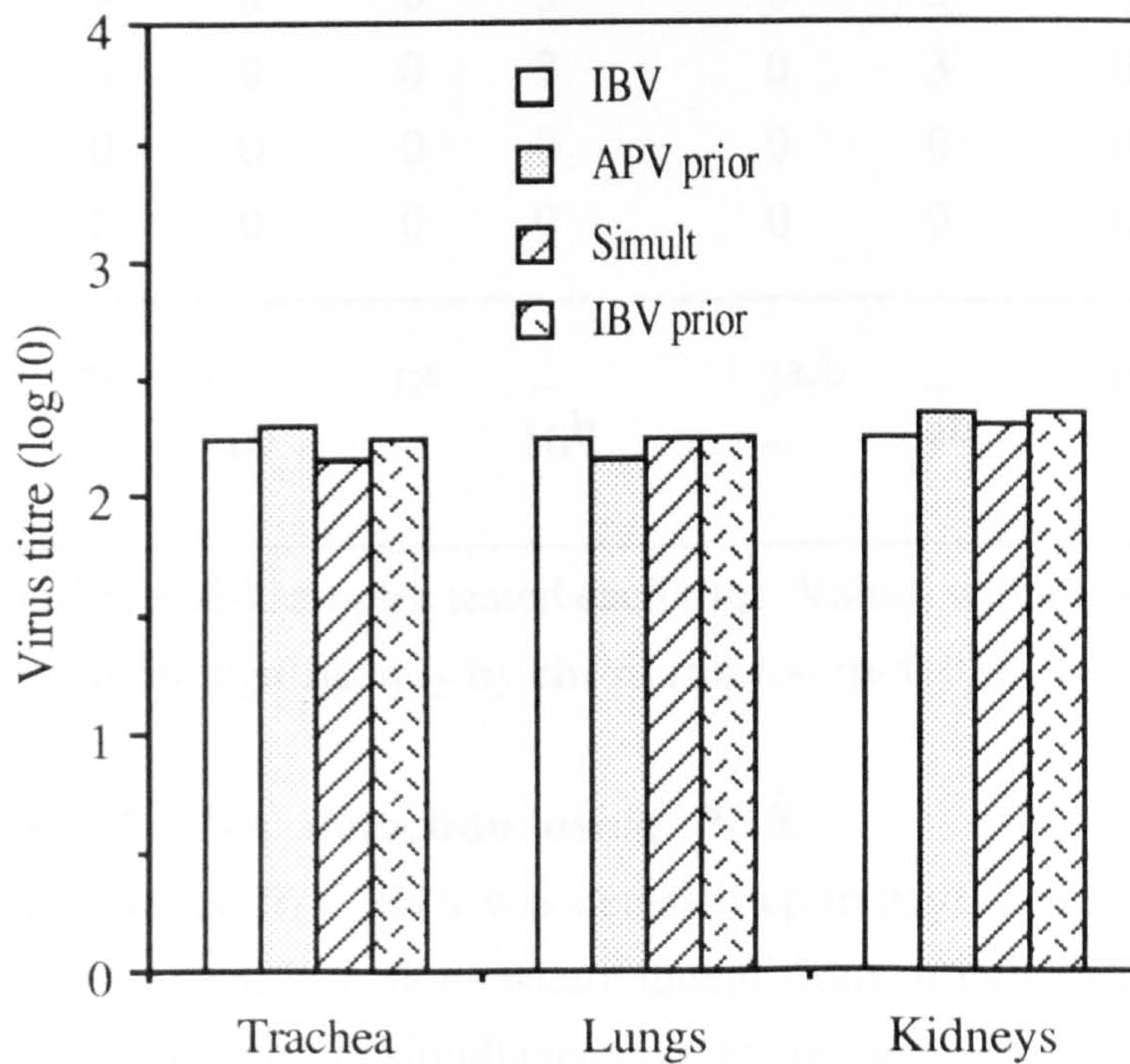

Fig. 8.24. Total ciliostatic virus titres in tissues of chicks following single IBV infection or dual infections with APV and IBV on day 7 pi.

8.5.3. Immunofluorescence

IBV-specific antigens in tracheas were detectable up to day 7 pi similarly in both IBV controls and dual infections simultaneously or with APV preceding IBV, but they were detected only up to day 5 pi in the group where IBV infection preceded that of APV (Table 8.7). No IBV was detected in the APV or uninfected control.

APV-specific antigens in tracheas were detected on days 3 and 5 pi in the APV controls but not in dual infections, with exception of APV infection preceding that of IBV, where antigens were detected only on day 1 pi (Table 8.7). No APV was detected in the IBV or uninfected control groups. Unlike the virus isolation results, APV and IBV antigens were never detected at the same time in any of the dual infected groups.

Table 8.7. Detection of APV and IBV antigens in tracheas by IF staining(s)

Days pi	Controls				Dual infections					
	APV control		IBV control		APV prior		Simult		IBV prior	
	APV	IBV	APV	IBV	APV	IBV	APV	IBV	APV	IBV
1	0*	0	0	2	3	0	0	2	0	3
3	3	0	0	3	0	3	0	3	0	3
5	4	0	0	3	0	3	0	3	0	3
7	0	0	0	2	0	3	0	3	0	0
9	0	0	0	0	0	0	0	0	0	0
12-21	0	0	0	0	0	0	0	0	0	0
Totals										
APV	7^{b}	–	0^{a}	–	$3^{a,b}$	–	0^{a}	–	0^{a}	–
IBV	–	0^{a}	–	10^{b}	–	9^{b}	–	11^{b}	–	9^{b}

*: No. positive of 4 samples tested each time. Values with different superscripts within virus type differ significantly by chi-square test ($p<0.05$).

8.5.4. Viral RNA detection using PCR

In tracheal swabs, IBV RNA was detected up to day 12 pi in IBV controls and up to day 9 pi in the dual infections where inoculations of two viruses preceded each other (Table 8.8). However, in simultaneous infection, IBV RNA was detected considerably longer, for up to day 21 pi. No IBV was detected in the APV or uninfected controls.

PCR detected APV RNA in tracheal swabs on days 3 and 5 pi in the APV controls but not in dual infections, with the exception of APV infection preceding that of IBV,

where APV was detected only on day 1 pi (Table 8.8). No APV was found in the IBV or uninfected controls. PCR never detected the two viruses at the same time unlike with virus isolation results.

IBV RNA was present in swabs from lower trachea, lungs and kidneys taken on day 7 pi similarly from all the dual infected groups and IBV control (Table 8.9). APV was not detected in tissues of any of the groups including APV controls (not shown).

Table 8.8. PCR-based detection of APV and IBV in pooled samples of tracheal swabs

Days pi	Controls				Dual infections					
	APV control		IBV control		APV prior		Simult		IBV prior	
	APV	IBV	APV	IBV	APV	IBV	APV	IBV	APV	IBV
1	–	–	–	+	+	–	–	+	–	+
3	+	–	–	+	–	+	–	+	–	+
5	+	–	–	+	–	+	–	+	–	+
7	–	–	–	+	–	+	–	+	–	+
9	–	–	–	+	–	+	–	+	–	+
12	–	–	–	+	–	–	–	+	–	–
15	–	–	–	–	–	–	–	+	–	–
19	–	–	–	–	–	–	–	–	–	–
21	–	–	–	–	–	–	–	+	–	–

Table 8.9. PCR-based detection of IBV in selected tissue swabs on day 7 pi

Tissue	Controls		Dual infections		
	APV	IBV	APV prior	Simult	IBV prior
Trachea	–	+	+	+	+
Lungs	–	+	+	+	+
Kidneys	–	+	+	+	+

8.5.5. Histopathology

The nature and persistence of histopathological lesions in tracheas examined at various intervals from IBV control and all the dual infected groups were of a similar order. On day 3 pi, the changes typical of IBV included loss of cilia, degeneration of epithelial cells, depletion of mucous secreting cells and mild infiltration of heterophils and mononuclear cells in the submucosa. In some cases, desquamated epithelial cells and heterophils were seen in the lumen. At this stage, in the group where IBV infection preceded APV, the lesions were more advanced relating to earlier IBV inoculation.

By day 7 pi, there was hyperplasia and hypertrophy of epithelial cells with loss of cilia and depletion of goblet cells. Heterophilic and mononuclear cell infiltration of epithelial cells was also evident. In some birds, there was inflammatory exudate in the lumen. The hyperplastic changes in the epithelium were still evident on day 12 pi but the regeneration process had also started and was completed between days 15 and 21 pi. There was reappearance of ciliated columnar epithelial cells and goblet cells, although mild mononuclear cell infiltration persisted in the submucosa.

In contrast to IBV controls and dual infected groups, lesions observed in tracheas from APV controls were very mild. These included slight thickening of the mucosa, focal deciliation and mononuclear cell infiltration on days 3 and 7 pi. By day 12 pi, a complete regeneration of the ciliated epithelium was observed.

8.5.6. Serology

IBV antibodies

On day 7 pi, the ELISA antibody titres in the IBV controls were similar to those in the group with simultaneous infection, but significantly higher than in the group where APV infection preceded IBV, indicating APV interference in the initial development of antibodies (Fig. 8.25). Where IBV infection preceded APV, the titres were significantly higher compared to the IBV controls, likely due to earlier IBV inoculation. On day 14 and 21 pi, the titres in all the dual infected groups reached similar levels to those in the IBV controls.

The neutralising antibody responses to IBV following single or dual infections of chicks were very similar to those obtained by ELISA (Fig. 8.26).

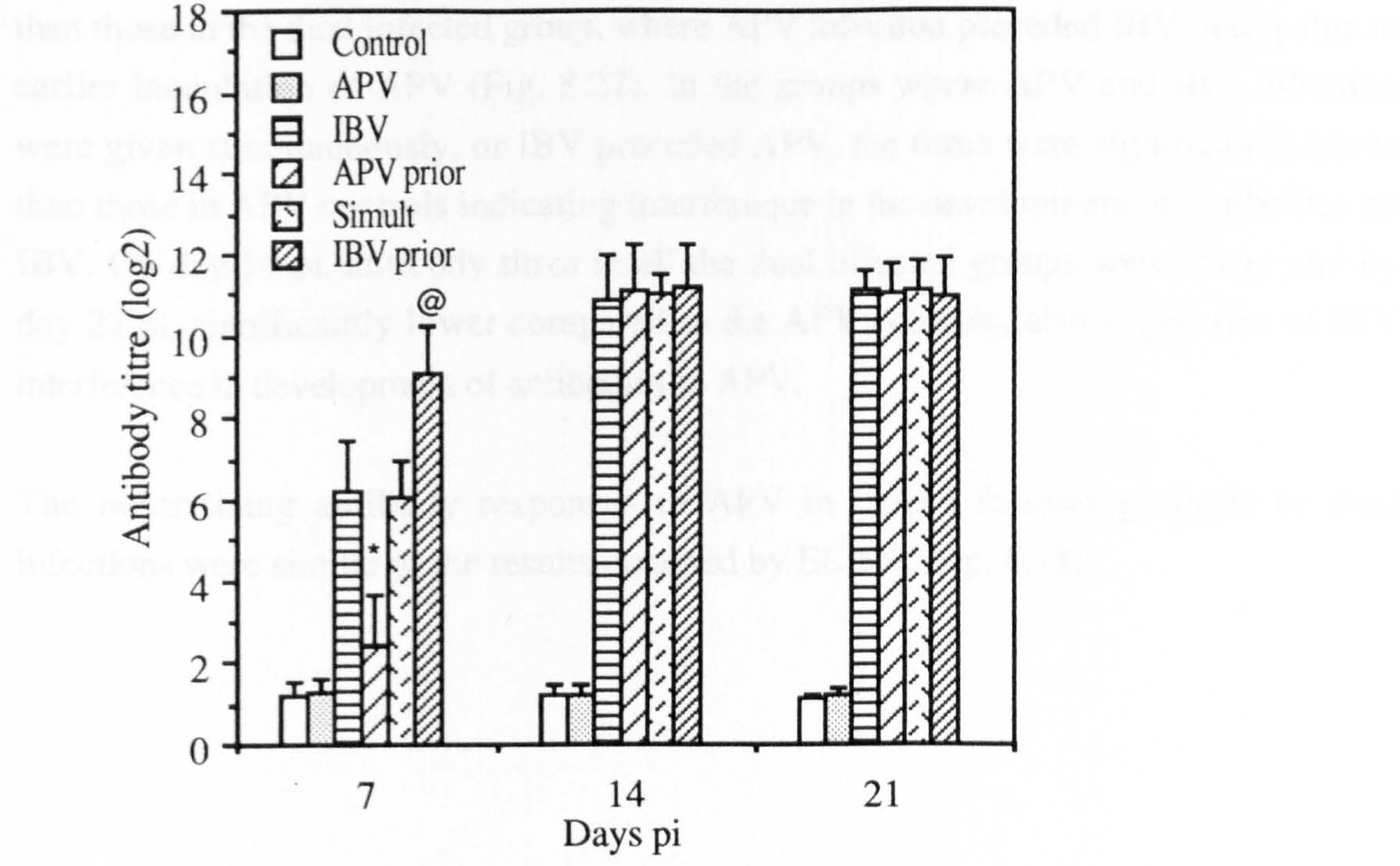

Fig. 8.25. ELISA antibodies titres (mean ± sd) to IBV in chicks after single or dual infections with APV and/ or IBV. *: Significantly lower ($p<0.05$) titres compared to IBV control. @: Significantly higher ($p<0.05$) titres compared to IBV control.

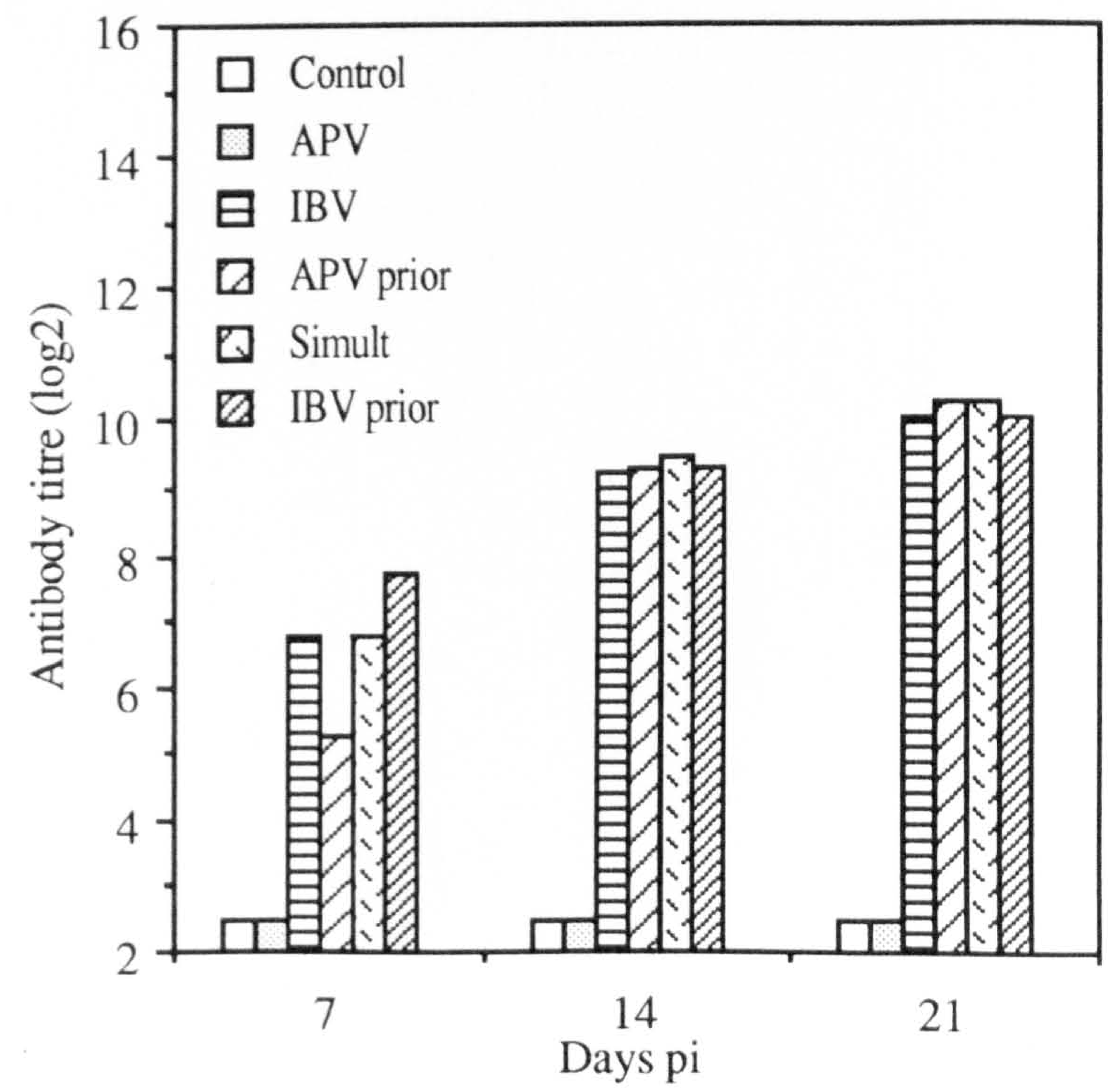

Fig. 8.26. Neutralising antibodies to IBV in pooled sera from chicks after single or dual infection with APV and/ or IBV. Lowest detectable concentration <2.5 $\log_2$.

APV antibodies

On day 7 pi, the ELISA antibody titres in the APV controls were significantly lower than those in the dual infected group, where APV infection preceded IBV, likely due to earlier inoculation of APV (Fig. 8.27). In the groups where APV and IBV infection were given simultaneously, or IBV preceded APV, the titres were significantly lower than those in APV controls indicating interference in the development of antibodies by IBV. On day 14 pi, antibody titres in all the dual infected groups were lower and by day 21 pi, significantly lower compared to the APV controls, also suggestive of IBV interference in development of antibodies to APV.

The neutralising antibody responses to APV in chicks following single or dual infections were similar to the results obtained by ELISA (Fig. 8.28).

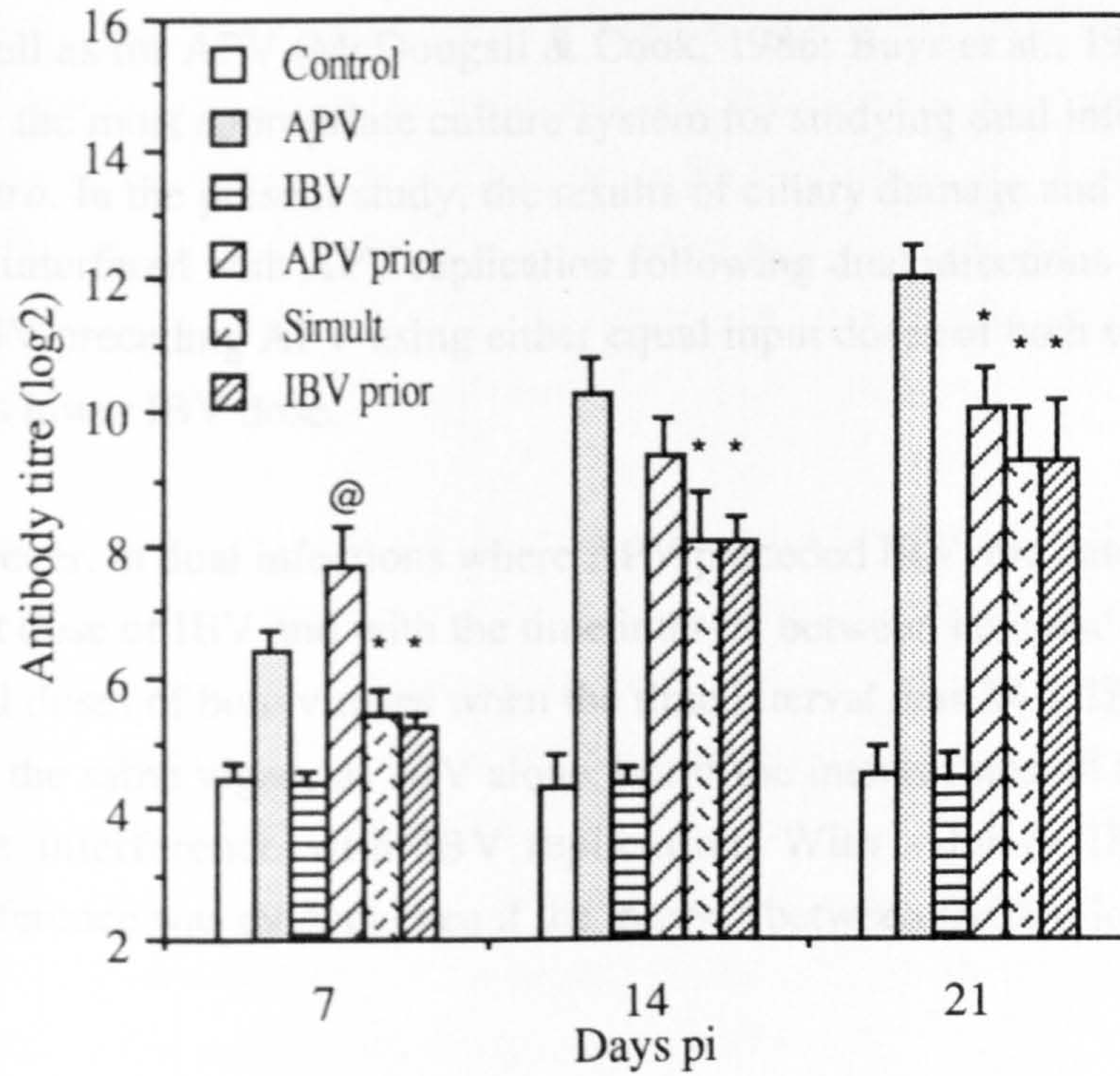

Fig. 8.27. ELISA antibodies titres (mean ± sd) to APV in chicks after single or dual infections with APV and/ or IBV. *: Significantly lower ($p<0.05$) titres compared APV control. @: Significantly higher ($p<0.05$) titres compared to APV control.

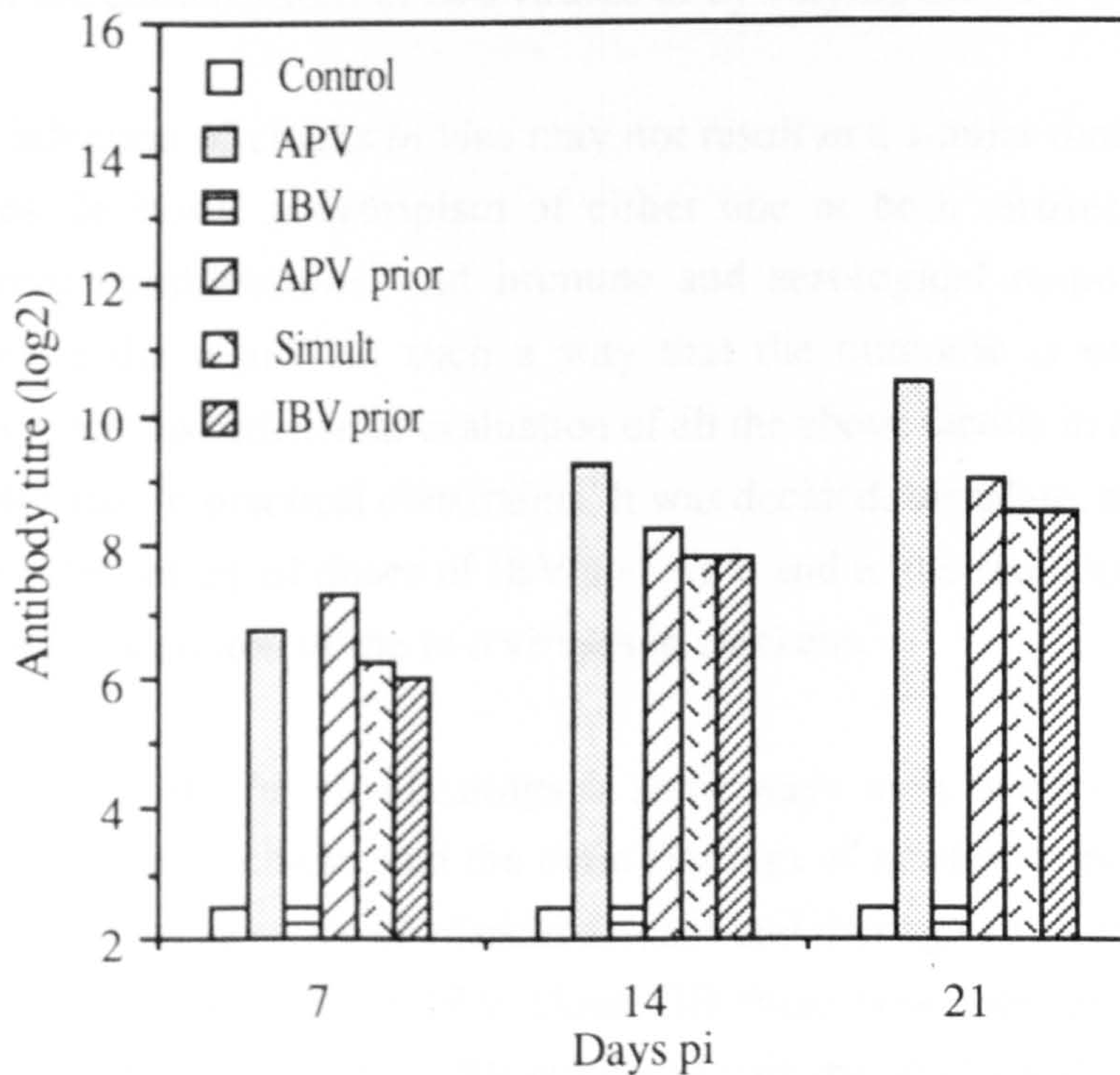

Fig. 8.28. Neutralising antibodies to APV in pooled sera from chicks after single or dual infection with APV and/ or IBV. Lowest detectable concentration $<2.5\ log_2$.

8.6. DISCUSSION

TOC has been commonly used in the cultivation and assay of IBV (Cook et al., 1976) as well as for APV (McDougall & Cook, 1986; Buys et al., 1989a). This was thought to be the most appropriate culture system for studying dual infection of APV and IBV *in vitro*. In the present study, the results of ciliary damage and IF staining showed that IBV interfered with APV replication following dual infections of TOC simultaneously or IBV preceding APV using either equal input doses of both viruses or a one hundred times lower IBV dose.

However, in dual infections where APV preceded IBV, the outcome was dependent on input dose of IBV and with the time interval between inoculation of APV and IBV. At equal doses of both viruses when the time interval was 24 h, IBV was able to replicate with the same vigour as IBV alone, but if the interval was 48 h or 72 h, APV showed some interference with IBV replication. With a lower IBV dose, APV-related interference was evident even if the interval between the two infections was as short as 24 h.

In conclusion of *in vitro* work, IBV was the predominant virus but APV could interfere with its replication when its infection preceded it by a sufficient time interval, especially with a lower IBV dose. However, on comparing with IBV infection alone, there was no evidence of an increase in ciliary damage to TOC as result of dual infection using any of the combinations of two viruses or by varying the IBV dose.

Dual infection of chicks *in vivo* may not result in a similar outcome to that of *in vitro* studies. In birds, polytropism of either one or both viruses, at varying doses, in different combinations, and immune and serological responses of the host may influence the results in such a way that the outcome is exacerbation instead of interference. Experimental evaluation of all the above factors in a single attempt was not feasible, due to practical constraints. It was decided, therefore, to limit the *in vivo* study to the effect of equal doses of IBV and APV and to the fixed time interval of 48 hours between inoculation of the two viruses in chickens.

Nonetheless, the *in vitro* findings in some ways were borne out by results of in *vivo* dual infection of chicks and the main findings of *in vivo* work were: (i) there was no increase in the severity of clinical disease and damage to tracheas or infectious virus persistence compared to IBV alone (ii) there was interference by IBV with the replication of APV (iii) and IBV interfered with the serological response to APV.

Although the clinical signs and tracheal lesions in IBV or APV infections are not pathognomic, these were used to assess disease severity. The clinical signs and tracheal lesions following single IBV infection or all the three dual infections were similarly more severe and longer in duration than in the single APV infection. However, there was no detectable difference in either severity or duration of signs and lesions between the IBV controls and the dual infected groups. In a previous dual infection study in chickens with IBV and NDV, Spradbrow et al. (1995) also found that there was no increase in severity of clinical disease.

Furthermore, there was no difference in total number or duration of IBV isolations from tracheal swabs from IBV controls and the dual infected groups, but APV isolations were either absent or markedly reduced in dual infected groups compared to the APV controls. This suggested the IBV interference with APV replication *in vivo*. When selected tissues were examined on day 7 pi, only IBV was isolated from dual infected groups. This indicated that there was no increased invasiveness of APV as the result of dual infections, nor indeed of the IBV, since virus titres in tissues in IBV controls and dual infected groups were similar.

The virus isolation, IF staining and PCR proved to be of broadly similar sensitivity for APV, all detecting this virus up to day 5 pi in tracheal swabs from chicks. This finding was in contrast to a previous report (Jing et al., 1993), where PCR was found to detect APV RNA in tracheal swabs from poults for two weeks longer than recovery of viable virus following experimental infection. With regard to IBV detection, as reported earlier (Kwon et al., 1993), PCR was the most sensitive method and this virus was detectable in tracheal swabs up to day 12 pi, followed by virus isolation (up to day 9 pi) and then IF staining (up to day 7 pi).

Like virus isolation, the results of IF staining and PCR did not demonstrate increased persistence of either virus following dual infections, except where both APV and IBV were inoculated simultaneously. In this case, PCR detected IBV for 12 days longer than for recovery of infectious virus. The reason for the increase in the persistence of IBV genome following simultaneous inoculation of two viruses is not known.

The results of virus isolation, IF staining and PCR all point to the interference of IBV with APV replication in tracheas of chicks following dual infection of APV and IBV at equal doses and in different combinations. This interference of IBV over APV replication was also observed in *in vitro* studies. The phenomenon of *in vitro* IBV interference with growth of another respiratory virus of chickens, NDV has been reported in embryonated chicken eggs (ECE) (Raggi et al., 1963) and chicken kidney

cell cultures (Beard, 1967). Raggi et al. (1963) showed that an egg-adapted strain of IBV (DA) and the GB (Texas) strain of NDV when inoculated into ECE in varied amounts and different combinations, the IBV component in excess consistently interfered with the growth of NDV. IBV has been also shown to interfere with the rate of multiplication of NDV in experimentally infected chickens as judged by a prolonged incubation period for nervous symptoms (Raggi & Lee, 1964).

During the *in vitro* study, the dual infection of TOC using equal doses of either agents and in some instances with the lower IBV dose, a predominance of IBV over APV replication was observed. This might have been due to rapid replication of IBV leading to the occupation and destruction of receptors sites or simply by rapid degeneration of the epithelial cells. While describing the mechanism of interference of IBV with NDV multiplication, Raggi and Lee (1964) suggested that IBV being capable of tremendous invasiveness, rapidly invades the cells and becomes a part of the metabolic patterns of a cell and thus prevents NDV multiplication in the same cells. This might be the case also in the present study.

The role of interferon in the interference of IBV over APV and *vice versa* was not an objective of this study. To-date, there are no reports on interferon induction by APV and only gamma-interferon has been studied in relation to IBV, but results are conflicting. Otsuki et al. (1988) detected variable levels of gamma-interferon in chickens infected with various strains of IBV, whereas other workers (Lomniczi, 1974; Holmes & Darbyshire, 1978) could not detect gamma-interferon in serum or organ cultures from chickens infected with this virus. Further studies are necessary to elucidate the role of interferon in the mechanism of interference between IBV and APV.

APV replicates primarily in the upper respiratory tract i.e. nasal turbinates and trachea of chickens (Cook et al., 1993a). In *in vivo* work of the present study, replication of APV in the nasal turbinates was not investigated. It is not known whether IBV would also interfere with APV replication in turbinates as it did in tracheas of the dually infected chicks. However, this outcome should not affect the results of the clinical disease and the serological response.

In the present work, there was no overall affect of APV on the development of ELISA and neutralising antibodies to IBV in any of the dual infected groups. However, IBV did interfere with the development of both ELISA and neutralising antibodies to APV. The interference of IBV in the development of antibodies to APV was more when IBV was inoculated either simultaneously or prior to APV. This might have been due to IBV

not allowing APV to replicate sufficiently and thus allowing less or shorter antigenic stimulus to the immune system.

A similar interference of IBV with antibody development to NDV in chickens has been reported previously (Raggi & Lee, 1964). It has been also shown that when live IBV and NDV vaccines were administered simultaneously, IBV interfered with NDV vaccines by resulting in less protection against Newcastle disease challenge (Raggi & Lee, 1964; Thornton & Muskett, 1975). It will require additional studies using live APV and IBV vaccines to assess the importance of IBV interference in antibody response to APV. Furthermore, since *in vitro* work with the lower IBV dose showed that a preceding infection of APV could interfere with IBV replication, its importance needs to be evaluated in other ways, for example in a dual infection of field APV strain and a vaccine IBV strain.

CHAPTER 9

DUAL INFECTION OF TURKEY POULTS WITH AVIAN PNEUMOVIRUS AND *MYCOPLASMA SYNOVIAE*

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CHAPTER 9

DUAL INFECTION OF TURKEY POULTS WITH AVIAN PNEUMOVIRUS AND *MYCOPLASMA SYNOVIAE*

9.1. INTRODUCTION

Mycoplasma synoviae (Ms) infection most frequently occurs as a subclinical upper respiratory tract infection and at other times, it becomes systemic and results in infectious synovitis, an acute to chronic infectious disease of chickens and turkeys (Kleven, 1997). Although field evidence suggests that Ms can produce respiratory disease in chickens and turkeys, it has been found difficult to reproduce experimentally, and furthermore, the organism can inhabit the respiratory tract of apparently healthy birds for long periods (Taylor-Robinson & Bradbury, 1998). Thus, it appears that some complicating factors may be necessary to trigger disease.

The severity of respiratory tract disease of turkeys caused by APV is much affected by managemental and environmental factors and involvement of secondary infections agents (see Naylor & Jones, 1993). Secondary agents including *Bordetella avium* and *Pasteurella*-like organisms (Cook et al., 1991) or *Mycoplasma gallisepticum* (Naylor et al., 1992) and *Mycoplasma imitans* (Ganapathy et al., 1998) have been shown to exacerbate and prolong the clinical disease caused by APV in turkeys. This chapter describes an attempt to investigate the possibility of Ms as a complicating factor following APV infection of turkey poults.

9.2. MATERIALS AND METHODS

9.2.1. Poults

Turkey eggs free of mycoplasmas and maternal antibodies to APV were obtained from a commercial source and hatched in this laboratory. The poults were maintained in complete isolation with food and water *ad libitum.*

9.2.2. Virus

APV strain # 8544 (Wilding et al., 1986) described in Chapter 3 was used. It was titrated in the chicken embryo tracheal organ culture (TOC) (Chapter 3) before use in this study.

9.2.3. Mycoplasma

A recent field Ms isolate B91/96 798-3S, available in this laboratory was used. It had undergone eight *in vitro* passages after isolation from the sinus of turkeys showing respiratory signs.

9.2.4. Experimental design

One hundred and twenty-eight poults were divided into 4 groups on hatching (Table 9.1). At one day of age, two groups were inoculated with APV with a dose of 3.0 $\log_{10}$ CD_{50}/bird in the right eye and nostril. Three days later, one APV-infected and one uninfected group was inoculated with Ms with a dose of 1.1 x 10^7 colony forming units/bird in the left eye and nostril. For single infections, the uninfected eye and nostril was inoculated as appropriate with TOC medium without antibiotics or mycoplasma broth (MB) (see Appendix). The controls were similarly inoculated with TOC medium and MB.

Table 9.1. Experimental design

	Inoculum			
	1 day-old		4 day old	
Group	Right EN*	Left EN	Right EN	Left EN
APV	APV	–	–	Mycoplasma broth
APV+Ms	APV	–	–	Ms
Ms	TOC medium	–	–	Ms
Control	TOC medium	–	–	Mycoplasma broth

*: eye and nostril.

Following infection, 10-15 birds in each group were examined daily for clinical signs. Five birds from each group were identified for repeated tracheal swabbing, each for virus and mycoplasma isolation. This was done on days 1, 4, 7, 11, 15, 18 and 21 post Ms infection. On days 3, 6, 10, 14 and 21 post Ms infection, 4 poults from each group were euthanased, necropsied, examined for gross lesions and sampled for mycoplasma and virus recovery. For mycoplasma isolations, swabs were taken from the turbinates, trachea, lungs and thoracic air sacs. Pieces of turbinates, trachea and lungs were collected for virus isolation and histopathology. Sera were collected from 6 poults in each group on days 7, 14 and 21 post Ms infection for assessment of antibody response to both APV and Ms.

9.2.5. Clinical signs

The severity of clinical signs was scored on 0 to 3 scale by the method of Jones et al. (1992) as described in Chapter 3.

9.2.6. Histopathology

Tissue samples collected in 10% formalin were processed by the conventional methods and the sections stained with haemotoxylin and eosin.

9.2.7. Virus isolation and titration

Swabs or tissues after collection were processed for virus isolation and titrations in TOC as described in Chapter 3. For virus isolations, a minimum of three passages was given in TOC and the ciliostatic virus was identified by immunofluorescence (IF) staining (Chapter 3).

9.2.8. Mycoplasma culture and identification

The swabs were processed within 1 hour of collection. Briefly, the swabs were plated onto mycoplasma agar (see Appendix) and then agitated in 1.0 ml of MB to increase the sensitivity of mycoplasma isolation. The MB was incubated at 37°C for 7 days. As soon as any broth changed colour, a 20 µl aliquot was plated onto mycoplasma agar and the remaining broths were plated out 7 days post incubation. Plates were incubated at 37°C in a CO_2 incubator and were examined for mycoplasma growth daily for three weeks post-incubation. All isolates were identified by IF staining (Rosendal & Black, 1972) using rabbit-raised Ms antiserum (Aarhus, Denmark).

9.2.9. Serology

Antibodies to APV were measured using enzyme linked immunosorbent assay (ELISA) (Naylor et al., 1992) and serum neutralisation test (SNT) (Cook et al., 1988) as described in Chapter 3. Serum samples were tested individually by ELISA and for SNT, pooled samples were used.

Antibodies to Ms were determined by the rapid serum agglutination (RSA) test and an Ms ELISA. RSA was performed using stained Ms antigen Nobilis (Intervet, Boxmeer, Holland) according to the manufacturer's protocol. The ELISA was performed using the commercial Ms-antibody enzyme-immunoassay test kit (Svanova Biotech, Uppsala, Sweden) and the test conducted strictly as per manufacturer's instructions. All the sera were tested individually for both RSA test and ELISA.

9.2.10. Statistical analysis

The ELISA antibody and virus titres were analysed using student's t-test ($p<0.05$). The mean clinical scores and other data were analysed by chi-square test ($p<0.05$).

9.3. RESULTS

9.3.1. Clinical signs

The clinical signs typical of APV infection were evident as early as 2 days following APV infection in the two virus-infected groups (Fig. 9.1). The nature of clinical disease in the dual infected group was similar to that for the APV control. Peak signs in the APV and the dual infection group were seen around days 2 and 3 post Ms infection and all birds in both groups recovered by days 11 or 12 post Ms infection. There were no significant differences in mean clinical scores between single APV and the dual infection group. However, the scores in the dual infection group were slightly higher on days 7 to 11 post Ms infection. Following single Ms infection, no signs were recorded at any time nor were they observed in the uninfected control group.

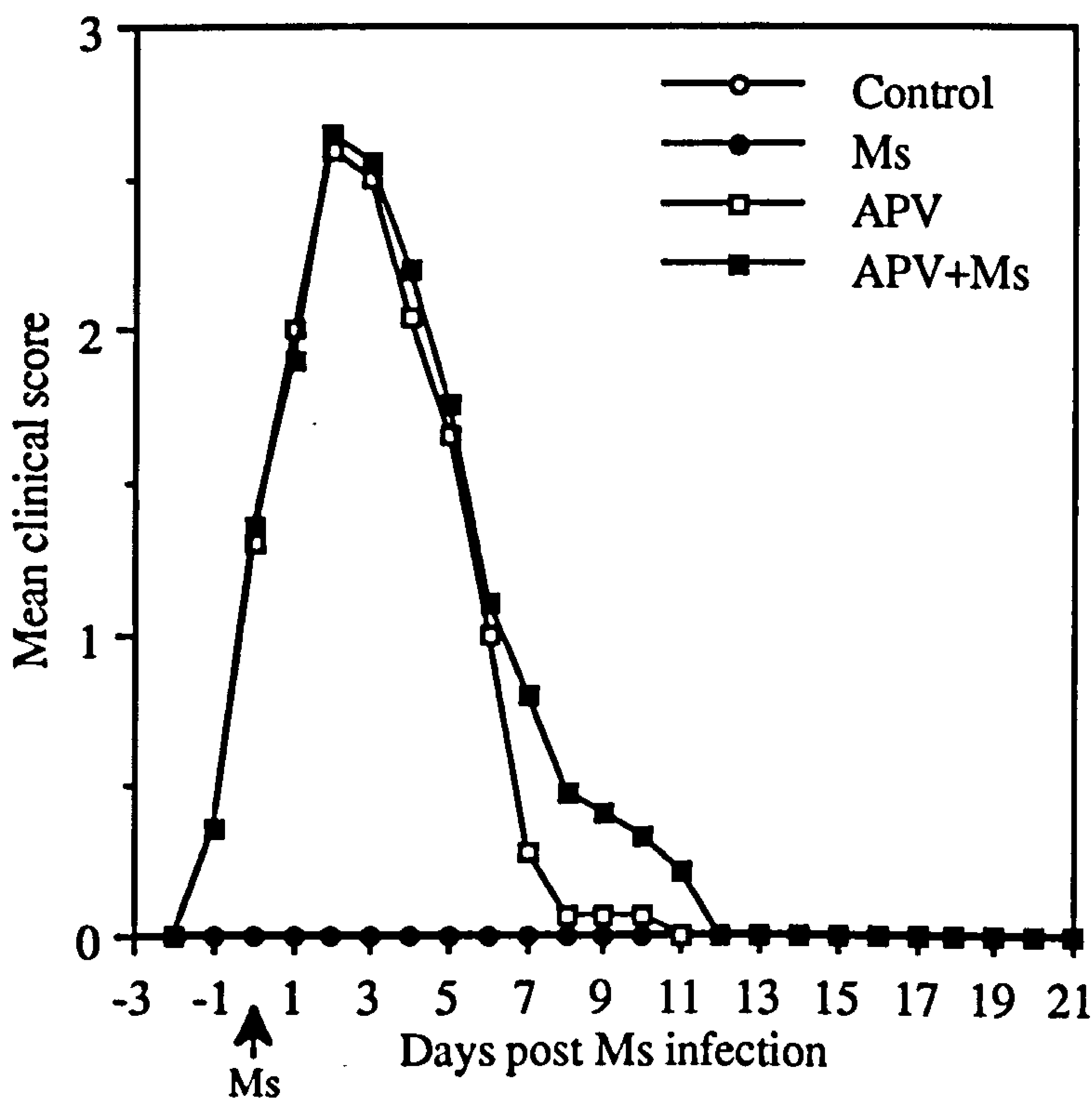

Fig. 9.1. Mean clinical scores of poults following single or dual infection with APV and/ or Ms.

9.3.2. Gross lesions

At necropsy, nasal and tracheal exudate were the only abnormalities seen similarly in the APV control and the dual infection group on days 3 and 6 post Ms infection (Table 9.2). The Ms and uninfected controls had no macroscopic lesions (not shown).

Table 9.2. Gross lesions in poults infected with APV or APV and Ms

		Days post Ms infection			
Lesion	Group	3	6	10-21	Total
Nasal exudate	APV	4*	4	0	8
	APV+Ms	4	4	0	8
Tracheal exudate	APV	1	2	0	3
	APV+Ms	1	1	0	2
Lungs congestion	APV	0	0	0	0
or air sacs cloudy	APV+Ms	0	0	0	0

*: No. positive of 4 birds examined each time.

9.3.3. Histopathology

On day 3 post Ms infection, microscopic lesions in turbinates and trachea of both single APV and dual infection groups included focal epithelial degeneration, inflammatory cells in the epithelium and inflammatory exudate and mucus in the airways. The lungs showed the presence of mucous gland hyperplasia. By day 10, mild focal inflammation of the turbinates and tracheas was observed in both the Ms infected groups but not in APV controls. No abnormality was seen in lungs of any group. No microscopic lesions of significance were recorded in tissues of any group on day 14.

On day 21 post Ms infection, bacterial colonisation of the surface epithelium of the turbinates together with mild heterophilic infiltration was observed in both the APV inoculated and the dual infection group. Scanty mucus in the tracheal lumen was seen in both groups which received Ms infection. No abnormality was recorded in lungs of any group. The tissues of uninfected birds showed no lesions on any occasion.

9.3.4. Virus isolations and titration

APV was isolated similarly from tracheal swabs of both APV and the dual infected group on days 1, 4 and 7 post Ms infection (Table 9.3). Virus recovery from turbinates, trachea and lungs was also made similarly in both the virus infected groups on day 3 post Ms infection (Table 9.4). On day 6, APV was isolated similarly from

trachea of both the virus infected groups and from lungs of only one bird in the dual infected group. There were no significant differences between virus titres of tracheal swabs (Table 9.3) or tissues (Table 9.4) from these two groups. No virus was isolated from tracheal swabs or tissues of either Ms or uninfected controls (not shown).

Table 9.3. Virus isolations and mean titres in tracheal swabs of poults following infection with APV or APV and Ms

Days post Ms infection	Group	
	APV	APV+Ms
1	5* (2.4 ± 0.7)[a]	5 (2.7± 0.3)
4	5 (1.7 ± 0.1)	4 (1.8 ± 0.6)
7	1 (< 0.5)[b]	1 (< 0.5)
11-21	0	0
Total	11	10

*: No. positive of 5 samples tested each time. a: Virus titre $\log_{10}$ CD_{50} (mean ± sd) per 0.1 ml. b: Minimum detectable virus concentration per 0.1 ml.

Table 9.4. Virus isolations and mean titres in tissues from poults following infection with APV or APV and Ms

Tissue	Group	Days post Ms infection 3	6	10-21	Total
Turbinates	APV	4* (2.6 ± 0.3)[a]	0	0	4
	APV+Ms	4 (2.5 ± 0.4)	0	0	4
Tracheas	APV	4 (4.3 ± 0.4)	1 (< 1.5)[b]	0	5
	APV+Ms	4 (4.2 ± 0.5)	1 (< 1.5)	0	5
Lungs	APV	4 (3.2 ± 0.3)	0	0	4
	APV+Ms	4 (3.3 ± 0.4)	1 (< 1.5)	0	5

*: No. positive of 4 samples tested each time. a: Virus titre $\log_{10}$ CD_{50} (mean ± sd) per gram of tissue. b: Minimum detectable virus concentration per gram of tissue.

9.3.5. Mycoplasma isolations

Ms isolations were made similarly from tracheal swabs from both the single and dual Ms infected groups between days 4 and 21 post Ms infection (Table 9.5). The organism was isolated from swabs from one or two birds up to day 18 but on day 21 it was present in five out of five birds in both Ms infected groups.

Ms was recovered inconsistently between days 3 and 21 in both the single and dual Ms infected groups, but only from turbinates and tracheas without any significant differences in their total number of isolations (Table 9.6). No mycoplasma was isolated from tracheal swabs or tissues from either APV or uninfected controls (not shown).

Table 9.5. Ms isolations in tracheal swabs of poults following infection with Ms or APV and Ms

	Days post Ms infection							
Group	1	4	7	11	15	18	21	Total
Ms	0*	1	1	1	1	2	5	11
APV+Ms	0	1	2	2	1	2	5	13

*: No. positive of 5 samples tested each time.

Table 9.6. Ms isolations in tissues from poults following infection with Ms or APV and Ms

		Days post Ms infection					
Lesion	Group	3	6	10	14	21	Total
Turbinates	Ms	2*	1	0	2	1	6
	APV+Ms	1	1	0	1	0	3
Tracheas	Ms	0	1	0	0	1	2
	APV+Ms	0	1	0	0	2	3
Lungs	Ms	0	0	0	0	0	0
or air sacs	APV+Ms	0	0	0	0	0	0

*: No. positive of 4 samples tested each time. No significant difference ($p<0.05$) in total number of isolations within tissue and between two groups.

9.3.6. Serology

The ELISA antibody titres to APV in both single APV and the dual infected group rose consistently from day 7 to 21 post Ms infection without any significant differences (Fig. 9.2). The neutralising antibody response to APV following single APV or dual infection was similar to the results obtained by ELISA (Fig. 9.3).

No antibody response to Ms was detected on any sampling occasion by either the RSA test or ELISA, neither in Ms infected groups nor in non Ms groups (not shown).

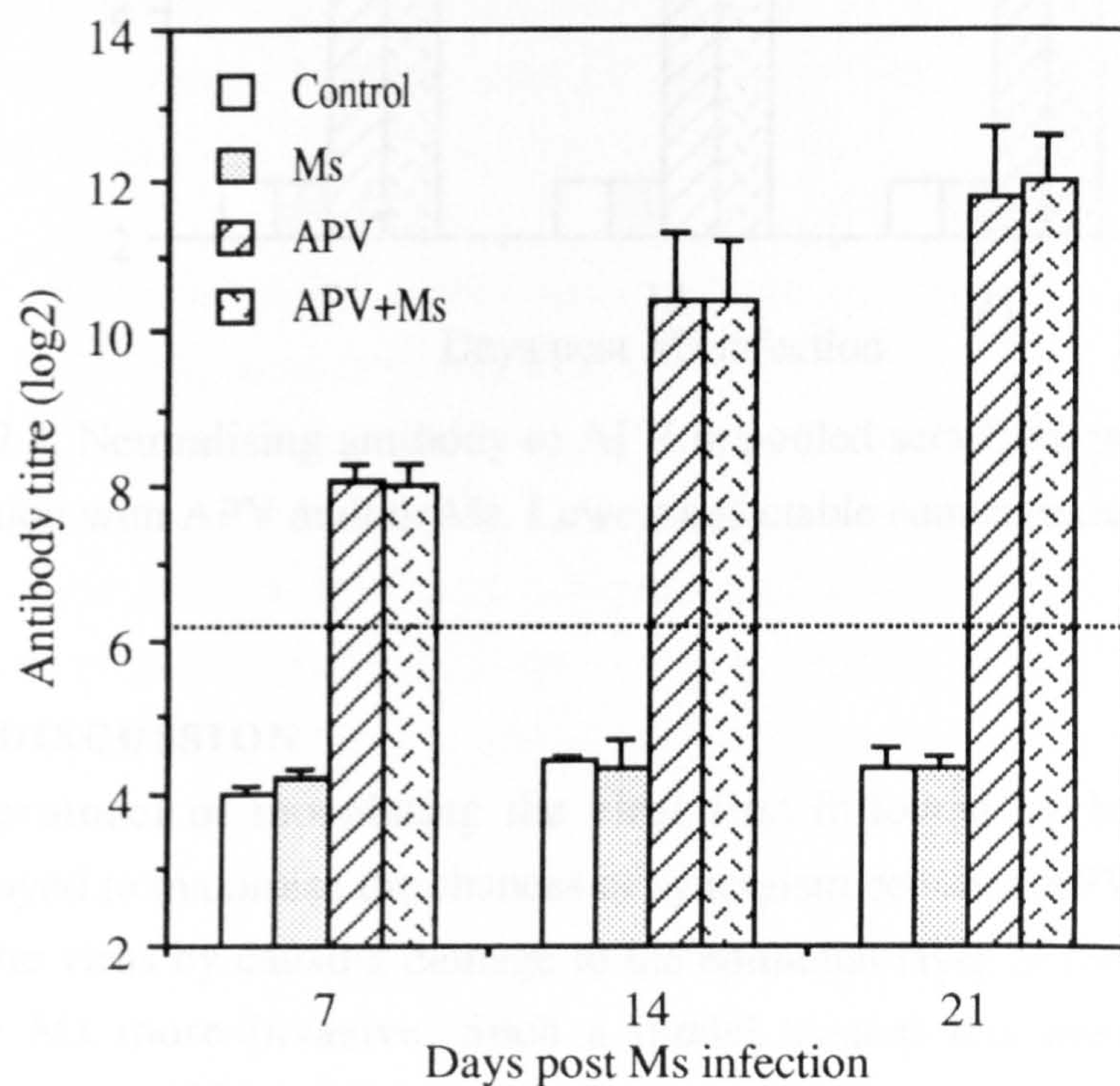

Fig. 9.2. ELISA antibody titres (mean ± sd) to APV in poults after single or dual infection with APV and/ or Ms. Level of significance >6.1 log_2.

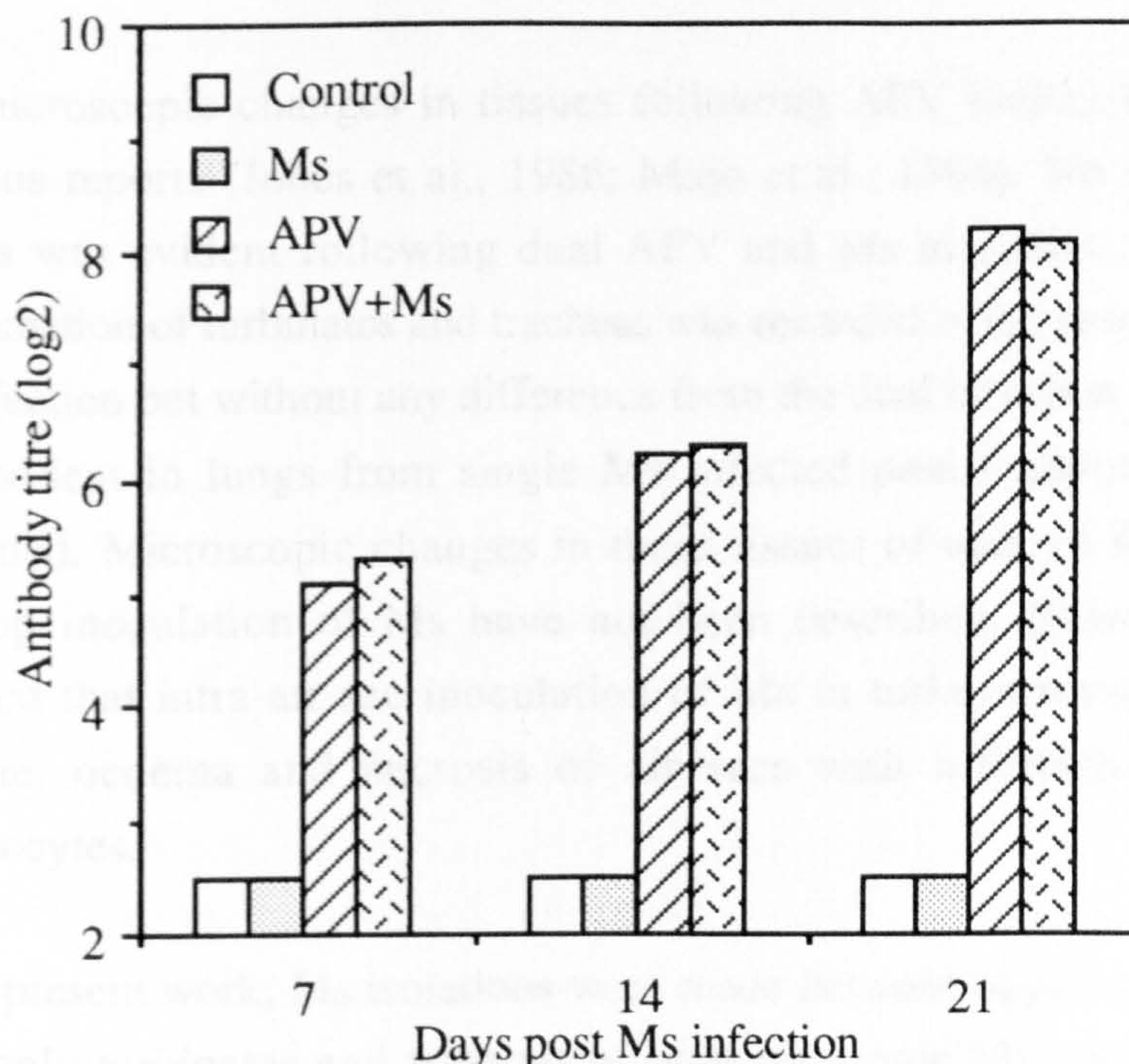

Fig. 9.3. Neutralising antibody to APV in pooled sera from poults after single or dual infection with APV and/ or Ms. Lowest detectable concentration <2.5 log_2.

9.4. DISCUSSION

The protocol of inoculating the virus first followed by Ms three days later was employed to maximise the chances of synergism between APV and Ms. It was thought that the virus by causing damage to the epithelial layer of the respiratory tract, might make Ms more invasive. Such a model system has been used successfully to demonstrate IBV and Ms synergism in chickens (Hopkins & Yoder, 1982). Dual infection of poults with APV and Ms in the present study did not result in detectable synergism.

The clinical disease following APV infection was similar to the experimental studies described previously (McDougall & Cook, 1986; Naylor et al., 1992). Signs in the dual infection group were sustained hardly a day longer than for APV controls, and there were no differences in gross lesions between the two virus infected groups. Following single Ms infection, no signs or gross lesions were seen in poults. Respiratory signs are not usually observed in turkeys following Ms infection, but Ms has been isolated from sinus exudate obtained from turkeys flocks exhibiting a very low incidence of sinusitis (see Kleven, 1997). However, direct inoculation of Ms into

air sacs of turkeys has been shown to result in air sacculitis (Ghazikhanian et al., 1973; Rhoades, 1987).

The microscopic changes in tissues following APV infection were also similar to previous reports (Jones et al., 1986; Majo et al., 1995). No increase in severity of lesions was evident following dual APV and Ms infection. Mild occasional focal inflammation of turbinates and tracheas was recorded in the group of poults with single Ms infection but without any difference from the dual infection group. No abnormality was evident in lungs from single Ms infected poults although air sacs were not examined. Microscopic changes in these tissues of turkeys following intranasal or eyedrop inoculation of Ms have not been described. However, Rhoades (1987) reported that intra-air sac inoculation of Ms in turkeys results in fibrosuppurative exudate, oedema and necrosis of air sacs with infiltration of heterophils and lymphocytes.

In the present work, Ms isolations were made between days 3 to 21 post Ms infection from only turbinates and tracheas of both the single Ms and dual infection groups without any significant differences, but no attempt was made to enumerate the Ms organisms. There was a sudden and surprising increase in the number of Ms isolations from tracheal swabs at the end of experiment but the increase was equal in both the Ms infected groups. No such increase in Ms isolations was evident from tracheal tissues. The birds used for repeated tracheal swabbing were not sampled for collecting tissues at necropsy. The increase in number of Ms isolations from tracheal swabs might have been due to local damage of tracheal surface epithelium caused by repeated swabbing. There were no significant differences in either the number of virus isolations or titres between single APV and the dual infection group.

The antibody response to APV was demonstrable in both the virus infected groups by ELISA and SNT as reported previously (Baxter-Jones et al., 1989; Naylor et al., 1992) but there were no significant differences between antibody titres of these groups indicating that Ms infection did not alter the humoral antibody response to APV. Antibodies to Ms in both the Ms infected groups were not detected either by the RSA test or ELISA. In a previous study, intra-ocular inoculation of Ms in 4-week old turkeys was also shown to result in lack of antibody response in 58% birds although 89% of challenged birds were found to be infected by isolation of Ms from the trachea (Ortiz & Kleven, 1992). The complete failure of the antibody response to Ms in the present study may be attributed to the younger age of the poults.

Dual infection of poults with APV and Ms did not result in detectable synergism except the minor difference in clinical signs between single APV and dual infection groups. Air sac inoculation of Ms in turkeys has been shown to result in air sacculitis (Ghazikhanian et al., 1973; Rhoades, 1987). It is possible, that the use of air sac route for Ms inoculation in poults might have resulted in synergism between Ms and APV.

In chickens, aerosol exposure (a natural means of inoculation) of certain isolates of Ms has been found to cause exacerbation in respiratory disease especially in the form of airsacculitis when combined with vaccination against Newcastle disease or infectious bronchitis (Kleven et al., 1972; Springer et al., 1974). It might be worthwhile in exploring APV and Ms synergism in chickens.

Hopkins and Yoder (1982) reported that the stress of low temperature produced a higher incidence of, and more severe Ms airsacculitis in, chickens previously infected with IBV, and the exposure to IBV field isolates generally resulted in more air sac lesions than did high passaged laboratory strains and vaccine strains. The strain of APV used in this study had undergone 24 passages in TOCs. However it has been previously shown to remain virulent even after 98 passages in TOC and induce the disease in turkeys resembling that caused by the low-passage challenge virus (Williams et al., 1991a). In the present work, infection of turkey poults with this strain of APV was followed by inoculation of a recent field isolate of Ms but no stress factor such as low temperature was included. Before a final conclusion can be made, it requires further work to investigate the effect of cold stress or perhaps some other environmental factors, species difference and other routes of inoculation.

CHAPTER 10

GENERAL DISCUSSION AND FUTURE WORK

This chapter reviews the main results of all the experimental work described in this thesis and highlights the directions for possible future work.

The role of humoral antibodies in APV infections is not clearly understood. Several reports have described the development of humoral antibodies following infection or vaccination and protection against virulent challenge (Cook et al., 1989b; Williams et al., 1991b; Naylor et al., 1997a). However, there have been certain instances which indicated a poor correlation between the presence of circulating antibodies and protection of the respiratory tract. Naylor et al. (1997b) recently reported that the presence of high levels of maternal antibodies in turkey poults did not prevent the development of clinical disease. In an earlier experimental study, Jones et al. (1992) found that chemically (cyclophosphamide) bursectomised vaccinated turkey poults were protected against virulent challenge of APV, despite the fact that there were no significant ELISA antibodies detectable in the serum.

Therefore, it was thought that the study of local and isotype-specific antibody might reveal better correlation with protection against APV infection. In Chapter 4, attempts were made to demonstrate local antibody production and to characterise local and systemic antibody responses following reinfection.

Following APV infection of 4-week old chickens with the virulent strain, local production of IgA and IgG antibody was demonstrated to a greater extent in the harderian gland (HG) and to a much lesser extent in the trachea, using an *in vitro* assay for detecting active local antibody secretion (Zigtermann et al., 1993). High levels of local production of IgA and IgG in the HG correlated with their corresponding secretion in large amounts in lachrymal fluid. Higher levels of virus neutralising (VN) antibodies were detected in lachrymal fluid than in serum particularly on day 7 pi, at the time of virus clearance from the HG itself, and turbinates and trachea. This suggested that the lachrymal fluid antibodies might have an important role in clearance of virus from tissues of chickens following primary infection.

The significance of the class-specific local and systemic antibodies in resistance to reinfection was evaluated in challenge protection trials in 1-week old chicks and poults. In chicks, only the virulent strain elicited local and systemic antibodies and induced

complete protection against reinfection but it turkeys both the virulent and attenuated strains induced these antibodies and conferred complete protection.

The reason for lack of complete protection in chicks primed with the attenuated strain is not clear. It could be related to undetectable levels of both local and serum antibodies elicited by this attenuated strain at time of challenge, or absence of memory antibody response. When 4-week old chicken were infected with a higher dose of this strain, IgA and VN antibodies in lachrymal fluid were detected but the lack of IgG antibody response was consistent. There is no published work describing the correlation of even circulating antibodies with protection against virulent APV challenge of chicks. In contrast to the findings of this study, Cook et al. (1995) observed a good protection against respiratory signs resulting from virulent challenge of chicks vaccinated with an another commercial vaccine strain. This indicates that vaccine efficacy with different strains may differ in chickens.

Following reinfection of chicks primed with a virulent strain and poults primed with either of the strains, a significant elevation in IgA and IgG antibodies in lachrymal fluid and IgG in serum was found to be associated with resistance against APV infection. However, following challenge of poults, there was a secondary boost in virus-specific IgA prior to IgG both locally in lachrymal fluid and tracheal washes, and serum indicating that IgA might have an initial role in inhibition of virus replication. A secondary elevation in VN antibodies in lachrymal fluid of poults occurred prior to that in serum further suggesting the importance of the lachrymal antibodies.

In chicks, challenge resulted in higher levels of VN antibodies in lachrymal fluid than serum also indicating their relative significance in protection against APV infection. In order to arrive at a definitive conclusion concerning lachrymal antibodies being an indicator of protection, further work is required involving larger data and monitoring lachrymal antibody response from individual birds in relation to protection against challenge.

Protection against APV infection like other viral infections, may involve different specific and non-specific immune mechanisms. The importance of cell-mediated immunity (CMI) in APV infections was another area which has been not investigated previously. In Chapter 5, cyclosporin A (CSA), a drug with well-characterised cell mediated immunosuppressive activity (Schreiber & Crabtree, 1992; Ho et al., 1996) was used for studying the role of T-cells in resistance to APV infections.

Primary infection of T-cell suppressed birds with APV resulted in an increase in severity of disease compared to untreated birds. The effect was more marked in turkeys, although some differences were evident in chickens. An increase in severity and prolongation of respiratory signs and tracheal lesions, together with a delay in APV clearance from the tissues of CSA-treated poults indicated that T-cells play a significant role in the recovery of birds from primary APV infection. However, the CSA treatment at the time of both primary and secondary infections was not found to abrogate protective immunity to APV infection and was accompanied by high levels of ELISA and VN antibodies to APV.

In a previous study by Jones et al. (1992), cyclophosphamide (CP) induced B-cell suppressed and vaccinated poults remained protected following virulent challenge despite the absence of significant levels of humoral antibodies. These findings suggested the importance of CMI in protection against APV infection. However, in that report, it was also shown that the CP-treated but unvaccinated poults experienced more severe respiratory disease and higher virus titres in tracheal swabs compared to untreated and unvaccinated birds. In this instance, antibodies appeared to play a role in limiting the severity of primary infection. The role of CMI in recovery from primary infection with APV was also evident in the present study, using CSA-induced T-cell suppression. Thus, from our present state of knowledge, it seems that both B-cell and T-cell immunity are involved in limiting primary APV infection and both arms of the adaptive immune response appear to be necessary for protection against APV challenge.

In consideration of these findings, however, the possible limitations in use of CP- and CSA-based immunosuppression should not be overlooked. The immunosuppression afforded by these drugs might have been incomplete. Also, in addition to its primary effect on B cells, CP has been shown to have a transient effect on T-cells (Elmubarak et al., 1981). In the CSA work, the drug was given at the time of both primary and secondary infections but not between, a similar protocol used successfully by Lillehoj (1987) for studying the role of CMI in the protective immunity to an avian coccidia. It is not known whether continuous administration of CSA throughout the experiment would have produced different results. For further studies more precise immune depletion could be achieved by treatment with selective monoclonal antibodies against T- and B-lymphocytes.

With regard to infectious diseases, a knowledge of modes of transmission and maintenance of reservoir of infection in nature is essential to adopt the most accurate control measures (Thrusfield, 1995). One of the important strategies for maintenance of reservoir of infections is persistence of the infectious agent within the host. In Chapter

6, an attempt was made to investigate long term persistence of APV in chickens and turkeys. The finding of a role for T-cells in recovery from primary APV infection (Chapter 5) was exploited to study APV persistence. The birds were subjected to CSA-based T-cell suppression in an attempt to induce virus re-excretion, on the assumption of APV persistence within the host.

No long-term persistence could be demonstrated in either chickens or turkeys and virus was only isolated during the acute phase of infection. Even the CSA treatment failed to elicit any evidence of virus re-excretion. Dhinakar Raj & Jones (1997) successfully used CSA to demonstrate the long-term persistence of infectious bronchitis virus (IBV) in chickens and age at infection of birds was found to play an important role for IBV persistence. It was found that CSA treatment of IBV-infected chicks at day-old but not at 2 week of age resulted in virus re-excretion.

In the present work, CSA treatment of day-old poults did not reveal persistence of APV. Perhaps APV does not persist, or if it does, the persistence is not under T-cell control. Alternatively, it may persist in other sites not examined in this study. PCR-based techniques have been shown to be more sensitive than virus isolation in detecting APV in experimentally infected poults (Jing et al., 1993). Thus for future studies of this kind, use of PCR with routine virus isolation method should be considered.

One of the most important economic consequence of APV infection in the turkeys is the effect on egg production in breeders. It has been confirmed that APV infects the reproductive tract of turkey hens by replicating in the epithelial cells of the oviduct (Jones et al., 1988; O'Loan & Allan, 1990). Field evidence suggests that the APV can cause reduced egg production in chickens as it does in turkeys, but little is known whether it is capable of infecting the reproductive tract of chickens. In Chapter 7, pathogenicity of APV for the chicken reproductive tract was studied.

The results of *in vitro* experiments on oviduct organ cultures (OOC) showed the intrinsic susceptibility of the chicken oviduct to both the chicken and turkey strain of APV. APV grew relatively poorly in OOC prepared from chicken magnum compared to OOC from uterus. Thus, the *in vitro* finding that the chicken magnum is apparently less susceptible to APV than the uterus would suggest that in the adult hen, if APV does indeed replicates in the oviduct *in vivo*, the virus would be more likely to affect shell quality of eggs than components produced by magnum (albumin).

The use of an *in vivo* precocious oviduct model failed to reveal any evidence of replication of APV in the reproductive tract of either chickens, or surprisingly, turkeys.

But virus indeed replicated in the tracheas of such birds. The reasons for failure to detect APV replication in precocious oviducts even of poults are not clear. One possibility is that oestrogen treatment might have affected replication of APV in the oviducts. However, Ganapathy (1997) successfully used such a model to demonstrate replication of IBV in precocious chicken oviduct and its increased invasiveness following a mixed infection with *Mycoplasma gallisepticum*.. Recently, in this laboratory, C.E. Savage (personal communication) found that a vaccine strain of APV was difficult to grow in rapidly dividing vero cells which otherwise multiplied in confluent monolayer. In the present work, failure to detect APV replication *in vivo* from precocious oviducts even of turkeys might have been due to oestrogen-induced rapid multiplication of oviducal epithelium.

In vivo replication of APV in the chicken ovary or oviduct could not be demonstrated even in 22 month old female chickens despite using two routes of infection with the turkey strain. One route was oculonasal, simulating the natural means of infection and the other, intravenous (IV), was intended to guarantee high levels of virus in the blood stream for the distribution to all sites. Despite these two approaches, virus replication in the oviduct could not be demonstrated even though respiratory infection did occur in both groups.

In a recent study conducted elsewhere (J.K.A. Cook, personal communication), the pathogenicity of a chicken strain of APV for oviduct of hens at peak of lay was evaluated in a similar manner. Although, APV was not detected in the oviduct following natural infection, IV inoculation resulted in its replication in the oviduct epithelium. In the present study, the hens were markedly older; the age difference might be one explanation for our failure to demonstrate the virus in oviducts, even after infection via the IV route. The difference in these findings could also be related to the virus strain used. Cook et al. (1993a) reported some differences in biological behaviour of a chicken and a turkey strain, that these strains caused more marked disease in their homologous species. Perhaps the use of the chicken strain would have resulted in virus replication in the reproductive tract of the chicken.

It may be concluded from the results of this and the Cook study, that while the epithelium of the chicken oviduct is innately susceptible to APV infection with strains of both the chicken and turkey origin, it may remain unaffected after infection via the natural route. What prevents the APV multiplying in the reproductive tract of chickens upon natural infection is not known. Perhaps one factor is the amount of virus circulating in the bloodstream following respiratory infection.

Secondary infectious agents like *E. coli*, *Bordetella* and *Pasteurella* spp are found to be frequently associated with APV infection in nature, resulting in septicaemic lesions thus contributing to the enhanced mortality (see Naylor & Jones, 1993). Under experimental conditions, *Bordetella avium*, *Pasteurella*-like organisms (Cook et al., 1991), *Mycoplasma gallisepticum* (Naylor et al., 1992) and *Mycoplasma imitans* (Ganapathy et al., 1998) have been shown to exacerbate and prolong the clinical disease in turkey poults. Chapter 8 and 9 were devoted to study experimental dual infection of APV with (i) IBV in chicks and (ii) *Mycoplasma synoviae* (Ms) in poults.

There has been evidence of occurrence of both APV and IBV together in respiratory infections of chickens (Gough et al., 1994; Capua et al., 1994; D. Cavanagh & others, personal communications) but there is no published work describing interaction of APV and IBV. In Chapter 8, the effects of these two agents were investigated in dual infections of tracheal organ cultures (TOC) in *vitro* and of chicks in *vivo*.

Dual infection of TOC with APV and IBV simultaneously or IBV preceding APV using either equal doses of both viruses or a lower IBV dose, revealed IBV to be the predominant virus, interfering with APV replication. However, APV interference with IBV replication was also observed when APV infection preceded that of IBV, especially using a lower IBV dose. As compared to single IBV infection, dual infection of TOC in any combination of two viruses or by varying IBV dose did not result in an increase in rate of ciliary damage to organ cultures.

Dual infection of chicks *in vivo* reflected the *in vitro* results and the main findings were: (i) no increase in the severity of clinical disease and damage to tracheas or infectious virus persistence compared to single IBV infection (ii) interference by IBV with the replication of APV in the trachea (iii) and IBV interference with the serological response to APV, but not vice versa.

The role of interferon in interference of IBV over APV was not investigated, although it appears that IBV may simply have interfered owing to its faster replication in TOC *in vitro* and in tracheas of chicks *in vivo*, exhausting the epithelial receptor sites. Further studies could be undertaken to investigate the role of interferon in the mechanism of interference between IBV and APV.

IBV is also known to interfere with rate of multiplication of and antibody development to Newcastle disease virus in experimentally infected chickens (Raggi & Lee, 1964). It has been also shown that IBV and NDV act as live vaccines when administered simultaneously: IBV interfere with NDV vaccine by resulting in less protection against

Newcastle disease challenge (Raggi & Lee, 1964; Thornton & Muskett, 1975). The results of the present study clearly have implications not only for natural multiple infections, but also for live vaccine application. However, it requires additional studies using live APV and IBV vaccines to further evaluate the importance of IBV interference in antibody response to APV in terms of protection.

In Chapter 9, the possibility of Ms as a complicating factor following APV infection of turkey poults was investigated. To maximise the chances of synergism between APV and Ms infections, the protocol of inoculating the virus first followed by Ms three days later was employed. It was thought that the virus by causing damage to the epithelial layer of the respiratory tract, might make Ms more invasive.

However, this protocol of dual infection of APV and Ms did not result in detectable synergism except minor difference in clinical signs. There was no increase in severity of gross and microscopic lesions in tissues of the respiratory tract of poults, and no increased persistence in either APV or Ms. The poults did not seroconvert to Ms and Ms inoculation did not affected the development of antibodies to APV.

Hopkins & Yoder (1982) reported that stress of low temperature produced higher incidence of and more severe Ms airsacculitis in chickens previously infected with IBV. Although an unnatural means of infection, air sac inoculation of Ms alone in turkeys have been shown to result in air-sacculitis (Ghazikhanian et al., 1973; Rhoades, 1987) unlike the oculonasal route used in the present study. Thus, before a final conclusion can be drawn concerning the synergism between APV and Ms, studies involving the use of environmental stress factors such as low temperature and other routes of inoculation should be considered.

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APPENDIX

BUFFERS, SOLUTIONS AND MEDIA

Antibiotic stock solution

600 mg of Crystapen (1000,000 IU benzyl penicillin sodium, BP)
(Glaxo Lab. Ltd., Greenford, England)
1 g streptomycin sulphate BP (Sigma)
40 ml of sterile distilled water

4 ml of this stock solution was added to one litre of medium for a 1x solution. For preparing 10x medium, 4 ml was added to 100 ml of medium.

Phosphate buffered saline (PBS)

80 g of sodium chloride
2 g of potassium chloride
11.5 g of sodium hydrogen phosphate
2 g of potassium dihydrogen phosphate
800 ml of distilled water

TOC medium

100 ml of 10x Minimum Essential Medium-Eagle's (Life Technologies)
20 ml of 7.5% sodium bicarbonate (Life Technologies)
900 ml of sterile distilled water
4 ml of stock antibiotic solution

OOC medium

85 ml of 10x Medium 199 (Life Technologies)
100 ml of tryptose phosphate broth (Sigma)
10 ml of 7.5% sodium bicarbonate (Life Technologies)
14 ml of HEPES (Life Technologies)
625 ml of sterile distilled water
4 ml of stock antibiotic solution

VERO CELLS

Culture medium

40 ml of Minimum Essential Medium (Life Technologies)
50 ml of lactalbumin hydrolysate (Life Technologies)
7.5 ml of 7.5% sodium bicarbonate (Life Technologies)
412 ml of sterile distilled water
2.5 ml of gentamicin (10 mg/ml) (Life Technologies)

Growth medium

10% foetal calf serum (Life Technologies) was added to culture medium immediately prior to use.

Maintenance medium

5% foetal calf serum (Life Technologies) was added to culture medium immediately prior to use.

PBS/EDTA

1000 ml of PBS
0.2 g of EDTA
Autoclaved.

Trypsin solution

100 ml of PBS/EDTA
10 ml of 2.5% Trypsin (Life Technologies)
Aseptically prepared.

GTNE BUFFER FOR VIRUS PURIFICATION

15 g of 200mM glycine (Sigma)
6.05 g of 50mM tris base (Sigma)
5.84 g of 100mM sodium chloride (Merck Ltd, Leicestershire, UK)
0.292 g of 1mM EDTA (Merck Ltd)

Dissolved in approximately 500 ml of distilled water. Dilute hydrochloric acid added whilst stirring till pH is 7.5. Made up to 1 litre with distilled water.

ELISA REAGENTS

CBC buffer pH 9.6

1.5 g of sodium carbonate
2.93 g of sodium bicarbonate
1 litre of distilled water

Wash-dilution buffer

292.2 g of sodium chloride
5.35 g of disodium hydrogen phosphate
1.95 g of sodium dihydrogen phosphate dihydrate
2.5 ml of tween 80
5 litres of distilled water

Phosphate-citrate buffer

243 ml of 0.1M citric acid
0.2M disodium hydrogen phosphate added till pH 5.0
Made up to 1 litre with distilled water.

OPD substrate

0.4 g of OPD (sigma) dissolved in 1 litre of phosphate-citrate buffer was divided into 12 ml aliquots and stored in the dark at - 20°C. Prior to use, 6 µl of 30% hydrogen peroxide was added to 12 ml of thawed substrate for activation.

Stop solution

5 N of sulphuric acid was used.

CULTURE MEDIA FOR MYCOPLASMA ISOLATION

Mycoplasma broth (modified Chanock medium)

To prepare 100 ml of broth:

Part A 1.47 g of PPLO broth powder (Difco, Detroit, USA)
70 ml of distilled water
Autoclaved at 15 lb/sq in for 15 min

Part B 15 ml of heated swine serum

10 ml of yeast extract

1ml of 1% NAD (Boehringer, Mannheim, Germany)

1ml of 10% glucose solution

1ml of 5% thallium acetate solution

100,000 IU of penicillin

2 ml of 0.1% phenol red solution

Part B was assembled from already sterile ingredients and mixed with Part A after cooling.

Mycoplasma agar

To prepare 100 ml of agar

Part A 1.47 g of PPLO broth powder (Difco)

1 g of purified agar (Amersham, Bury, England)

70 ml of distilled water

Autoclaved at 15 lb/sq in for 15 min

After autoclaving, kept molten by placing in a water bath at 56°C.

Part B As for broth but without phenol red. Part B was warmed to 56°C in water bath and mixed with Part A. It was then poured into sterile petri dishes and after solidifying stored in an airtight container at 0-4°C.