SOME IMMUNOLOGICAL ASPECTS OF *TAENIA HYDATIGENA* INFECTIONS IN SHEEP.

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Science at Massey University.

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ABSTRACT

The literature on the biology, distribution, prevalence and importance of *T.hydatigena*, the biology of its life cycle, and the immunology of the relationships between taeniids and their hosts, is reviewed.

In three experiments, serum was transferred from immune donors to non-immune recipients before the latter were given a homologous challenge infection. Highly significant protection was achieved in recipients of serum from lambs given three immunizations of solubilized *T.hydatigena* oncospheres or three oral infections, but not in recipients of serum from lambs given a single low-level oral infection. Comparison of sera of recipients by ELISA using solubilized *T.hydatigena* oncosphere antigen, revealed that unprotected recipients had substantially lower levels of anti-*T.hydatigena* antibodies than protected recipients. The donors of the sera which did not protect the recipients also had low ELISA absorbances but were, themselves, immune to a challenge infection.

The importance of antibody in causing death of oncospheres was examined *in vitro*. Oncospheres were cultured in the presence or absence of antibody, complement, and leukocytes from immune or non-immune animals, and their effects and interactions on larval survival assessed after 10 days culture. No reduction of larval survival occurred when antibody was absent. The major effect of antibody was mediated by complement. In the presence of antibody and complement, leukocytes further reduced larval survival but in the absence of complement, their influence was unclear.

The involvement of colostral antibody from orally infected ewes in protecting neonatal lambs was also examined. A significant, short-acting, immunity was transferred from ewes which had received either three oral doses of 150 activatable oncospheres, or an initial dose of 100 activatable oncospheres followed by two of 10 000. The correlation between the number of cysts resulting from the challenge infection and the level of anti-*T.hydatigena* antibody in their serum at the time of challenge, was highly significant. There appeared to be a critical level of antibody, above which virtually complete protection resulted and below which, there was very little. Significant relationships existed between the levels of antibody in the sera of the one-week-old lambs and their dams on the day of parturition, and in the whey of colostrum collected on the same day.

The duration of the colostral immunity suggested that IgG_2 might be more effective than IgG_1 against *T.hydatigena* oncospheres. Culturing oncospheres with fractionated IgG from serum and colostrum indicated that increasing levels of both IgG_1 and IgG_2 resulted in decreasing levels of larval survival. However, the effect of increasing levels of IgG_2 was much more marked than with IgG_1.

A preliminary attempt to identify antigens able to induce protection against a challenge infection in sheep, indicated that antigens of less than 30 kDa molecular weight significantly protected recipients of them. The antigens on a Western blot of *T.hydatigena* oncospheral antigen which were recognized by immune sheep serum did not correspond with the antigens stained by the protein stains, Coomassie blue or silver stain. This suggests that protective antigens may be predominantly carbohydrate rather than protein.

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CHAPTER 1
INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 GENERAL INTRODUCTION TO *TAENIA HYDATIGENA*.

1.1.1 Classification and General Features.

The true cestodes are platyhelminths classified in the Subclass Eucestoda within the Class Cestoidea. With few exceptions, their life cycles are indirect. Eggs are produced which contain an embryo which is infective to the appropriate intermediate host(s). In some cycles (e.g. in the Pseudophyllidea) a second intermediate host is required before the larvae (metacestodes) can become infective to the definitive host.

The Eucestodes of greatest importance medically and economically belong to the Family Taeniidae (Ludwig, 1886) in the order Cyclophyllidea. The larval stages of those species presenting significant public health and meat hygiene problems mainly develop in domesticated animals, especially ruminants and pigs. In the normal life cycles of these species, the adults mature in canids (e.g. *Taenia ovis*, *Taenia hydatigena*, and *Echinococcus granulosus*) or man (e.g. *Taenia saginata* and *Taenia solium*). Humans can also become seriously affected by the larval stages of some taeniid species (including *T.solium*, *Echinococcus multilocularis* and *E.granulosus*) although humans are not the normal intermediate hosts.

T.hydatigena (Pallus, 1766) is a large, 750 - 5000 mm, (Soulsby, 1982) tapeworm residing in the small intestine of canids (Table 1.1). Anteriorly, a scolex, or holdfast, bearing 4 suckers (acetabula) and 2 rows of hooks, attaches the parasite to the wall of the definitive host's intestine. The elongate body, comprising a chain of proglottids each containing male and female reproductive organs, lies free within the intestine. It possesses no gut and acquires its nutritional requirements by absorption across the syncytial outer covering, the tegument.

The life cycle of *T.hydatigena* will be described in detail in Section 1.2. Briefly, gravid proglottids, filled with mature eggs, are passed out in the faeces of the definitive host. These eggs, ingested by an intermediate host, hatch and the oncospheres become activated in the small intestine. These enter the blood stream through the intestine wall and are carried to the liver where they begin to develop

and burrow through the liver tissue before emerging into the peritoneal cavity. Here each larva, or metacestode, develops into a large, fluid-filled cysticercus into which projects an invaginated scolex. These cysticerci, often referred to as *Cysticercus tenuicollis*, are found enclosed in host tissue primarily attached to the mesentery and omentum in the peritoneal cavity of a wide range of intermediate hosts (Table 1.1). Many of the hosts listed in Table 1.1 would be regarded as accidental or abnormal hosts.

The definitive host becomes infected by ingesting mature metacestodes from the carcass of an infected intermediate host.

1.1.2 Distribution, Prevalence and Importance in the World.

T.hydatigena can generally be found to be cycling wherever sheep, goats or pigs live in association with dogs. In Canada and the USA *T.hydatigena* is common in wild ruminants. In the USA the prevalences shown in Table 1.2 have been recorded. Prestwood *et al.* (1976) found both white tailed deer and domesticated sheep, which share a common range in eastern West Virginia, were infected with *T.hydatigena* cysticerci; no prevalence data were given. Occasionally lambs from widely separated parts of Canada have been reported to be infected (Sweatman and Plummer, 1957). In one survey, 0.5% of slaughtered lambs were infected and in another, 3%. The increasing numbers of domesticated animals in frontier settlements at that time provided the means of transfer of the parasite into the domesticated animal population through the close association of these settlements with wild animals. Sweatman and Plummer showed that it was possible to transfer *T.hydatigena* from moose to dogs and that the eggs from the subsequent infection were infective to lambs and pigs. They also reviewed the wild intermediate hosts of *T.hydatigena* in Canada, showing that moose, reindeer, wapiti, and caribou scattered all over Canada were infected with the prevalence ranging from 14-85%. Timber wolves are commonly infected and are an important definitive host in Canada; 49% of wolves in Yukon and the North-west Territories were found infected with *T.hydatigena* (Choquette *et al.*, 1973). Since these wander over land where domesticated animals graze they are a potential source of infection for them.

The United Kingdom appears to have a relatively high prevalence of adult and larval *T.hydatigena* in domesticated animals as indicated in Table 1.3 and the following reports. Several studies have been carried out in the past decade in various areas of the United Kingdom and although a relatively high prevalence has been recorded, Trees *et al.* (1985) believe there is a lack of awareness of the importance of

T.hydatigena. This is supported by the fact that in many other surveys in the United Kingdom *T.hydatigena* is not categorized as a separate entity but is often included in the "other causes" category (Evans and Pratt, 1978; Blamaire *et al.*, 1980; Cuthbertson, 1983).

Table 1.1 Hosts of *T.hydatigena.*

Intermediate Hosts.	References.*
Moose (*Alces alces, A.alces gigas*)	a,d,e
Alces americana	b
Barren-ground caribou (*Rangifer articus*)	b,d
Reindeer (*Rangifer tarandus*)	a,b
Rangifer tarandus caribou, R.tarandus stonci	a
Roe deer (*Capreolus capreolus*)	a,e
Black-tailed deer/Mule deer (*Odocoileus hemionus*)	a,b,f,g
Whitetail deer (*Odocoileus virginianus*)	a,b,f,h
Musk deer (*Moschus moschiferus*)	a
Spotted deer (*Cervus axis*)	a
Red deer (*Cervus elaphus*)	a
Manchurian wapiti (*Cervus elaphus xanthopygos*)	a
Wapiti (*Cervus canadensis*)	Love cited by b
Axis deer (*Cervus nippon*)	a
Fallow deer (*Dama dama*)	a
Giraffe (*Giraffa camelopardalis*)	a
Buffalo (*Babalus bufellus*)	a,c,i
Four-horned Antelope (*Tetracerus quadricornis*)	a
Bison (*Bos bonasus*)	a
Yak (*Bos grunniens*)	a
Cattle	j
Waterbuck (*Kobus ellipsiprymnus*)	a
Mountain Reedbuck (*Redunca redunca*)	a
Oryx (*Oryx gazella*), Arabian oryx (*O.leucoryx*)	a
Bontebok (*Damaliscus dorcas*)	a
Springbok (*Antidorcas marsupialis*)	a
Blackbuck (*Antilopa cervicapra*)	a,k
Dorcas gazelle (*Gazella dorcas*)	a
Zeren (*Gazella gutterosa*)	a
Persian/Goitered Gazelle (*Gazella subgutterosa*)	a
Saiga (*Saiga tatarica*)	a
Musk Ox (*Ovibos moschatus*)	a
Chamois (*Rupicapra rupicapra, R.rupicapra caucasica*)	a
Goats	c,i,j,l,m,n,o
Goat (*Capra hircus*)	a
Ibex (*Capra ibex*)	a
Siberian goat (*Capra siberica*)	a
Mountain Goat (*Oremnos americanus*)	p
Sheep, domestic (*Ovis aries*)	a,h,Section 1.1.2
Argali (*Ovis ammon*)	a
Bighorn sheep (*Ovis canadensis*)	a,f
O.canadensis mexicana, O.canadensis dalli	a

Ovis vinei	a
Mouflon (*Ovis musimon*)	a
Red sheep (*Ovis orientalis*)	a
Bactrian Camel (*Camelus bactrianus*)	a
Arabian Camel (*Camelus dromedarius*)	a
Pigs (*Sus scrofa*)	a,c,i,o,q
Wart hog (*Phacochoerus aethiopicus*)	a
Bush pig (*Potomochoerus porcus*)	a
Horse (*Equus caballus*)	a,c
Donkey, Mule	c
Jird (*Meriones unguiculatus*)	z
Field Mouse (*Apodemus sylvaticus*)	a
House Mouse (*Mus musculus*)	a
Norway Rat (*Rattus norvegicus*)	a
Common Hamster (*Cricetus cricetus*)	a
Yellow Vole (*Evatomys glareolus*)	a
Common Vole (*Microtus arvalis*)	a
Tree Squirrels (*Sciurus nigeran, S.niger rufiventer*)	a
Ground Squirrel	r
Red Squirrel (*Sciurus vulgaris*)	a
Rabbit (*Oryctolagus cuniculi*)	a
Hares (*Lepus californicus deserticola*)	a
Dog (*Canis familiaris*)	a
Cat (*Felis ocreata domestica*)	a
Monkeys	q
Mona Monkey (*Cercopithecus mona*)	a
Cercopithecus ruber	a
Green Monkey (*Cercopithecus sabaeus*)	a
Barbary ape (*Macaca cynomolgus, M. innuus*)	a
Baboons (*Papio* sp)	a,q
Man (*Homo sapiens*)	a,q

Definitive Hosts.

Red Fox (*Vulpes vulpes*)	a,s,t,u
Arctic Fox (*Alopex lagopus*)	a
Dog (*Canis familiaris*)	a, Section 1.1.2
Wolf (*Canis lupus*)	a,d,v,w
Steppe wolf (*Canis lupus campestris*)	a
Asiatic Jackal (*Canis aureus*)	a
Black-backed Jackel (*Canis mesomelas*)	a
Coyote (*Canis latrans*)	a,w
Dingoes (*Canis familiaris dingo*)	x
Raccoon Dog (*Nyctereutes procyonoides*)	a
Cat (*Felis ocreata domestica*)	a
Felis euptilura	a
Lion (*Panthera leo*)	a
Leopard (*Panthera pardus*)	a

Pine Marten (*Martes martes*)	a
Stoat (*Mustela erminea*)	a
Weasel (*Mustela nivalis*)	a
Polecat (*Mustela putorius*)	a
Tasmanian Devil (*Sarcophilus harrisi*)	y

*References for Table 1.1.

The majority of the information presented is taken from Abuladze (1964) (reference a).

a = Abuladze, 1964
b = Sweatman and Plummer, 1957
c = Soulsby, 1982
d = Choquette *et al.*, 1973
e = Nilsson, 1971
f = Boddicker and Hugghins, 1969
g = Worley and Eustace, 1972
h = Prestwood *et al.*, 1976
i = Varma and Ahluwalia, 1986
j = Dada and Belino, 1978
k = Thornton *et al.*, 1973
l = Opasina, 1985
m = Sanyal and Sinha, 1983
n = Dajani and Khalaf, 1981
o = Pathak and Gaur, 1982
p = Boddicker *et al.*, 1971
q = Flynn, 1973
r = Jenkins and Grundmann, 1973
s = Edwards *et al.*, 1979b
t = Dent, 1974
u = Williams, 1976a
v = Mobedi *et al.*, 1973
w = Samuel *et al.*, 1978
x = Durie and Riek, 1952
y = Gregory, 1976b
z = Williams and Colli, 1970

Table 1.2 Prevalence of *T.hydatigena* in wild ruminants of N. America.

Intermediate host	Proportion Infected	Area	References
White tailed deer	6%	S. Dakota	Boddicker and Hugghins, 1969.
Black tailed deer	13%	S. Dakota	"
Bighorn sheep	1/3	S. Dakota	"
Mountain goats	58%	S. Dakota	Boddicker *et al.*, 1971.
Black tailed deer	14%	Montana	Worley and Eustace, 1972.
Blackbuck	2/3	Texas	Thornton *et al.*, 1973.

Table 1.3 **The Prevalence of *T.hydatigena* in Domestic Animals.**

Period	Country/Area	Dog Type	Mean (Max.) Worms/dog	% Infected					References*
				Dogs	Goats	Pigs	Cattle	Sheep	
	EUROPE.								
Mar 1963	Builth Wells, Mid-Wales	Farm	4	55.2	--	--	--	--	a
	Mid-Wales			--	--	--	--	21.6 (livers)	b
?	Lake District	Farm		26.8	--	--	--	--	c
	North Pennines	Farm		11.9	--	--	--	--	
	East Anglia	Farm		0	--	--	--	--	
?	Dyfed, Wales			--	--	--	--	40	d
1973-75	Dyfed, Wales	Farm, all year	2.7 (37)	46	--	--	--	--	d
		Farm, winter		22.6	--	--	--	--	
Nov '74-Mar '75	Dyfed, Wales	Foxhounds	3.5 (37)	49	--	--	--	--	e
Jan 1975	Mid-Wales	Farm		28	--	--	--	--	f
May 1975			3.2	15	--	--	--	--	
Oct 1975				10	--	--	--	--	
Jan '76-Jun '78	Snowdonia,	Farm	2.1 (8)	11.3	--	--	--	6.4 (livers)	g
	North Wales			--	--	--	--	30.6	

Period	Country/Area	Dog Type	Mean (Max.) Worms/dog	% Infected					References*
				Dogs	Goats	Pigs	Cattle	Sheep	
1977-78	Snowdonia, North Wales	Foxhounds and Kennelled dogs	2.3 (21)	18.2	--	--	--	--	h
				--	--	--	--	25.2 (lambs)	
1980-83	Clwyd, North Wales	Farm	1.8 (2)	4.3	--	--	--	--	i
		Foxhounds		11.7	--	--	--	--	
		Gundogs		4	--	--	--	--	
Dec 1985	North Yorkshire			--	--	--	--	73.4 (livers)	j
Jan				--	--	--	--	35.7 (livers)	
Dec, 1981-83	Colne			--	--	--	--	23 (livers)	j
Jan				--	--	--	--	30 (livers)	
Feb				--	--	--	--	11 (livers)	
Mar				--	--	--	--	19 (livers)	
Apr - Nov				--	--	--	--	5 (livers)	
Jan '85-Jan '86	Germany			--	--	--	--	16.7	k
	INDIA.								
Aug '69-Mar '70	Uttar Pradesh			--	55.34	--	--	44.6	l
Apr '77-Mar '79	Uttar Pradesh			--	28.01	4.4	15.76 (buffalo)	29.17	m
Jun '78-Jun '79	West Bengal			--	10.22	--	--	--	n

Period	Country/Area	Dog Type	Mean (Max.) Worms/dog	% Infected					References*
				Dogs	Goats	Pigs	Cattle	Sheep	
?	Uttar Pradesh	Stray		37.5	--	--	--	--	o
		Indoor		29	--	--	--	--	
				--	27.29	8.3	--	37.03	
	AFRICA.								
May '80-Apr '82	Zambia	Stray and owned	4 (19)	17.64	--	--	--	--	p
Jan - Jun '78	Jordan			--	6.2	--	--	9.2	q
?	Jordan			45.6	--	--	--	--	r
Apr '79-Dec '80	South-West Nigeria			--	11.6	--	--	23.5	s
?	Northern Nigeria			--	34.2	--	0.1	21.4	t
Sep '85-Jun '86	Ethiopia			--	--	--	--	37	u
?	Ethiopia		Intermediate host prevalence			=	20%		v
	Ivory Coast		"				=	49%	w
	Sudan		"				=	32.4%	x
	AMERICA.								
Aug '73-May '74	Colorado			--	--	--	--	28 (lambs)	y
1990	South America			High Prevalence					z

* References for Table 1.3

a = Cook, 1984
b = Cook, 1965
c = Cook and Clarkson, 1971
d = Williams, 1976b
e = Williams, 1976c
f = Hackett and Walters, 1980
g = Edwards *et al.*, 1979a
h = Edwards *et al.*, 1979b
i = Stallbaumer, 1985
j = Trees *et al.*, 1988
k = Hasslinger, 1988
l = Varma and Rao, 1973
m = Varma and Ahluwalia, 1986
n = Sanyal and Sinha, 1983
o = Pathak and Gaur, 1982
p = Islam and Chizyuka, 1983
q = Dajani and Kahlaf, 1981
r = Saliba and Ajluni, 1979
s = Opasina, 1985
t = Dada and Belino, 1978
u = Bekele *et al.*, 1988
v = Anonymous, 1971; Graber, 1978; FAO, 1968;
w = El-Badawi *et al.*, 1978
x = Mishra and N'depo, 1978
y = Jensen and Pierson, 1975
z = Heath, 1990 Personal Communication.

1.1.3 Prevalence and Importance in New Zealand.

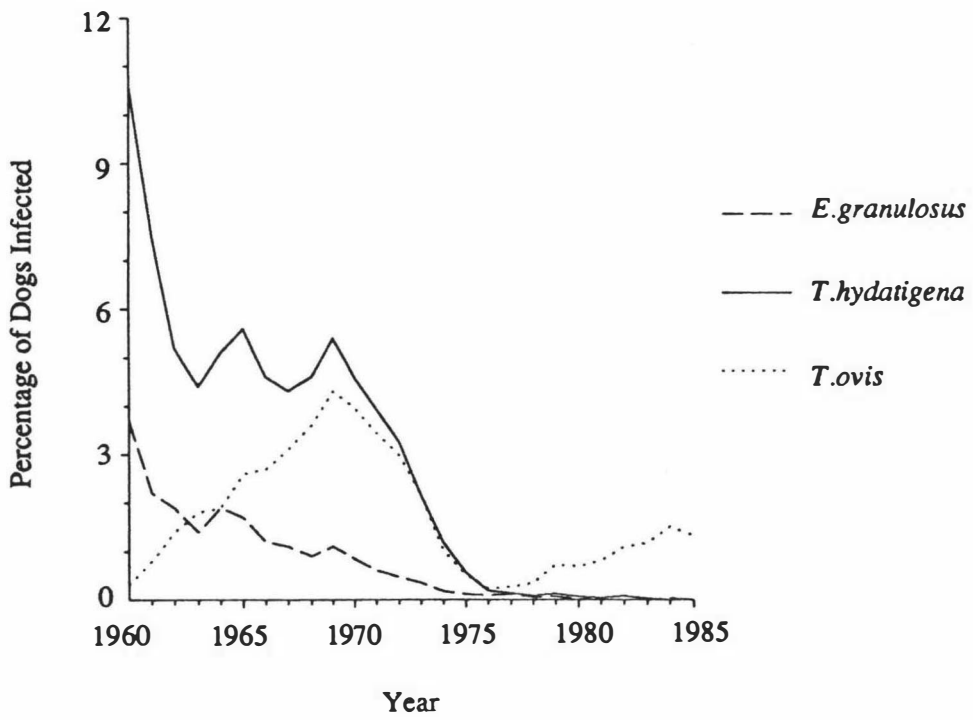
In 1958-1959 (just prior to the initiation of the official national campaign to control *E.granulosus* and *T.hydatigena*) a survey of the livers of domesticated food animals in New Zealand revealed that 60 - 65% of the livers of ewes and lambs had healed pits and scars or calcified lesions presumed to be caused by the cysticerci of *T.hydatigena* (Gemmell, 1961a).

In the early 1940's the prevalence of *T.hydatigena* in dogs in New Zealand was 37.7% (Gemmell, 1973). Public education and the administration of purgatives to dogs at risk (i.e. working or rural) reduced this to 10.6% in 1960 and to 4.7% in 1972 (Figure 1.1; Gemmell, 1973). A further reduction in the prevalence of *T.hydatigena* began in 1972 when the 6-weekly treatment of dogs with niclosamide was introduced as part of the National Hydatids Control Programme. This treatment was introduced in an attempt to control the rise in prevalence of *T.ovis* which occurred in association with the reports of increased feeding of sheep meat to dogs and a decline in the prevalence of *T.hydatigena* and *E.granulosus* (Figure 1.1; Gemmell, 1973). However, niclosamide is not effective against *E.granulosus* and from about 1978 dogs were treated with praziquantel which is also effective against *E.granulosus*.

The prevalence of *T.hydatigena* in sheep is now very low and the parasite is no longer officially reported at meat inspection (Heath, personal communication). Very few cysts are found in adult sheep and only occasional outbreaks of infection

("cysticercosis storms") occur. These are characterized by a high percentage of lambs on a single property becoming heavily infected over a short period of time. This reflects the low prevalence of infection which prevents lambs from acquiring a small, immunizing dose of eggs when they begin to graze. Consequently they are vulnerable to an intake of large numbers of eggs arising, for example, from a dog on the property being infected (Gemmell, 1978; Gemmell *et al.*, 1986b).

Figure 1.1 **Percentage of dogs infected with tapeworms in New Zealand.**



The 6-weekly dosing of working dogs with a cestocidal drug has apparently contributed to the marked decrease in prevalence of *T.hydatigena*, but the policy for eradication of *E.granulosus* and control of *T.ovis* has recently been revised. While good progress is being made towards the eradication of *E.granulosus*, the prevalence of *T.ovis* is not declining, in part because 6-weekly dosing is encouraging illegal feeding of dogs. It has now been decided that 6-weekly dosing of dogs will cease except on specified properties. The National Hydatids Council has stated:

> "For *Taenia ovis* control purposes ... better results can be obtained by monitoring effectiveness of infection preventive measures taken by dog owners rather than continue to treat their dogs for them." (National Hydatids Council 29th Annual Report, 1989)

Serological testing of dogs has been introduced and this will detect the occurrence of recent infection even if treatment is given (29th National Hydatids Council Annual Report, 1989). A survey carried out by the National Hydatids Council revealed evidence of widespread feeding of offal to dogs under the protection of the 6-weekly dosing scheme. It did not specify whether the offal was cooked or uncooked (National Hydatids Council Senior Executive Officer, 1989, personal communication). The removal of the dosing scheme could result in a return to the prevalence of *T.hydatigena* which existed in 1958-59 unless sheep scavenging and the feeding of untreated offal to dogs can be controlled. This level of infection could cost the New Zealand meat industry more than $10 million annually in lost liver sales (Appendix 1).

1.2 BIOLOGY OF THE PARASITE

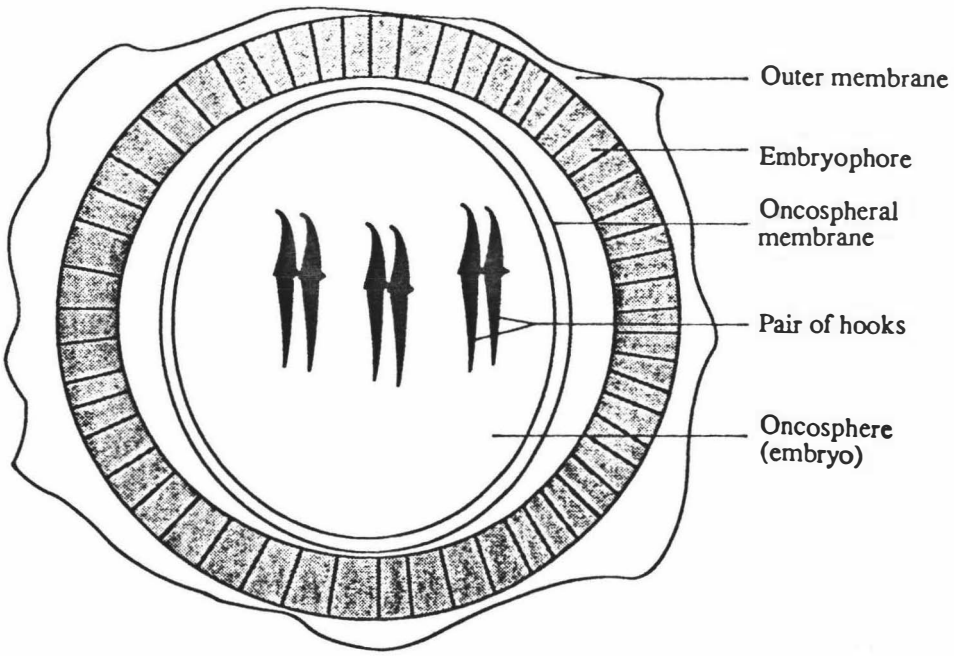
1.2.1 The Egg.

1.2.1.1 Structure.

The eggs of taeniid species are morphologically indistinguishable by light microscopy (Figure 1.2). They are spherical to ellipsoidal in shape and usually range from 30 - 50 μm x 22 - 44 μm (Thompson, 1986). Their outermost layer, or yolk sac, is delicate, containing a few small vitelline cells (Lethbridge, 1980) which are stripped from the egg before it is expelled from the tapeworm proglottid (Section 1.2.3).

Inside the outer membrane is the embryophore, a thick protective structure made up of polygonal blocks (Morseth, 1965; Thompson, 1986), the chemical composition of which is stated to be a keratin-type protein in *Taenia pisiformis*, *T.hydatigena* and *T.ovis* (Morseth, 1966). These blocks are held together by a cementing substance (Nieland, 1968) the composition of which is unknown, but which is susceptible to the proteolytic enzymes of the small intestine. On the inside of the embryophoric blocks is a granular layer and, separating this from the oncosphere itself, is the oncospheral membrane (Figure 1.2).

Figure 1.2 Simplified diagram of a taeniid egg.

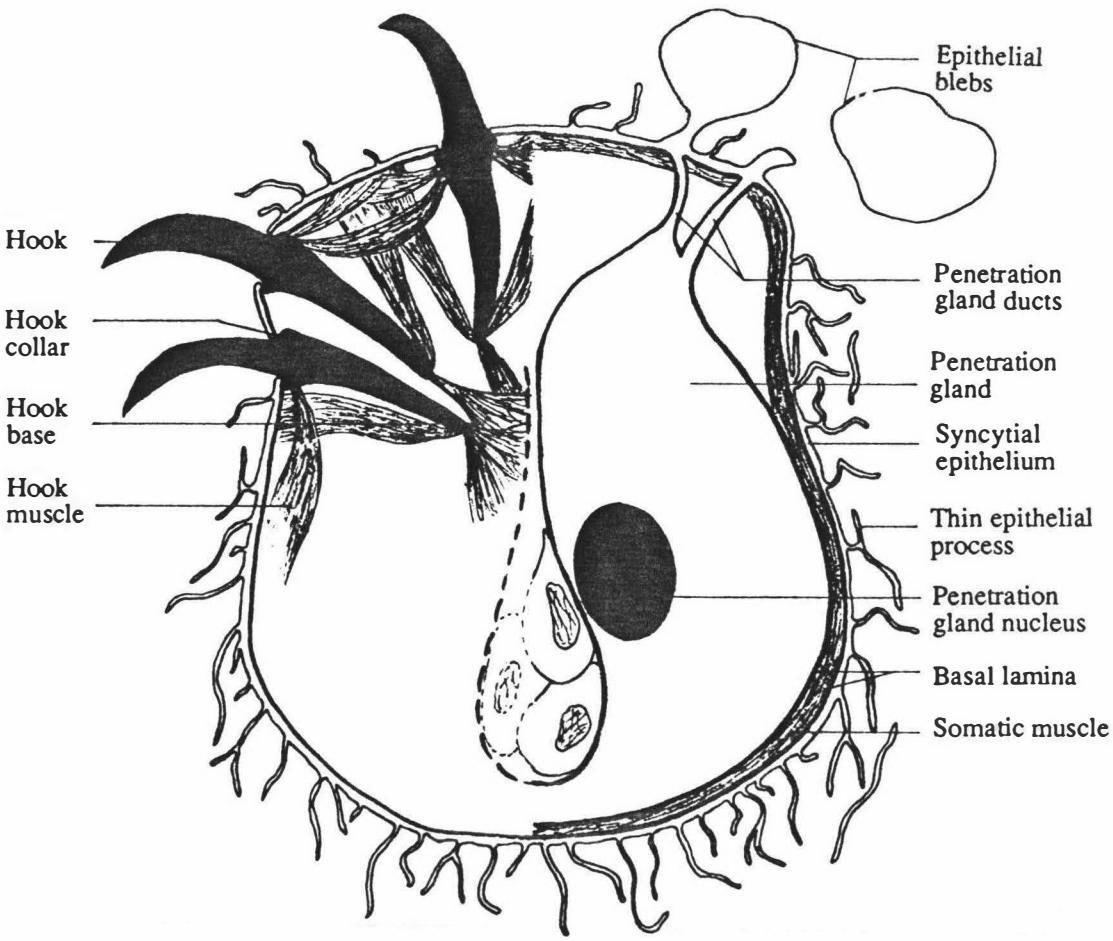


The contents of the oncosphere are contained within a syncytial epithelium, the outer surface of which is thrown into many filamentous projections (Figure 1.3). A complex array of muscles attach to the basal lamina which lies below the epithelium, and to the collar and base of each hook, while bands of somatic muscle encircle the oncosphere beneath the basal lamina (Swiderski, 1983).

A structure which may occupy at least a third of the volume of newly-hatched cyclophyllidean oncospheres is the penetration gland. The glands from various species of cyclophyllidean oncospheres are of similar general structure but vary in some details (Reid, 1948; Lethbridge, 1980; Fairweather and Threadgold, 1981; Swiderski, 1983; Harris *et al.*, 1987). The ultrastructure of the penetration gland of *T.hydatigena* has not been described but those of *T.ovis* and *E.granulosus* have been reported as comprising a large U-shaped, 4-nucleated syncytial gland associated with one pair (*T.ovis*) or 2 pairs (*E.granulosus*) of other, smaller, glandular structures (Swiderski, 1983; Harris *et al.*, 1987). The glands open out into the syncytium of the epithelium via convoluted ducts leading from the apex of each

lobe. The precise nature of the contents of the gland is unknown and some authors suggest that the granules contained in the glands may be of more than one type and have more than one function (Silverman and Maneely, 1955; Lethbridge, 1980; Swiderski, 1983; Thompson, 1986; Harris *et al.*, 1987). For example, one type of granule may be involved in activation and another in penetration of host tissues (Heath, 1971).

Figure 1.3 Simplified diagram of a typical taeniid oncosphere.



1.2.1.2 Hatching and Activation of the Embryo.

Taeniid eggs hatch, and the embryos become activated, in response to the chemical milieu of the stomach and small intestine of the host. It is a two-step process involving, firstly, disintegration of the embryophore and, secondly, activation of the embryo and its release from the oncospheral membrane.

The disintegration of the embryophore follows the dissolution of the cementing substance which binds the keratin blocks together. Various species of taeniid eggs have been hatched *in vitro* using artificial hatching media which simulate the conditions of the stomach and small intestine. It appears that for different taeniid species, the susceptibility of the cementing substance to dissolution varies, depending on the proteolytic enzymes present, (Silverman, 1954; Smyth, 1969; Heath and Smyth, 1970; Mitchell and Armour, 1980). *T.hydatigena* can be successfully hatched and activated after incubation for one hour at 37-39°C in an artificial gastric fluid (AGF) consisting of 1% pepsin and 1% hydrochloric acid in 0.15 M NaCl in distilled water, followed by 30 minutes in an artificial intestinal fluid (AIF) consisting of 1% (w/v) pancreatin, 1% (w/v) $NaHCO_3$ and 5% (v/v) lamb bile in distilled water (Heath and Smyth, 1970).

An alternative to using proteolytic enzymes for inducing taeniid eggs to hatch *in vitro* is chemical removal of the blocks using a solution of sodium hypochlorite (Osborn *et al.*, 1982b; Desplazes and Eckert, 1988). This also acts by dissolving the cementing substance between the blocks but if incubation in this solution is continued, the blocks themselves will eventually be dissolved.

Activation of the embryo *in vitro* results from the combined action of bile salts and pancreatin which initially causes an increase in the permeability of the oncospheral membrane; this is followed by the oncosphere becoming motile (Silverman, 1954). This motility results in the extrusion of some of the contents of the penetration gland which in turn causes a weakening of the oncospheral membrane (evident by the latter ballooning out) allowing vigorous movements of the hooks to tear through the weakened region and free the oncosphere from the oncospheral membrane (Heath, 1971).

Smyth and Haslewood (1963), noting that the chemical composition of bile varies from species to species, suggested this may be an important factor in the host specificity shown by many taeniids. Their studies involved the effect of bile on the development of the adult stage of *E.granulosus in vitro.* They reported that the salts, or concentrations of salts, contained in the bile from certain species in which the parasite cannot develop, caused the death of the parasite within 10 minutes. It is conceivable that a similar situation might exist for oncospheres in the bile of species which are unable to harbour the larval stage of the parasite, the bile salt concentrations or composition causing death of the oncospheres or failing to activate them. However, bile composition is not necessarily associated with host specificity

as shown by Coman and Rickard (1975) who found that eggs of *T.pisiformis*, *T.ovis* and *T.hydatigena* could be successfully hatched and activated in the small intestine of a dog. The very large range of intermediate hosts recorded for *T.hydatigena* (see Table 1.1) also indicates that for this species, host specificity of the larval stage is low, suggesting that hatching and activation of its oncospheres can occur under a wide variety of conditions.

While proteolytic enzymes would appear to be the most likely means of hatching, and bile the most likely means of activation of taeniids *in vivo*, they are not vital for these processes since viable eggs of many species, when injected subcutaneously or intramuscularly into sheep, are able to develop into larvae at the site of injection, a process which requires both hatching and activation. This has been demonstrated with *T.hydatigena* (Gemmell, 1966; Gemmell, 1970; Heath, 1978), *T.ovis* (Gemmell, 1966), *E.granulosus* (Gemmell, 1966) and *T.pisiformis* (Heath and Chevis, 1975). Other instances where taeniid eggs have developed into cysts without the action of gastrointestinal proteolytic enzymes include the development of *T.hydatigena* and *E.granulosus* eggs into larvae when injected intraperitoneally into jirds (*Meriones unguiculatus*) (Williams and Colli, 1970), and the development of pulmonary hydatid cysts in the lungs of sheep following the administration of *E.granulosus* eggs there by tracheostomy (Borrie *et al.*, 1965).

1.2.2 Development of the Larva.

1.2.2.1 Intestinal Penetration and Transport to the Site of Development.

After hatching and becoming activated in the intestine of the intermediate host, taeniid oncospheres penetrate the intestinal epithelial layer and enter the lamina propria. Both the hooks and the contents of the penetration glands are involved in this process although the precise nature of their involvement is uncertain. The hooks have been described by different authors as devices for attachment to the cells of the epithelium and lamina propria, for tearing through the cells, or for separating the cells so that the oncosphere can pass between them. The role of the penetration gland secretions has been suggested to be a means of adhesion to tissues to assist the hooks penetrate them, a lubricant to facilitate passage through the tissues, a coating for protection against the host's immune system, or as a host-cell lysing agent (Reid, 1948; Silverman and Maneely, 1955; Barker, 1970; Heath, 1971; Lethbridge, 1980; Harris *et al.*, 1987). The actual functions of the hooks and penetration gland secretions remains to be unequivocally established.

Taeniid oncospheres also release their penetration gland contents when cultured *in vitro* (Heath, 1971; Heath and Smyth, 1970). Indeed, it is probable that the penetration gland secretions released into the culture media are the substances which have demonstrated immunological properties when used as antigens for immunization against *Taenia taeniaeformis* (Rickard and Bell, 1971a), *T.ovis* (Rickard and Bell, 1971a and 1971b; Rickard and Adolph, 1977; Rickard *et al.*, 1977b), *T.saginata* (Rickard and Adolph, 1976; Rickard *et al.*, 1977a; Rickard and Brumley, 1981; Rickard *et al.*, 1981a), *T.pisiformis* (Rickard and Outteridge, 1974; Heath, 1976), and *T.hydatigena* (Rickard and Bell, 1971b; Rickard *et al.*, 1981a).

Penetration of the intestinal mucosa is a rapid process. Oncospheres of *Taenia serialis*, *T.hydatigena*, *T.pisiformis*, *T.saginata*, *T.taeniaeformis*, *T.ovis* and *E.granulosus* have been found to have penetrated the epithelium and entered the lamina propria of their host within 30 - 120 minutes of entering the lumen of the small intestine (Silverman and Maneely, 1955; Banerjee and Singh, 1968; Barker, 1970; Heath, 1971). Once through the epithelium, some oncospheres enter subepithelial capillaries and are carried to the liver via the portal system (Barker, 1970; Heath, 1971).

It is not known why some species of taeniid oncospheres which are transported by the portal system pass through the liver while others stop migrating there and start development. A comparison of the diameter of the oncospheres with that of the capillaries shows that the size of the oncospheres cannot be the cause, since *T.pisiformis* and *T.serialis* oncospheres have similar diameters and yet the oncospheres of *T.pisiformis* stop in the liver while those of *T.serialis* pass through and continue to the muscles (Heath, 1971). Heath (1971) suggested that a species-specific stimulus may be present in the organ of predilection of the host and that this may cause the oncospheres to stop there, but this has yet to be investigated.

Although the peritoneal cavity is the location in which mature *T.hydatigena* cysticerci are most commonly found, pulmonary infections have also been reported in sheep (Whitten and Batham, 1945; Sweatman and Plummer, 1957; Gemmell, 1964b; Edwards and Herbert, 1980; Heath, 1990 personal communication) and in pigs (Gemmell, 1961b). The route taken by the oncospheres from the lamina propria of the intestinal villus to the lungs is probably via the lymphatic system after entering a lymphatic lacteal rather than a capillary or venule, although this has not been demonstrated (Heath, 1971). The alternative route is via the portal system, with the oncospheres passing through the liver and continuing in the blood stream to the lungs. Since the oncospheres are in a continuously moving blood stream, it

would not be surprising if some were carried through the liver to the next major capillary bed.

1.2.2.2 **Post-oncospheral Development and Larval Migration.**

Post-oncospheral development *in vitro* is similar in *T.hydatigena*, *T.pisiformis*, *T.serialis*, *T.ovis* and *E.granulosus*, and the form and rate of development are similar with that occurring *in vivo* (Heath and Smyth, 1970). Freshly-activated *T.hydatigena* oncospheres have a diameter of approximately 22 µm (Heath and Smyth, 1970). On the second day *in vitro*, microvilli surround the oncosphere and the internal structures begin to become reorganized; for example, the hooks are withdrawn and resorbed, and clearly outlined, circular structures appear. By day 3, the diameter of the larva is more than twice that of the activated oncosphere, the microvilli disappear and the larva continues to increase in size. Heath (1973a) reported that by day 10, muscle systems have developed, the larvae have become elongated and gently undulating contractions occur.

In vivo, the earliest sign of the presence of an infection with *T.hydatigena* has been observed 7 days after lambs were orally infected (Sweatman and Plummer, 1957). At necropsy, 0.5 - 2 mm lesions immediately below the liver capsule were reported. The time after infection when these lesions occur corresponds with the time when they first become motile *in vitro* (Heath and Smyth, 1970) or soon after (personal observation) (Section 1.2.2.1) and are presumably beginning to burrow and cause damage to the liver tissue. Clinical signs, which only occur in animals which receive a large oral infection, have been reported to occur first about 9 or 10 days after infection (Edwards and Herbert, 1980). These clinical signs have been described as including pyrexia, increased pulse and ventilation rates, diarrhoea, anaemia, jaundice and severe depression of growth rate (Sheplev, 1959, cited by Smyth and Heath, 1970). Edwards and Herbert (1980) noted that the severity of the clinical signs was directly related to the degree of liver damage and the numbers of larvae seen at necropsy. Deaths have also been recorded approximately 2 weeks after a heavy infection (Schiefer, 1966 cited by Smyth and Heath, 1970; Edwards and Herbert, 1980).

Experimental infections indicate the following sequence of events. Macroscopically visible larvae are first recovered from the livers of lambs 10 days after infection (Sweatman and Plummer, 1957). Between days 10 and 20 post-infection, haemorrhagic tracks containing blood coagula are caused by the larvae as they burrow throughout the liver parenchyma. The area surrounding the tracks becomes

infiltrated with eosinophils, lymphocytes, plasma cells, fibroblasts, neutrophils and reticuloendothelial cells (Pullin, 1955; Sweatman and Plummer, 1957; Jensen and Pierson, 1975). About 2-3 weeks after infection, the first cysticerci are found in the peritoneal cavity (Pullin, 1955; Sweatman and Plummer, 1957) though with a very heavy infection, larvae may emerge as early as 12 days after infection (Edwards and Herbert, 1980).

By 25 days post-infection many of the tracks still harbouring larvae contain yellow or white caseous material (Sweatman and Plummer, 1957) consisting of neutrophils, macrophages, multinucleate giant cells, lymphocytes, plasma cells, small numbers of eosinophils and connective tissue (Pullin, 1955; Jensen and Pierson, 1975). No evidence of larvae emerging from the liver capsule is reported to occur later than 25 days after infection, and it is possible that larvae which have not emerged from the parenchyma by then would eventually degenerate (Sweatman and Plummer, 1957). A detailed account of the development and healing of lesions resulting from a heavy infection is given by Pullin (1955).

Animals have been found to harbour mature *T.hydatigena* cysticerci while no grossly visible liver damage is apparent. If the larvae emerge from the liver early in the infection before a severe granulomatous reaction occurs, the lesions may heal with little or no scar formation. But if the developing host reaction is able to surround the larvae while in the parenchyma, calcium salts deposit around them and persistent lesions result (Edwards and Herbert, 1980; Heath, 1990 personal communication).

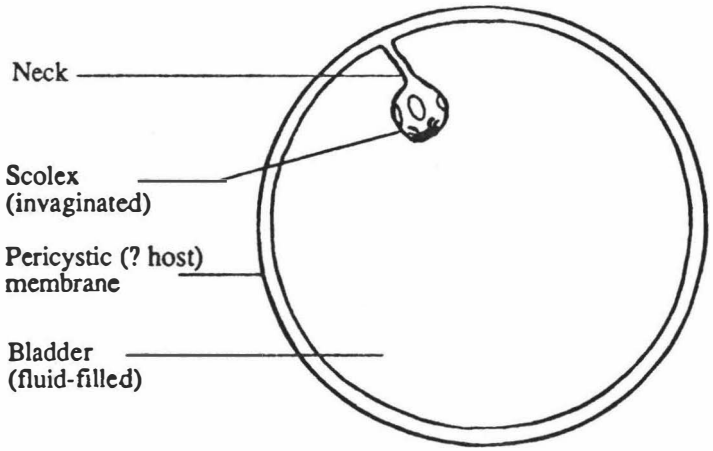
1.2.2.3 **Completion of Development of the Cysticerci.**

Having emerged from the liver, the cysticerci increase in size and the rostellar hooks and suckers develop on the larval scoleces. By day 34 most cysticerci contain fully developed hooks and exceed 10 mm in length (Sweatman and Plummer, 1957). It is likely that at least some of these are infective to dogs. Heath (personal communication) has noted that cysticerci recovered 42 days after infection of the intermediate host are infective to dogs. Cysticerci which emerge from the liver parenchyma but do not penetrate the liver capsule are able to continue development to maturity between the liver capsule and the liver parenchyma.

The oncospheres which enter the peritoneal cavity become attached to the peritoneum approximately 4 weeks after infection. Each mature cysticercus contains one invaginated protoscolex which has a long narrow neck attaching it to the bladder which consists of the cyst wall enclosing a fluid-filled cavity (Figure

1.4). Cysticerci are surrounded by 2 translucent hyaline membranes, the origin of which has not been investigated but is presumed to be from the host (Smyth and Heath, 1970). Often the attached cysticercus becomes enclosed in peritoneum. Although cysticerci can become attached in almost any part of the peritoneum, observations have shown that the areas around the rectum and bladder, uterus and distal large intestine are common sites for adherence. Cysticerci may survive the lifetime of the host (Gemmell, 1978) and are often up to 6 cm in diameter (Soulsby, 1982) and can grow even larger.

Figure 1.4 Simplified diagram of a typical cysticercus.



Of those larvae which emerge into the peritoneal cavity, not all develop to maturity. Some of those that reach maturity do not remain infective. These larvae become surrounded by a thick fibrous capsule, the bladder of the larva eventually collapses and the cysticercus dies. A fibrous nodule results which may subsequently become calcified (Pullin, 1955; Sweatman and Plummer, 1957).

1.2.3 Infection of the Definitive Host.

When a definitive host eats a mature *T.hydatigena* cysticercus and it reaches the small intestine, the scolex evaginates and attaches to the intestinal mucosa using hooks and suckers (Featherston, 1971). Featherston showed that dog bile together with trypsin, pancreatin, or both, was able to induce the immediate evagination of 90 - 100% of scoleces *in vitro*. Although a pretreatment of pepsin-HCl occurs *in vivo*

in the stomach, this was not required *in vitro*. Although it appears that evagination of *E.granulosus* scoleces can occur in some circumstances in the absence of specific enzymes and bile (Smyth, 1967; Thompson, 1986), it is not clear if this is the case with *T.hydatigena*.

As the evaginated scoleces develop into mature worms, they do not remain at their initial position of attachment. Featherston (1969) found that in the first week after infection the worms attached in the proximal quarter of the intestine, but during the third week (days 15 - 25) they were mainly in the second quarter. By day 40, however, the worms had returned to the proximal quarter. To account for these movements, it has been suggested that different physical and physiological conditions may be required by the worms at different stages of development. Featherston observed that when the worms were most distally located in the intestine, the testes were beginning to develop in the terminal proglottids and fertilization commenced.

The length of time required for the first eggs or gravid proglottids to be detected in faeces is 7-8 weeks (Featherston, 1969; Gregory, 1976a; Harris *et al.*, 1980).

The length of the strobila of *T.hydatigena* tapeworms is affected by the number present in the dog's intestine. An increase in the worm burden results in smaller specimens with fewer segments (Parmeter *et al.*, 1981). Estimates of the numbers of eggs present in a gravid proglottid when it is still attached to the strobila range from $17x10^3$ - $62.3x10^3$ (Featherston, 1969; Coman and Rickard, 1975; Gregory, 1976a), but *in vitro* experiments suggest that, even in the terminal gravid segments, not all eggs can be activated and so may not be infective (Gemmell, 1977). The majority of proglottids excreted in the faeces have been found to contain only a few hundred to a few thousand eggs (Sweatman and Plummer, 1957; Featherston, 1969; Coman and Rickard, 1975; Gregory, 1976a; Deplazes and Eckert, 1988) with most presumably being lost from the proglottid as a result of the segment's muscular contractions (Sweatman and Plummer, 1957; Featherston, 1969).

Two gravid *T.hydatigena* proglottids may be released daily from each cestode (Featherston, 1969) so that each may release between $34x10^3$ and $124x10^3$ eggs per day. The longevity of an infection with a *T.hydatigena* tapeworm is between about 4 - 11.5 months (Sweatman and Plummer, 1957; Gregory, 1976a; Heath *et al.*, 1980). Allowing for the prepatent period, it can be calculated that an infection of a dog with a single cestode could potentially result in the release of $2x10^6$ - $35x10^6$ eggs onto pastures in the lifetime of one worm. The actual number of infective eggs

released is likely to be less than this since some eggs released from proglottids in the proximal half of the dog intestine may hatch and the oncospheres become activated prematurely (Coman and Rickard, 1975) which would effectively render them incapable of infecting an intermediate host.

1.2.4 Egg Survival and Dispersal.

The survival of taeniid eggs stored in laboratory conditions is found to be affected by high temperatures, desiccation and the presence of tapeworm debris (Gemmell and Johnstone, 1977).

T.hydatigena eggs survive longest at 7°C in water or 0.15 M NaCl. Using *in vitro* hatching as a test of viability, Gemmell (1977) found that the viability of eggs stored at 7°C in water declined over time with a small percentage of the original number of viable eggs still able to hatch after almost 300 days of storage. In desiccated conditions (stored over phosphorus-pentoxide) the percentage of viable eggs present was substantially less than that of eggs stored in water. With increasing temperatures, the viability of the eggs declined more rapidly, both in water and desiccated conditions until, at more than 45°C in water, all eggs were killed in less than one day. There is some disagreement about the effect of freezing temperatures on the survival of *T.hydatigena* eggs. Some have claimed that storage of eggs at temperatures of -9 and -28°C substantially reduces the number of eggs which remain viable over time (Gemmell, 1977; Deplazes and Eckert, 1988), while Heath (1982) found no difference in the ability of eggs to be activated after storage for 6 months at -20°C with that of eggs stored at 4-7°C for the same length of time. While there were different temperatures and storage media used by these three authors, it is difficult to explain the differences in the results. However, another possibility is the quantity of eggs stored in each experiment since high concentrations of eggs are detrimental to their viability (Heath, personal communication).

Gemmell (1977) also reported that failure to wash *T.hydatigena* eggs free from parasite debris before storage results in a reduced percentage of viable eggs over time.

On pasture, some *T.hydatigena* eggs survived one year of cold-dry and hot-dry conditions in Central Otago, and one year of very wet conditions but moderate temperatures on the West Coast of the South Island, and were able to complete development into cysts when lambs were experimentally infected with them (Sweatman and Williams, 1963).

The dispersal of *T.hydatigena* eggs from where the faeces of an infected dog lie, can occur over considerable distances. Lambs grazing approximately 80 metres from a chained, infected dog became infected 10 - 19 days after the dog was introduced (Gemmell and Johnstone, 1976). The rapidity with which a pasture can become contaminated is illustrated by an experiment of Gemmell's (1976a) in which all 10 lambs were infected with a high mean of 46.5 cysts 10 days after 2 dogs, each with 4 patent tapeworms were chained in the pasture.

Proglottids have been reported to move up to one metre from the faeces by means of their own motility. Within a pasture, the sheep themselves may be involved in the dispersal of the eggs as they walk through them, but the transfer of eggs over longer distances requires alternative dispersal mechanisms. Taeniid eggs are known to be transported from the faeces of infected dogs by blowflies (*Calliphoridae*). Eggs of *E. granulosus* and *T.pisiformis*, ingested by flies, have been shown to infect lambs and rabbits, respectively (Schiller, 1954; Heath and Bishop, 1985). Lawson and Gemmell (1986) found in laboratory experiments, that flies ingested up to several thousand *T.hydatigena* eggs, most of which were voided within 48 hours. Eggs which had been ingested by flies in field experiments were reported to be viable and caused infection when ingested by lambs.

Sweatman and Williams (1963) showed that in a dry climate, with a rainfall of 13.87 inches in the year of the experiment, eggs had been transferred from an experimentally contaminated plot to an adjacent plot on the leeward sides 10 months after the former was sprayed with *T.hydatigena* eggs. Assuming that care was taken to ensure that eggs did not drift onto the leeward plot as the eggs were being sprayed, the transfer of eggs was attributed mainly to the strong winds which are typical of the region, although birds and insects had access to the contaminated plot. Lawson and Gemmell (1986), however, claim from their experiments that wind is not an important agent of dispersal.

Gemmell summed up this subject in his report on the Styx field trial (1968b):

> " The widespread infection rate in the lamb population even when few dogs harboured worms is consistent with a high egg production, an efficient egg dispersal mechanism and a long survival time for eggs of *T.hydatigena*."

1.3 IMMUNOLOGICAL ASPECTS OF THE TAENIID HOST-PARASITE RELATIONSHIPS.

Studies of the immunological aspects of the host-parasite relationship of *T.hydatigena* have been comparatively few. On the other hand, a considerable amount of information is accumulating relating to other taeniid infections and much of this is relevant to understanding the immunology of *T.hydatigena* infections. Consequently, in this section, literature relating to species other than *T.hydatigena* will also be reviewed.

1.3.1 Immunity in the Definitive Host.

Early investigators considered lumen dwelling cestodes to be "outside" the body and therefore isolated from the host's immune system. This explanation was given for the lack of resistance to superinfection found in many of these authors' experimental infections. The taeniids in these experiments included *T.taeniaeformis*, *T.pisiformis* and *T.hydatigena* (reviewed by Gemmell and Soulsby, 1968; Rickard, 1983).

Apart from those relating to *E.granulosus*, the majority of reports on the immunity of the definitive host to cestodes has concentrated on the hymenolepids (for reviews see Gemmell and Soulsby, 1968; Weinmann, 1970; Williams, 1982; Rickard, 1983). The work which has been reported on the taeniids other than *E.granulosus* is limited and largely inconclusive. The infection of dogs with *T.hydatigena*, *T.pisiformis* or *E.granulosus* was found to induce the production of antibodies which react with homologous egg and scolex excretory/secretory antigens (Heath *et al.*, 1985; Jenkins and Rickard, 1985). Jenkins and Rickard showed that despite the presence of these antibodies, the dogs were not immune to a second infection after the first worms were removed. Other experiments which have been reported include an attempt to immunize dogs against *T.hydatigena*. Antigens secreted from *T.hydatigena* scoleces were collected in culture medium and were injected with Freund's complete adjuvant into dogs without inducing immunity (Heath *et al.*, 1980). Rickard *et al.* (1977c) studied *T.pisiformis* infections in beagles and found that the number of segments and the length of the worms infecting the puppies decreased as the age at which the puppies were infected increased, but the authors were unable to show resistance to superinfection indicating that acquired immunity was not operating.

As mentioned above, *E.granulosus* has received more attention in relation to definitive host infection than have other taeniids. It has been reported that the susceptibility of dogs to *E.granulosus* is variable (Herd, 1977; Gemmell *et al.*,

1986a) but apparently independent of the age of the dog (Gemmell *et al.*, 1986). Lubke (1973) claimed that there was an age-dependent resistance to infection, but the validity of this claim has been questioned since Lubke infected the youngest dogs with the smallest doses of scoleces (Heath, 1986).

Partial protection of dogs against a challenge infection of *E.granulosus* has been achieved by immunization with a variety of preparations (reviewed by Heath, 1986). Significant reductions in the numbers of worms or a lack of development of eggs in the terminal segments of worms have been reported after immunization with dried, powdered scoleces, germinal membranes and cuticular membranes, killed germinal membranes, freeze-dried tapeworm tissue and scoleces or infection with irradiated larvae or protoscoleces (Turner *et al.*, 1933; Forsek and Rukavina, 1959 cited by Rickard 1983; Gemmell, 1962b; Movsesijan *et al.*, 1968). Herd *et al.* (1975) achieved a highly significant suppression of egg production in *E.granulosus* and a significant decrease in the number of proglottids per worm by immunizing dogs with secretory antigens combined firstly with Freund's complete adjuvant and subsequently with Freund's incomplete adjuvant. However, in later experiments the adjuvant alone induced significant resistance in some dogs; the reasons for this were not established (Herd, 1977).

Rickard (1983) has suggested that the success achieved in inducing a protective immune response against *E.granulosus* is due to the very close association of the scolex with the intestinal crypts. This close association has been clearly demonstrated, both histologically and ultrastructurally (Thompson *et al.*, 1979) while Smyth's studies (1967) on the *in vitro* cultivation of *E.granulosus* also suggest this. Smyth showed that evaginated scoleces would develop into the strobilate stage only in the presence of a solid base to which they could attach and from which nutrients could be absorbed. Such a close relationship between the host and the parasite would provide the opportunity for the host's immune system to become activated as has been demonstrated with *Hymenolepis diminuta*, where the scolex, as opposed to the strobila, has been identified as the source of antigens which induce protection against challenge infections of *H.diminuta* in mice (Hopkins and Barr, 1982). The association between the intestine and the scoleces of other taeniids has not been investigated in detail in dogs.

1.3.2 Immunity in the Intermediate Host.

1.3.2.1 General Features of Innate and Acquired Immunity.

No evidence of innate resistance to the larval stage of *T.hydatigena* has been reported. The effect of sex, age and genetics on the innate resistance of intermediate hosts to taeniids has largely been studied in laboratory animals. Significant effects of host strain on the larval development of *E.granulosus*, *E.multilocularis* and *T.taeniaeformis* have been reported. The innate immunity of the hosts of these tapeworms has been reviewed by Rickard and Williams (1982) who suggest that similar genetic effects will also occur in the domesticated hosts of taeniids, including those of *T.hydatigena*. Significant effects of sex on the susceptibility of cattle to *T.saginata*, and of age and sex on the susceptibility of laboratory hosts to many taeniid infections have also been described (see Rickard and Williams, 1982).

While no innate immunity against *T.hydatigena* infections is apparent in sheep, the oncospheres of this parasite stimulate strong immune responses capable of destroying a challenge infection while the parasites established from the immunizing infection are not affected. This concomitant immunity to *T.hydatigena* was first reported by Sweatman (1957 cited by Heath, 1978) when a high level of protection against an oral challenge was induced by an oral infection of 3-month-old lambs. Gemmell and his collegues (Gemmell, 1964a, 1964b, 1969a, 1970, 1972, Gemmell *et al.*, 1968a, 1969, 1990) have described 2 phases of immunity in sheep against a challenge infection with *T.hydatigena*. The first phase is said to be directed against the oncosphere before it becomes established as a metacestode in the peritoneal cavity and the second phase against the parasite after it has become established. A similar phenomenon was first described by Campbell (1936) with *T.taeniaeformis* infections in rats.

Immunization of sheep with *T.hydatigena* oncospheres results in complete protection against challenge infection in approximately 2 weeks (Gemmell *et al.*, 1968) while partial protection is present 1 week after infection (Gemmell, 1969c) or immunization (Gemmell *et al.*, 1968).

Craig and Rickard (1982) analysed the antibody responses of lambs to an infection with *T.hydatigena* using an enzyme linked immunosorbent assay with various *T.hydatigena* antigens. Antibodies against *T.hydatigena* oncosphere excretory/secretory (E/S) antigens reached a peak approximately 2 weeks after the primary infection and had returned to background levels by 12 weeks post-infection.

A second infection resulted in an antibody response which peaked within 1 week and showed higher levels of antibody than in the primary response. Antibodies in lamb sera which reacted with a crude extract of *T.hydatigena* oncospheres peaked and declined with a time course similar to those reacting with the oncosphere E/S antigen. Both IgG_1 and IgG_2 gave similar patterns of response, although IgG_2 was of a lower magnitude. However, antibodies which reacted with a crude deoxycholate-solubilized sonicate of *T.hydatigena* oncospheres showed no distinct peaks but continued to rise in titre for approximately 8 weeks and then remained constant until the end of the 36 week experiment. This indicates that developing larvae produce antigens which stimulate the production of antibodies which also react with oncospheral antigens, but these are not normally exposed.

The duration of protective, acquired immunity depends on how frequently the sheep's immune system is boosted by further immunizations or by ingesting viable *T.hydatigena* eggs. Gemmell and Johnstone (1981) reported that if no eggs were ingested after the initial immunization, 12 months were required to pass before a challenge infection resulted in viable metacestodes in the peritoneal cavity. A challenge infection given between 6 and 9 months after the initial immunization resulted in lesions and scarring of the liver; a few metacestodes had emerged from the liver but had not remained viable.

Gemmell has suggested that lambs do not become fully resistant to infection with *T.hydatigena* until after 3 months of age (Gemmell, 1972). However, Heath (1978) showed that at one week of age, lambs could be immunized and, therefore, were able to recognize enough protective antigens to resist infection.

The number of *T.hydatigena* eggs needed to induce an immune response capable of preventing the development of cysts from a challenge infection, has been reported to be as few as 50 (Sweatman 1957 cited by Gemmell, 1969c) although Gemmell found that a weak challenge (10 eggs) could be resisted by lambs which had received an initial infection of only 10 eggs. As one might expect, the number of cysts developing in the peritoneal cavity increases as the size of the initial oral dose of *T.hydatigena* eggs increases. However, the proportion of the eggs which develop into cysts decreases as the number of eggs administered increases (Gemmell, 1969c).

1.3.2.2 **Immunization Against Taeniid Infections.**

Live Embryo Vaccines.

The parenteral administration of activated taeniid embryos is highly successful in immunizing the host against homologous challenge with many taeniid parasites, including *T.hydatigena*, *T.pisiformis*, *T.ovis* and *E.granulosus* (Gemmell, 1962a; 1964a; 1964b; 1965a; 1965b; 1966; 1969a). While the immunity which is induced by such vaccines is effective in preventing challenge infections from developing, the parasites used in the vaccine remain viable at the site of injection (Gemmell, 1964b) providing a potential source of infection for a definitive host. These vaccines would, therefore, be unacceptable for parasite control schemes. However, repeated treatment of vaccinated intermediate hosts with Mebendazole has been found to kill *T.pisiformis* and *T.hydatigena* metacestodes used to immunize rabbits (Heath and Chevis, 1975) and sheep (Heath, 1978), respectively. Nevertheless, the practicality of using this in the field is questionable. Another technique which may or may not leave viable post-vaccination metacestodes involves the use of irradiated oncospheres. Those of *T.saginata* and *T.pisiformis* have been shown to induce variable levels of protection (Dow *et al.*, 1962; Urquhart *et al.*, 1963; Wickerhauser, 1982).

Excretory/Secretory Antigens.

The concept that living, metabolizing oncospheres were necessary to induce immunity, suggested that protective antigens may be contained in the products excreted or secreted by the parasites.

The first evidence that this concept was correct for taeniids was demonstrated by Rickard and Bell (1971a). Diffusion chambers containing activated *T.ovis* or *T.taeniaeformis* oncospheres were implanted into the peritoneal cavities of lambs and rats, respectively. The developing oncospheres induced a highly significant degree of immunity in the hosts to an oral challenge infection of homologous eggs.

The immunization of lambs with E/S antigens of *T.hydatigena* has been successful in protecting against a homologous challenge (Onawunmi and Coles, 1980) and also against a challenge infection with *T.ovis* eggs (Rickard and Bell, 1971b).

Highly significant protection against homologous challenge has also been achieved in their respective intermediate hosts using E/S antigens from *T.pisiformis*, *E.granulosus*, *T.ovis*, *T.solium*, *T.taeniaeformis* and *T.saginata* (Rickard and Adolph, 1976; Rickard *et al.*, 1977a; Rickard and Adolph, 1977; Rickard *et al.*,

1977b; Lloyd, 1979; Ayuya and Williams, 1979; Rajasekariah *et al.*, 1980b; Rickard and Brumley, 1981; Rickard *et al.*, 1981a; Osborn *et al.*, 1982a; Pathak and Gaur, 1990). There have, however, been reports of E/S antigens from *T.saginata* preparations which have not induced protective immunity in cattle (Mitchell and Armour, 1980; Wickerhauser, 1982). Rickard and Howell (1982) suggested differences in breed or sex of the host, or of a lack of antigen standardization as the reason for these results which are contrary to numerous successful immunizations.

Vaccinations with Killed Oncospheres.

Gemmell's (1964a, 1964b) initial suggestion that the induction of a protective immunity in sheep to *T.hydatigena* required the presence of living and developing larvae was later found to be incorrect when highly significant protection was also achieved after immunizing with frozen, or formalinized, hatched *T.hydatigena* eggs (Gemmell, 1969a). However, in this same series of experiments, a preparation of sonicated oncospheres did not induce protection. The failure of the sonicated oncospheres to induce a protective immune response is difficult to explain in view of the success of whole dead oncosphere preparations and since highly significant protection was achieved with this type of preparation in experiments described in this thesis (Chapters 2 and 6). One possibility is that the preparation used by Gemmell may have become overheated during the sonication process resulting in denaturation of the protective antigen. Another possibility is that enzymes released from the ruptured oncospheres may have caused degradation of the protective antigens.

Sonicated oncospheres of *Taenia multiceps*, *T.saginata*, *T.ovis*, *T.taeniaeformis* and *T.pisiformis* have all been shown to protect their natural hosts against a challenge infection (Rajasekariah *et al.*, 1980a; Rickard and Brumley, 1981; Osborn *et al.*, 1981; Edwards and Herbert, 1982; Lightowlers *et al.*, 1984; Rajasekariah *et al.*, 1985).

1.3.2.3 **The Nature and Origins of Antigens Stimulating Protection.**

The precise nature of the antigens mentioned above has yet to be established and reports of investigations into their nature are fragmentary. Lightowlers and Rickard (1988) have reviewed the nature and origins of E/S products of helminth parasites and the effects of these antigens on the host's immune system, but few attempts have been made to characterize the antigens themselves.

A likely source of the antigen in the E/S preparations is the secreted contents of the penetration glands of the oncospheres. Experiments involving the centrifugation of E/S antigen preparations of *T.taeniaeformis* and *T.saginata* suggest that the protective antigens are either membrane-bound or particulate (Rajasekariah *et al.*, 1980b, 1980a; Rickard and Brumley, 1981). It has been suggested that E/S antigens may include surface antigens actively released by the oncosphere (Harris *et al.*, 1987). Indirect evidence for this is also provided in *in vitro* studies on trematodes and nematodes where surface particles are found to be present in the culture medium (see review by Lightowlers and Rickard, 1988).

Rickard and Adolph (1977) have shown that culturing *T.ovis* oncospheres for only 24 hours is sufficient for the production of antigens able to induce complete protection in lambs, suggesting that these protective antigens are not formed in the oncospheres after activation but are present in the unactivated oncosphere. It is possible, therefore, that the oncospheral antigen preparations contain the same protective antigens as E/S preparations.

Completely different stages of some taeniid parasite life cycles can also share antigens. Craig and Rickard (1982) reported that while sonicated oncospheres of *T.ovis* and *T.hydatigena* have antigens which are unique to the oncospheral stage, when these oncospheres are solubilized with deoxycholate they are found to contain antigens which are also present in the mature larval stages of the life cycles of the parasites. Common antigens of approximately 140 kiloDaltons (kDa) have also been identified in the E/S and somatic antigens of the strobilocerci of *T.taeniaeformis* and these induced protective immunity in rats (Kwa and Liew, 1977)

Work with *T.pisiformis* suggests some of the E/S antigens are rendered ineffective relatively easily. Heath (1976) separated 2 distinct bands of secretory molecules from *T.pisiformis* by double diffusion in gels. These protected rabbits when injected subcutaneously but were rendered ineffective when exposed to air. A characteristic of *T.pisiformis* oncospheral antigen is that it loses its ability to induce protection in rabbits after solubilization with butanol, lithium idiodosalicylic acid, KCl or sodium dodecyl sulphate (SDS) (Rajasekariah *et al.*, 1985).

It has been reported that some of the granules released from the penetration glands of *T.ovis* oncospheres become incorporated into the syncytial membrane of the oncosphere (Harris *et al.*, 1987). It was also reported that the epithelial membranes of oncospheres incubated in immune serum, rupture. From this information Harris

and his colleagues suggested that the immune response induced by the secreted antigens reacts with similar antigens incorporated into the outer membrane of the oncospheres of a challenge infection, resulting in destruction of the oncospheres. No conclusive evidence for the contents of the penetration glands being protective has been reported but Harris *et al.* (1987) point out that these antigens fulfil the established characteristics of protective antigens, namely, that they are present in unhatched oncospheres (see Section 1.3.2.2), released within 24 hours of activation (Rickard and Adolph, 1977), and are membrane-bound (Rajasekariah *et al.*, 1980a).

1.3.2.4 **The Transfer of Immunity via Colostrum.**

Lambs, *in utero*, do not receive maternal antibody across the placenta. For this reason the sera of new-born lambs contain only low levels of endogenous immunoglobulin (Curtain, 1975). The source of the immunoglobulin needed for protection against infectious agents for the lamb's first weeks of life is the ewe's colostrum.

The concentrations of IgG_1 and IgM in the sera of ewes increase during the first half of pregnancy and then remain relatively constant before declining suddenly about 2 weeks before parturition due to the selective transfer of these antibodies into the colostrum (McCarthy and McDougall, 1949, 1953; Ciupercescu, 1977). The majority, approximately 70%, of immunoglobulin in colostrum is IgG_1 (Lisowski *et al.*, 1975) which is selectively accumulated in the mammary gland (Janusz *et al.*, 1973 cited by Campbell *et al.*, 1977b). Other immunoglobulin classes are present in considerably smaller amounts (see Campbell (1977b) for review). The total concentration of IgG in colostrum may greatly exceed that in the ewe's serum (Reneau, 1973; Sawyer, 1977) but it declines rapidly in the first few days after lambing (Halliday, 1965).

In the newborn lamb absorption is non-selective with all immunoglobulins absorbed and also many other proteins (Simpson-Morgan and Smeaton, 1972). Generally, lambs are able to absorb immunoglobulins from the ingested colostrum across the small intestine wall for up to 24 - 48 hours after birth (McCarthy and McDougall, 1949; Cooper, 1967; Halliday, 1976). However, this appears to be affected by the time at which the lamb is first fed and how much colostrum it ingests (Lecce and Morgan, 1962).

Before suckling, the serum concentration of total IgG in lambs has been reported to range from 0.07 mg/ml to 0.22 mg/ml while in lambs one day after birth, mean

serum IgG levels of 23.3 - 47.5 mg/ml have been recorded (Reneau, 1973; Smith *et al.*, 1976; Sawyer, 1977). The concentration of IgG_1 in lamb sera is normally higher than in ewe sera 3 days after parturition (Cooper, 1967; Ciupercescu, 1977) but levels vary widely because many factors influence the amount absorbed (Halliday, 1974; Campbell *et al.*, 1977b). The level of immunoglobulin in lamb serum decreases over the next 4 - 5 weeks (McCarthy and McDougall, 1949; Halliday, 1974). Fourteen weeks after birth the levels of IgG_1, though increasing, have been found to still be significantly lower than in ewes, as are those of IgG_2 (Ciupercescu, 1977).

Conflicting evidence exists concerning the significance of litter size or sex of the lambs on the level of immunoglobulin present in the lamb's serum. Some claim that there is no effect of litter size or sex on the amount of immunoglobulin absorbed (Reneau, 1973; Sawyer, 1977; Ciupercescu, 1977) while others have reported significantly lower immunoglobulin concentrations as litter size increased (Halliday, 1976; Logan and Irwin, 1977) and a higher concentration in serum immunglobulin in male lambs compared with females (Halliday, 1976). The situation is complicated by the number of factors affecting the amount of colostrum absorbed; these include variations in the abilities of lambs to absorb immunoglobulin across the intestinal wall (Simpson-Morgan and Smeaton, 1972; Larson *et al.*, 1974; Halliday, 1976; Sawyer, 1977; Logan and Irwin, 1977), the amount of colostrum produced and the concentration of immunoglobulin in the colostrum (Halliday, 1976; 1978).

The ability of colostral antibody to protect against taeniid infections has been studied in a number of species. Immunization of cows with E/S antigens from *T.saginata* induces considerable protection and the colostrum from cows immunized in this way transfer immunity to the calves resulting in 95% protection (Lloyd, 1979). Rickard *et al.* (1977a) confirmed the colostral transfer of immunity to this parasite. In the later report the number of cysts which were able to develop in the calves was reduced but those cysts which did become established, remained viable. In Lloyd's experiments, however, the established cysts did not survive.

The successful transfer of immunity to *T.saginata* in all cases involved cows immunized with a variety of antigens. Attempts to transfer immunity from naturally infected cows to their calves via colostrum have not been successful (Urquhart, 1961).

In rats (Musoke *et al.*, 1975) and in mice (Lloyd and Soulsby, 1978) the passive transfer of colostral IgA (but not IgG) provides protection against *T.taeniaeformis* infections.

Ewes infected or immunized with *T.ovis* are able to transfer protection against an homologous infection to their lambs via the colostrum which lasts for 6 - 16 weeks (Rickard and Arundel, 1974; Rickard *et al.*, 1977b; Heath *et al.*, 1979c; Sutton, 1979). However, Sutton (1979) also found that if the ewes were vaccinated once, one month before parturition their lambs were not protected by the colostrum in spite of the ewes themselves being significantly protected from the challenge infection.

Passive immunization through colostrum can apparently interfere with active immunization of the lambs as Heath *et al.* (1979b) were unable to immunize one-week-old lambs born to ewes hyperimmunized with *T.ovis* antigen.

The transfer of resistance to *T.hydatigena* by colostrum appears to be of a much lower order than is the case with *T.ovis*. Some experiments involving ewes naturally infected with *T.hydatigena* have shown no colostral transfer of immunity (Gemmell *et al.*, 1969; Heath, 1974) while ewes hyperimmunized with an intramuscular injection of activated embryos conferred a short-acting immunity to their lambs (Gemmell *et al.*, 1969). Such a difference between naturally infected and immunized ewes was not observed in experiments performed by Heath (1978) in which infected ewes conferred a short-acting, partial resistance to challenge infection of the lambs which was not enhanced by hyperimmunizing the ewes. The partial resistance of the lambs to oral challenge infection was not sufficient to prevent the development of eggs injected subcutaneously. This is consistent with earlier observations which indicated that it was possible to vaccinate lambs from infected ewes soon after birth (Heath, 1974). In an attempt to mimic a field situation, lambs born to infected ewes and run on infected pastures from birth to 3 weeks of age had fewer larvae at necropsy than did lambs grazed from 5 weeks of age (Gemmell, 1976b). Whether this was due to passive protection or the lack of ingestion of eggs by young lambs which were not eating much grass was impossible to determine.

The amount of immunoglobulin remaining in the circulation of the lambs in the first few weeks of life is likely to be important in protecting the lamb from taeniid infection and would depend on the amount of immunoglobulin transferred initially and the half-life of the particular immunoglobulin class in question.

1.3.2.5 **The Transfer of Immunity via Serum.**

Attempts have been made with several taeniid species to transfer immunity with serum.

Contrary to the reports of protection against *T.saginata* by colostrum, Froyd (1964) was unable to protect calves by intravenously injecting immune serum from naturally infected cows into naive recipients prior to challenge. This might have been because the amount of antibody transferred was insufficient to confer protection since Lloyd and Soulsby (1976) achieved significant protection by feeding immune serum to newborn calves. Monoclonal antibodies prepared against surface antigen of *T.saginata* oncospheres and transferred to calves also protected them against a challenge infection (Harrison and Parkhouse, 1986). This suggests that surface antigens are important in inducing a protective immune response. Interestingly, monoclonal antibodies reactive with a major secretory component of the penetration glands did not confer immunity.

Passive transfer experiments with *T.taeniaeformis* showed that serum from naturally infected mice was more efficient in protecting recipients than was serum from mice immunized with oncosphere antigen, although both groups of donors were resistant to challenge infection (Lightowlers *et al.*, 1986). The difference may have been due to different amounts of antibody present since an increase in the volume transferred improved the protection achieved.

In *T.hydatigena*, a 78% reduction in cyst numbers was achieved by transferring to recipients 100-120 ml of serum from sheep immunized with activated *T.hydatigena* oncospheres (Blundell *et al.*, 1968). Transferred leukocytes from the same immunized sheep did not protect the recipients from a homologous challenge.

1.3.2.6 **Possible Mechanisms of Immunity.**

Immune responses can involve complex interactions between antibody, antigen, complement and leukocytes. For the sake of clarity, these components will be considered separately as far as possible.

Antibody.

In vitro, immune serum has been shown to have a lethal effect on the oncospheres of *T.pisiformis*, *T.taeniaeformis*, *E.granulosus* (Silverman, 1955; Heath, 1970; Heath, 1973b; Rickard and Outteridge, 1974; Heath and Pavloff, 1975; Heath and

Lawrence, 1981) and *T.hydatigena* (Chapters 3 and 5). *In vivo*, host immunity becomes fully active and protective against many taeniid infections 10 - 14 days after an initial infection or immunization (Gemmell *et al.*, 1968b; Gemmell, 1969c; Heath, 1973b) and a similar length of time is required for an antibody response to become established. These two factors, together with the ability to passively transfer immunity from immune to naive hosts via serum, indicates the importance of antibody in the host's defence against larval taeniids.

It appears from both *in vitro* and *in vivo* experiments that antibodies are effective primarily against the very early stages of the oncospheral development with the oncosphere becoming more resistant to the effects of antibodies within 6 days of activation (Heath, 1973b; Musoke and Williams, 1975; Mitchell, Goding and Rickard, 1977a; Ito, 1977). Heath's observations of *T.pisiformis in vitro* (Heath, 1973b), suggest that in the early period after activation, antibody acts by inhibiting the reorganization and development of the oncosphere.

Craig and Rickard (1982) identified IgG_1 and IgG_2 as the main immunoglobulins produced by lambs against *T.hydatigena* or *T.ovis* infections. However, the majority of studies on the effects of antibodies have involved laboratory hosts and their taeniid parasites with *T.taeniaeformis* being the most common. IgG_{2a} has been reported to increase the number of circulating eosinophils in a *T.Taeniaeformis* infection (Ansari *et al.*, 1976), and although details of the other mechanisms in which IgG is involved have not been defined, IgG has been shown to be the principle component of protection against this parasite (Leid and Williams, 1974; Musoke and Williams, 1975; Musoke *et al.*, 1975; Lloyd and Soulsby, 1978). IgE has been shown to have no effect on the number of *T. taeniaeformis* larvae surviving from a challenge infection in rats, but it does accelerate the rate at which challenge oncospheres are killed. It has been suggested that IgE is involved in the translocation of specific IgG_{2a} antibodies (which fix complement and are opsonins, enhancing neutrophil phagocytosis) in *T.taeniaeformis* infections and in the attraction of non-specific killer cells (Leid, 1977). An increase in IgE-positive mucosal mast cells in rat infections with *T.taeniaeformis* has also been reported (Lindsay *et al.*, 1983). Secretory IgA is the principle active component of the mucosal immune defences. In mouse colostrum and intestinal secretions, this is the immunoglobulin which is protective against *T.taeniaeformis* infections (Lloyd and Soulsby, 1978). It has bactericidal properties in the presence of complement and lysosyme, and inhibits the adherence of bacteria to mucosal epithelia. The suggestion was made that IgA could prevent the adherence of oncospheres to the intestinal mucosa in the same way, thus preventing infection.

Complement.

In vitro, immune serum has been shown to be much more effective in killing the oncospheres of *T.pisiformis* (Heath, 1973b), *E.granulosus* (Heath and Lawrence, 1981), and *T.hydatigena* (see Chapter 3) when complement is present. Similarly, the effect of specific immunoglobulins on *E.multilocularis* protoscoleces *in vitro*, is complement-dependent and relies on the classical pathway (Kassis and Tanner, 1977). The death of adult *E.granulosus* worms, however, has been reported to occur via the alternative pathway *in vitro* (Herd *et al.*, 1984).

The importance of complement and antibody in the killing of taeniids has also been shown *in vivo*. Rats passively protected by an inoculation of immune serum were no longer protected when given a series of injections of cobra venom factor which results in the consumption of complement (Musoke and Williams, 1975 427). Mitchell *et al.* (1977a) showed that strains of mice which were particularly susceptible to *T.taeniaeformis* infections, were also found to have a genetic defect in their complement system.

There is also evidence for complement interacting with leukocytes. The adhesion of eosinophils and mast cells to *T.taeniaeformis* larvae is complement-mediated and leads to tegumental damage and the death of the parasite. While dependent on the presence of serum, the death of the larvae is independent of the presence of specific antibody (Englekirk *et al.*, 1981). Letonja and Hammerberg (1983) also reported the enhancement of leukocyte attachment by C3 to *T.taeniaeformis* larvae.

Leukocytes.

In many taeniid infections, eosinophils are prominent in both tissue and peripheral blood responses. A rapid peripheral blood eosinophil response occurs in both primary and secondary infections with *T.taeniaeformis* in the rat (Ansari and Williams, 1976). Eosinophils have also been shown to be involved in the cellular response in the liver to a challenge infection with *T.taeniaeformis* in rats which had been previously injected intravenously with immune serum (Heath and Pavloff, 1975). *T.taeniaeformis* larvae transplanted into the peritoneal cavity of rats were destroyed by the activity of eosinophils but this occurred only after removal of blocking antibody and proteins from the metacestode's surface (Kwa and Liew, 1978). The adherence of host immunoglobulins and proteins to the surface of established larvae, rendering them resistant to host cellular attack, may be an example of a mechanism of parasite evasion of the hosts defences. This is discussed in the next section. Eosinophils were the predominant leukocyte interacting with the

larval surface of *T.taeniaeformis in vitro*, with mast cells also adhering and some degranulating (Engelkirk *et al.*, 1981). Relationships between the rate of development of eosinophilia and the destruction of *Taenia crassiceps* cysticerci in mice (Freeman, 1964), and between the degranulation of eosinophils and the destruction of microtrichs of *T.crassiceps* in mice (Siebert *et al.*, 1979), have been described. The latter effect occurred only in the presence of mast cells and resulted in the disorganization and destruction of the parasite.

Considering the involvement of eosinophils in other taeniid infections it is reasonable to suppose that they may also be important in the immune response to a *T.hydatigena* infection. They have been described as being prominent in the lesions caused by *T.hydatigena* in lambs livers. In a primary *T.hydatigena* infection, lesions on the surface of the liver of lambs 2 weeks after oral infection, comprise proliferating fibroblasts and infiltrations of eosinophils with smaller numbers of plasma cells and lymphocytes. In the parenchyma of the liver, foci of eosinophilic infiltration occur in association with early scarring, and the liver lesions evident one month after infection are infiltrated by eosinophils (Pullin, 1955).

Following a second *T.hydatigena* infection, eosinophils are again the predominant granulocytes present at sites where larvae succumb to the immune response (Meeusen *et al.*, 1989). Aggregates of eosinophils are surrounded by large numbers of helper T lymphocytes while cytotoxic T lymphocytes are present in much smaller numbers and mainly located on the periphery of the lesions. Tightly packed B lymphocytes were also present in foci within the lesions suggesting local antibody production.

Neutrophils adhere to *T.hydatigena* oncospheres in large numbers in the presence of immune serum *in vitro* and have been reported to have a lethal effect on oncospheres under these conditions (Beardsell and Howell, 1984). However, identifying the viability of oncospheres which have a heavy covering of leukocytes presents some difficulties and while several techniques have been investigated (Section 3.4) a suitable method has yet to be found.

The information on the mechanisms of immunity against taeniid infections is fragmentary although experiments investigating the involvement of leukocytes in the death of schistosomules have provided information which might also be applied to taeniids.

Eosinophils have often been reported to be involved in antibody-dependent killing of schistosomules (Butterworth *et al.*, 1974; Butterworth *et al.*, 1977; James and Colley, 1978; McLaren and Ramalho-Pinto, 1979; David *et al.*, 1980) and also complement-mediated killing (Ramalho-Pinto *et al.*, 1978). Neutrophils are incapable of killing schistosomules in the absence of complement or antibody (Butterworth *et al.*, 1979; Vadas *et al.*, 1979) but in the presence of complement, the death of the schistosomules occurs (Incani and McLaren, 1981). Activated macrophages and sensitized basophils have also been reported to kill schistosomules *in vitro* (Gordon and McLaren, 1987) and an IgE-dependent killing of schistosomules occurs via platelets (Joseph *et al.*, 1983, Pancré *et al.*, 1988).

1.3.2.7 Site of Immune Protection.

Immunity against taeniid oncospheres has been suggested to occur in the intestinal tissue.

Leonard and Leonard (1941) described an experiment in which rabbits were injected intravenously with immune serum prior to receiving either an oral dose of *T.pisiformis* eggs, or hatched eggs injected into the mesenteric vein. Significantly fewer larvae were reported to survive and form lesions in the liver from the oral dose of eggs, suggesting to the authors that the oncospheres succumb to immune mechanisms while penetrating the intestine wall. However, the high numbers of liver lesions resulting from the hatched eggs which were injected into the mesenteric vein is inconsistent with the evidence of oncosphere death when incubated in immune serum *in vitro* (Silverman, 1955; Heath, 1970; Heath, 1973b; Rickard and Outteridge, 1974). The suggestion of the involvement of strong immunological reactions in the intestinal wall preventing oncospheral penetration was made by Banerjee and Singh (1968); however, further experiments are needed to verify their conclusions. Furthermore, the results of experiments by Musoke and Williams (1975), in which rats were completely protected against both oral and intravenous challenge of *T.taeniaeformis* by the passive transfer of immune sera, indicate that while the intestinal barrier may aid in the resistance of an immune host to taeniid infection, this is not the only important factor of the host defence.

1.3.2.8 Parasite Evasion of the Host Response.

All taeniid parasites require their larval stages to survive as long as its host in order for the life cycle to be continued. For such survival the parasite must be able to withstand or avoid the host's immunological defences. In the case of *T.hydatigena*

larvae, it is unlikely to be simply a case of sequestration or being walled off from the host. Contact between the surface of taeniid larvae and the host's inflammatory cells is intimate and host immunoglobulins have been shown to have access to the cyst wall of many taeniid larvae (Varela-Diaz and Coltorti, 1973; Chernin, 1982; Willms and Arcos, 1977; Kassis and Tanner, 1977; Kwa and Liew (1978); Siebert *et al.*, 1981) and to be present in the cyst fluid (Hustead and Williams, 1977; Chordi and Kagan, 1965; Kassis and Tanner, 1977). However, no investigations of this have been reported in relation to *T.hydatigena.*

While specific immunoglobulin in the presence of complement is destructive in the early stages of larval development, many authors have suggested that larvae which have survived this susceptible stage use host immunoglobulin bound to the tegument as a blocking mechanism or a disguise (Varela-Diaz *et al.*, 1972; Mitchell *et al.*, 1977b; Kwa and Liew, 1978; Rickard and Williams, 1982; Craig, 1988). Rickard (1974) presented some circumstantial evidence for this using *T.pisiformis* larvae. The ability of antibody to protect larvae against destruction in a rat peritoneal cavity, when they have passed the susceptible stage, is removed when the antibody-coated larvae receive an additional coating of goat anti-rabbit-immunoglobulin before being implanted into the rat. The extra coating of foreign protein (goat anti-rabbit-immunoglobulin) effectively removes the protective mask of rabbit antibody, thus attracting a cellular response.

Damian (1964) proposed that parasites synthesize surface molecules which mimic host molecules. Evidence supporting this hypothesis was provided by Willms *et al.* (1980) who reported that one of the proteins resulting from the translation, in the laboratory, of RNA extracted from a *T.solium* larva is recognized by antibody to pig IgG.

An alternative to the masking of parasite antigens with antibodies involves the enzymatic cleavage of adherent immunoglobulin into Fab and Fc portions by proteases released from the parasite. This process has been called "fabulating" and there is evidence for this occurring with trematodes, e.g. schistosomes (Auriault *et al.*, 1981) and with the protozoan *Tetrahymena pyriformis* (Eisen and Tallen, 1977) but has apparently not been investigated with larval taeniids. The trematode *Fasciola hepatica* also produces a papain- or cathepsin B-like proteolytic enzyme which cleaves immunoglobulin *in vitro* (Chapman and Mitchell, 1982).

Evasion of the host's immune system by parasites might also be achieved by changing the antigenic determinants on the larval surface, or shedding them. A mechanism displayed by *Schistosoma mansoni* schistosomules involve the shedding

of surface antigens which become bound to ligands (Kemp *et al.*, 1980). This ultimately removes antigens which are recognized by host immunoglobulins. Similarities between cestode and trematode teguments have been described by Lumsden (1975) and Rickard and Williams (1982) suggest that the pits and vesicles formed in the membranes at the base of the microthrix layer of larval cestodes could be involved in the uptake or externalization of membrane components. A loss of antigenic determinants in this way may partly explain the finding of a reduction in the antigenicity of the surface of *T.pisiformis* larvae as they mature (Craig, 1988). A change in the surface or excreted antigens has been described for *T.crassiceps* by Siebert *et al.* (1979) and there is also evidence that *T.taeniaeformis* in the intermediate host may show stage-specific antigens (Rajasekariah *et al.*, 1980b; Bogh *et al.*, 1988).

In vitro experiments with a number of species have suggested that complement might play an important part in the destruction of cestode larvae. As indicated earlier, in the first 6-8 days after activation, *T.taeniaeformis* larvae are susceptible to antibody-mediated damage *in vivo* and *in vitro* (Musoke and Williams, 1975), as are *T.pisiformis* and *Hymenolepis nana* (Heath, 1973b; Ito, 1977), but later are resistant to it. The cyst fluid of *T.taeniaeformis* larvae has been found to contain a substance which, *in vitro*, is able to fix complement in the serum of rats, humans and guinea pigs (Hammerberg *et al.*, 1976, Hammerberg and Williams, 1978a; Hammerberg and Williams, 1978b). This substance, which is secreted by the larva, is similar to cobra venom factor in that it depletes complement levels, blocking activation of both the alternative and classical pathways of the complement system. The larval stages of *E.granulosus*, *T.crassiceps*, *T.saginata*, *T.pisiformis* and *T.hydatigena* all contain soluble substances which produce complement inhibition (Hammerberg *et al.*, 1976).

T.taeniaeformis also seems to secrete factors such as anaphylatoxin inactivators and carboxypeptidase B-like enzymes. These could affect anaphylatoxins which may be generated by the non-immunological consumption of complement (Leid, 1977), thus interfering with inflammatory responses and mast cell degranulation in the vicinity of the parasite surface. Substances secreted by *T.pisiformis* include proteases which are capable of inactivating trypsin and chymotrypsin of host origin but not other enzymes, including papain or pepsin (Németh and Juhász, 1980).

A further possibility is that substances produced by larval cestodes have a direct effect on lymphocyte functions. E/S products from *T.taeniaeformis* inhibit lymphocyte functions through the induction of suppressor-cell populations (Burger

et al., 1986) and through a protease inhibitor which suppresses lymphocyte activity (Leid *et al.*, 1984; Leid *et al.*, 1986). There is also evidence that larval cestodes may secrete substances which affect host granulocytes and macrophages but the synthesis of these *in vivo* has not been established (see Rickard and Williams, 1982; Leid *et al.*, 1987b, 1987a).

1.3.2.9 Cross-Resistance Between Taeniid Species.

Antigens common to different taeniid species often result in cross-resistance between species. This resistance may be useful when vaccinating hosts against more than one taeniid species.

Significant protection of cattle against *T.saginata* challenge has been reported after vaccination with antigens of *T.hydatigena* (Rickard and Adolph, 1976; Rickard *et al.*, 1981a, 1982; Rickard and Brumley, 1981), *T.taeniaeformis* (Lloyd, 1979) and *T.ovis* (Rickard and Adolph, 1976). However, the level of protection is less than with homologous immunization and probably insufficient to be useful in the field.

Cross-resistance between *E.granulosus*, *T.ovis* and *T.hydatigena* has been investigated. Serological cross-reactions occur between these three taeniids (Sweatman *et al.*, 1963; Craig and Rickard, 1982; Yong *et al.*, 1984) to the extent that *T.hydatigena* cyst fluid is a possible source of antigen for the immunodiagnosis of hydatidosis (Monzon *et al.*, 1985) and of *T.solium* infections in both humans and pigs (Rhoades *et al.*, 1987). Despite the serological responses, infection or immunization with *T.hydatigena* did not result in a significant reduction in the number of hydatid cysts which became established following challenge (Gemmell, 1966; Heath *et al.*, 1979a) but the results of an immunization with *T.ovis* has produced variable levels of protection against *E.granulosus*. Heath *et al.* (1979a) reported a 67% reduction in the numbers of hydatid cysts established, all of which were viable, while Gemmell (1966) claimed no significant reduction in the establishment of cysts but a reduction of 97% in the survival of cysts which became established. Different immunization procedures may account for the differences in the results.

Immunization of rabbits with *T.ovis* or *T.hydatigena* induces significant protection against subsequent infection with *T.pisiformis* (Gemmell, 1964a; 1965a; 1969b). However, an immunizing injection of activated *T.pisiformis* embryos into sheep gives no significant protection against *T.hydatigena* establishment or survival (Gemmell, 1964b; 1969b) and with *T.ovis* the results are conflicting (Gemmell,

1965b; Gemmell, 1969b). *T.pisiformis* has been reported to protect mice against a *T.taeniaeformis* infection (Rickard *et al.*, 1981b).

Conflicting reports of cross-resistance between *T.ovis* and *T.hydatigena* have been published. Some experiments have shown that infections or immunizations with *T.ovis* can induce significant protection against *T.hydatigena* infections (Gemmell, 1964a, 1964b; Varela-Diaz *et al.*, 1972) while others have failed to do so (Gemmell, 1969c; 1970; Heath, 1979a). Similarly, on some occasions infection or immunization with *T.hydatigena* has induced protection against *T.ovis* (Gemmell, 1964a, 1965b, 1970; Heath, 1979a), and on other occasions, has not (Gemmell, 1968a, 1969c; Varela-Diaz *et al.*, 1972). To some extent these variable results may be attributable to differences in procedure but, for the most part, remain to be explained.

T.hydatigena infection has been shown to induce some protection against *T.taeniaeformis* in mice (Rickard *et al.*, 1981b), *T.saginata* in calves (as mentioned above) and *F.hepatica* in sheep (Campbell *et al.*, 1977a; Dineen *et al.*, 1978) although the later could not be confirmed by Hughes *et al.* (1978). Cross-resistance has also been reported by Ito *et al.* (1988) between *T.taeniaeformis* and *Hymenolepis nana.*

Although cross-resistance between *Taenia* species is incomplete, it does raise the theoretical possibility of producing vaccines containing heterologous antigens or which may protect against more than one species. However, whether or not this concept will prove of practical use remains to be seen.

1.3.2.10 **Taeniid Vaccines.**

Control of *E.granulosus*, *T.ovis* and *T.hydatigena* in New Zealand would be improved by the use of vaccines against these taeniids (Harris *et al.*, 1980) and since taeniid infections induce rapid and effective host immune responses against challenge infections this makes them prime candidates for the production of successful vaccines against infection in the intermediate host. Recently a protective *T.ovis* antigen has been genetically engineered enabling the rapid, cheap and safe production of parasite material to be used in a vaccine for sheep (Johnson *et al.*, 1989). The production of genetically engineered antigens of other taeniid parasites, whether of domesticated hosts or not, allows "model defined-antigen vaccines" to be developed against larval cestode parasites (Bowtell *et al.*, 1984) which then may be applied to other taeniid parasite systems.

Section 1.1.3 outlined the importance of control of *T.hydatigena* in New Zealand due to the potential monetary loss incurred if the prevalence were to return to previous levels while Rickard and Williams (1982) reviewed reports of substantial losses resulting from cysticercosis and hydatidosis in many areas of the world. This factor of economic loss of livestock industries together with the risk to human health from infection with zoonotic taeniids (e.g. *Echinococcus vogeli*, *E.granulosus*, *E.multilocularis*, *T.solium* and *T.saginata*), show that the control of these parasites is important.

Vaccines would ideally provide a means of rendering a host population immune to primary infection, thus assisting to break the life cycle. Chemotherapy may have been thought, at one stage, to be capable of achieving the same result. However, after 22 years of strict treatment of dogs in a field trial conducted in the Styx Valley (Gemmell, 1968b), outbreaks of infection were still found to occur. While vaccination results in an immune population of intermediate hosts, chemotherapy results in a susceptible population making eradication of the parasite more difficult.

1.4 THE AIMS OF THIS STUDY.

If one is to develop a vaccine it is necessary to have a detailed understanding of both the immune response of the host to the parasite and the mechanisms involved in the expression of effective immunity. It is then necessary to identify protective antigenic epitopes which induce the host response.

The main aim of the following study was to clarify the role of antibody in the protective immunity developed by sheep in response to infection or immunization with *T.hydatigena*. This was done *in vivo* in a series of experiments in which antibody was transferred either by serum or colostrum to 6-month-old or neonate lambs, respectively, and *in vitro* by recording the survival of freshly activated oncospheres cultured in the presence of immune serum, complement and leukocytes or in the presence of separated antibody classes and complement.

Western blots of *T.hydatigena* oncosphere antigen, probed with immune or non-immune serum were examined in an attempt to identify antigens which are recognized by immune serum. Protective antigens were also investigated by challenging sheep immunized with *T.hydatigena* oncosphere antigens of different molecular weights obtained by polyacrylamide gel fractionation.

CHAPTER 2

THE ABILITY OF ANTI-*T.HYDATIGENA* ANTIBODY, TRANSFERRED FROM IMMUNE TO NAIVE HOSTS VIA SERUM, TO PROTECT AGAINST A CHALLENGE INFECTION.

2.1 INTRODUCTION.

This investigation of the immunological relationship between *T.hydatigena* and its intermediate host, focuses on the importance of antibody in the protection of sheep against a challenge infection.

Blundell *et al.* (1968) showed that the sera from 4-month-old lambs immunized with 2 injections of 10 000 activated *T.hydatigena* oncospheres conferred significant protection against an oral challenge infection when intravenously injected into naive sheep. The experiments described in this chapter were intended to confirm these results and to see if the recipients of sera from sheep orally infected with high or low doses of *T.hydatigena* eggs were resistent to an homologous challenge infection. A parallel experiment used sera from sheep immunized with solubilized oncosphere antigens as the source of passively transferred antibody. Knowledge of the importance of antibody in the immunological defence of sheep against oral challenge with *T.hydatigena* would indicate the appropriateness of using antibodies for immunoprecipitation or to probe Western blots for the identification of antigens recognized by immune hosts. If antibodies were shown to be important, they could then be used to probe a *T.hydatigena* oncosphere gene library for antigens capable of inducing protection.

2.2 MATERIALS AND METHODS.

2.2.1 Experiment 1. The Transfer of Serum From Lambs Given a Single Oral Dose of *T.hydatigena* Eggs, to Naive Lambs.

2.2.1.1 Experimental Animals.

The sheep used in these experiments were born and reared on the Ministry of Agriculture and Fisheries farm at Kaitoke, Upper Hutt, New Zealand. Every

precaution was taken to prevent taeniid eggs from contaminating the pastures. While freedom from infection could not be guaranteed, this was the only source of experimental animals available. Natural infections occurred very rarely; in all the experiments described in this thesis, in only one animal was an infection acquired previously.

Ten 10-month-old Romney lambs, destined to be the donors of the serum, were taken from pasture and housed indoors in pens 2 weeks prior to the commencement of blood collection. Later, a further 15 lambs (the serum recipients) were taken from the same flock and housed 2 weeks prior to the transfer of serum.

The penned animals were fed commercial sheep nuts, lucerne pellets and hay *ad libitum*.

2.2.1.2 Harvesting *T.hydatigena* Eggs.

Eggs from mature *T.hydatigena* tapeworms were required for experimentally infecting sheep, preparing antigen extracts and for use in *in vitro* cultures. In all instances the eggs were harvested from the terminal segments of tapeworms recovered from the intestines of dogs 10 weeks after they had been experimentally infected. The dogs, of mixed breed, were bred and housed at Wallaceville Animal Research Centre. They were infected by feeding them 6 - 12 mature *T.hydatigena* cysticerci which had been removed from an infected sheep less than 24 hr previously and kept in McCoys modified 5A medium (Grand Island Biological Company (GIBCO)). Weekly 2 ml injections of Opticortenol 0.5%^{R} (5 mg/ml dexamethasone trimethylacetate, Ciba-Geigy) and Streptopen 250/250 Injection^{R} (aqueous solution of 250 mg/ml procaine penicillin, 250 mg/ml dihydrostreptomycin, Pitman-Moore Animal Health Ltd) in a ratio of 2:1 were given, starting a week before infection, to try to ensure that as many mature worms as possible would result from the infection. After recovery of the mature worms, each tapeworm strobila was submerged in water. Segments containing fully-formed eggs were identified by rupturing, with a pair of forceps, the most anterior segment in which an egg-filled uterus was clearly visible. Fully developed eggs (recognizable microscopically by the presence of a thick embryophore and parallel hooks) sink immediately in water whereas immature eggs sink relatively slowly. If the majority of the eggs in the examined segment did not sink immediately they were judged to be not fully developed and a more distal segment was ruptured and so on until the first segment was found in which the majority of eggs sank immediately. All segments distal to this were cut from the worm, finely chopped in

a food processor, and washed through a 100 µm sieve and the eggs retained on a 20 µm sieve. Eggs recovered were stored at 4°C in 0.15 M NaCl (saline) containing 100 U/ml penicillin, 100 ug/ml streptomycin and for every 200 ml of solution, two drops (approx 70 µl) of Tween-20 were added.

2.2.1.3 Collection of Serum from Donor Lambs Prior to Infection.

During the 2 weeks prior to their being infected, each donor lamb was bled 4 times. On each occasion approximately 130 ml of blood were collected from a jugular vein of each lamb using a sterile 60 ml syringe and an 18 G needle and transferred, without anticoagulant, into 25 ml sterile containers. The blood was allowed to clot at 4°C overnight. The clot was removed and the serum centrifuged at 1500 x g for 10 min at 4°C to remove any cells present. Twenty-five ml aliquots of serum from each animal were stored separately at -20°C. This serum is referred to as non-immune serum. All handling of blood or sera was carried out in a laminar flow cabinet (Gelman Sciences BH/AS series). Samples of this blood were used to probe Western blots and ELISAs.

2.2.1.4 Estimation of the Number of Eggs Required for Infecting Donor Lambs.

Due to variations in the maturity of the eggs contained in different batches of stored eggs, and due to the effects of storage, the percentages of the eggs which were capable of being activated varied between batches. To determine the number of stored eggs needed to give approximately the required number of activated oncospheres, 0.5 ml samples of the stored suspension were hatched and activated using the following method.

Eggs were washed once by suspending them in 10 ml of saline and centrifuging them at 700 x g for 5 min. They were resuspended in 5 ml of a 20% solution of sodium hypochlorite with 5% available chlorine (Chlorodux bleach, Clorogene Supplies) in saline (Osborn *et al.*, 1982). (When eggs were being hatched for the purpose of antigen preparation and more than $1\text{-}2\text{x}10^6$ oncospheres were required, the volume of sodium hypochlorite solution was increased to 10 ml). The suspension was gently mixed every minute and hatching was observed using an inverted microscope. As soon as all the embryophores had disintegrated (i.e. about 2 - 3 min), the reaction was stopped by adding an equal volume of 0.145 M sodium thiosulphate. The embryos were centrifuged at 700 x g for 5 min and washed twice

in McCoy's medium (GIBCO). After the second wash, the supernatant was removed leaving the oncospheres in a volume of approximately 500 µl.

The oncospheres were activated by adding 20 µl of concentrated (approximately 9.7 M) HCl followed immediately with 5 ml of artificial intestinal fluid (AIF) (Appendix 2.1) before capping the tube to retain the CO_2 produced. The oncospheres were incubated at 37°C on a roller for 30 min. They were then washed once in McCoy's medium at 37°C, the supernatant withdrawn down to 1-2 ml, and the number of activated and unactivated oncospheres counted in 10 x 5 µl samples using a haemocytometer slide. Activated oncospheres were identified as those which were free from their oncospheral membranes. If fewer than 20 oncospheres were present in each sample, the suspension was further concentrated by centrifugation. All oncospheres in the samples were examined and the mean number of activated oncospheres was multiplied by 200 to give the number of "activatable" oncospheres/ml of suspension. The total number of activated oncospheres present in the final suspension was calculated and multiplied by 2 to give the number of activatable oncospheres/ml in the original egg suspension.

2.2.1.5 **Experimental Infection of Serum Donors.**

The eggs were diluted to contain approximately 50 activatable oncospheres per 500 µl dose. This number was chosen since an infection was sought which was large enough to induce immunity but likely to produce numbers of cysts similar to those which may be found in natural infections. The eggs were thoroughly suspended immediately prior to being drawn into a 1 ml syringe and gently administered onto the back of the lamb's tongue. The mouth was then held closed until the animal had swallowed.

2.2.1.6 **Collection of Serum from Donor Lambs Orally Infected with *T.hydatigena***

Serum was collected from the infected lambs on days 16, 20, 24 and 28 post-infection. One hundred and thirty ml of blood were collected from each lamb on each occasion and the sera collected and stored as before. This serum is referred to as immune serum, and samples of this were used to probe Western blots and ELISAs.

2.2.1.7 Verification of the Presence of Anti-*T.hydatigena* Antibodies in Donor Sera by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting.

It was necessary to show that the sera collected from the donor lambs prior to their infection (i.e. the non-immune sera) did not contain antibodies to *T. hydatigena*, while sera collected after such infection (i.e. the immune serum) did. To demonstrate this change in immune status, oncosphere antigens were fractionated by SDS-PAGE (Laemmli, 1970) and blotted onto nitrocellulose (Towbin *et al.*, 1979). The blots were then probed with either non-immune or immune sera.

The preparation of the oncosphere antigens involved hatching an appropriate number of eggs as described in Section 2.2.1.4, removing the embryophore blocks by mixing the pellet of hatched eggs with 15 ml of isotonic Percoll^R (Pharmacia) in Eagle's minimum essential medium (GIBCO) followed by centrifugation for 10 min at 1500 x g. The pellet was discarded and the supernatant containing oncospheres was diluted volume/volume with McCoy's medium and centrifuged for 10 min at 1500g. The pellet was retained and resuspended in McCoy's medium and the mean number of embryos/ml of suspension in 10 x 5 µl samples were counted. The embryos were then concentrated to approximately 4 x 10^6/ml in 1 M Tris-HCl buffer, pH 6.8, containing enzyme inhibitors (Appendix 2.1) and were frozen overnight (-20°C), thawed at room temperature and sonicated using a Soniprobe^R 7532A (100 W, 20 KHz) on ice for 15 min (10 sec on and 10 sec off to prevent overheating). This preparation will be referred to a freeze, thaw, sonicate (FTS) antigen. SDS (10% w/v) was added to give a final concentration of 1% and after mixing on a Matburn blood mixer for 2 hr at room temperature, the suspension was centrifuged at 100 000 x g for 1 hr. The supernatant was removed and 4 volumes of supernatant added to 1 volume of sample buffer (Appendix 2.1) and placed in a boiling water bath for 5-10 min and then held at room temperature, or frozen at -20°C, until needed.

A 5-25% SDS-PAGE gradient gel, 0.75 mm thick, was poured using Bio-Rad equipment and according to the Bio-Rad^R (Richmond, CA) instructions for vertical slab gels. The stacking gel formed two troughs with a lane for the molecular weight markers on each side and between the troughs. Either Sigma^R prestained SDS molecular weight markers or Pharmacia^R (Pharmacia LKB Biotechnology, S-751 82 Uppsala, Sweden) low molecular weight markers, were loaded onto the 3 lanes and oncosphere antigen into the troughs (300 µl, approximately 1x10^6 oncosphere

equivalents, per trough). The gel was run for 1 hr at 100 V and then for 15 hr at 200 V. It should be noted that the estimated molecular weight of the markers, given by Sigma or Pharmacia do not entirely correspond. This could be because the Sigma markers used were prestained and the added molecular weight of the dye molecule attached to the marker proteins had not been accounted for in their estimations.

The separated proteins were transferred onto 0.2 μm nitrocellulose (Schiecher and Schuell) using 50 Watts for 4 hr at 5°C in carbonate buffer, pH 9.9, (Dunn, 1986; Appendix 2.1). After transfer, the nitrocellulose was washed free of any adhering gel and stained in Ponceau S (Appendix 2.1) for 5 min. After removing excess stain with distilled water, the lanes of antigen could then be marked for cutting. The stain was then completely removed by rinsing the nitrocellulose in distilled water.

After removing the molecular weight marker lanes for staining, the rest of the sheet was incubated in a blocking solution of 500 mM Tris-buffered saline (TBS, Appendix 2.1) containing 5% non-fat milk powder and 0.1% Tween 20. The sheet was blocked for 2 hr at 37°C and the areas containing separated antigen cut into strips 5 mm wide. Each strip, therefore, carried the separated antigens from approximately 80 000 oncospheres. The strips were probed at room temperature with both the non-immune and immune sera diluted 1:40 in blocking solution. After overnight incubation, the strips were washed 4 times for 10 min each in 500 mM TBS containing 0.1% Tween 20. The strips were then incubated in horseradish peroxidase (HRP)-conjugated rabbit anti-sheep antibody (Cappel Biotek, Cooper Biomedical Inc. PA) diluted 1:1000 in the blocking solution, for 4-6 hr at room temperature. The strips were washed again 4 times for 10 min each in 500 mM TBS containing 0.1% Tween 20 followed by 2 washes for 5 min each with 500 mM TBS without Tween 20.

Immediately before use, the HRP colour development solution was prepared by dissolving 60 mg of 4-chloro-1-napthol (stored dessicated at -20°C) in 20 ml of ice-cold methanol. This was combined with a solution of 60 μl of cold 30% H_2O_2 in 100 ml of 500 mM TBS. The strips were incubated in this solution in the dark until the bands had appeared to the required degree. The chosen endpoint was normally the time when the first band began to appear on the strips probed with non-immune sera. The development solution was then removed and the strips rinsed in distilled H_2O to stop colour development. The strips were placed on an acetate transparency sheet alongside of the molecular weight marker lanes, photographed and air dried in the dark.

A few of the Western blots presented in the results of this thesis failed to show any bands of antigen recognized by antibody after development with the chloro-napthol reagent. In these instances, the strips were then reacted with a more sensitive development system, amino-ethyl carbazole (AEC), and kept in the dark to prevent colouration of the background. The preparation of this solution involved adding 50 ml of 50 mM acetate buffer, pH 5.0, to 2 ml of stock AEC (Appendix 2.1) followed by 10 µl of 30% H_2O_2. When the bands had developed the strips were washed in distilled H_2O, photographed and dried as above.

2.2.1.8 Detection of Anti-*T.hydatigena* Antibody in Lamb Sera by ELISA.

FTS antigen was prepared as described in Section 2.2.1.7 except that the hatched eggs were concentrated to approximately $1x10^6$/ml by centrifugation and were resuspended in coating buffer (Appendix 2.1) containing enzyme inhibitors at a ratio of 1:100 (Appendix 2.1). The suspension was then frozen, thawed and sonicated as described previously, before being stored at -20°C until use.

Titertek (Flow Laboratories) 96-well, flat-bottomed plates were washed 3 times with 0.05% Tween-20 in distilled water and 3 times in distilled water without Tween-20. The plates were dried by tapping them onto clean paper towels.

FTS oncosphere antigen was diluted 1:90 in coating buffer and 100 µl dispensed into each well before the plates were covered and refrigerated overnight at 4°C.

To remove unbound antigen, the plates were washed 4 times in 0.05% Tween-20 in distilled water at room temperature by flushing and aspiration with 3 min incubations between each wash.

One hundred µl of blocking buffer (1% bovine serum albumin (BSA) in 0.15 M Phosphate buffered saline (PBS), pH 7.4, Appendix 2.1) were dispensed into each well and incubated for 30 min at room temperature. The plates were washed 3 times and dried as above.

The wells were probed with the test antibody diluted 1:40 with 0.5% BSA in PBS, pH 7.4, with 0.05% Tween-20. Control wells received 100 µl of diluent. The plates were incubated at room temperature for 1 hr before being washed 3 times and dried. One hundred µl of HRP-conjugated rabbit anti-sheep antiserum (Cappell^R) diluted 1:30 000 in 0.5% BSA in PBS, pH 7.4, were dispensed into each well. After one hr the plates were washed 3 times and dried as above.

Fresh substrate was prepared (Appendix 2.1, no. 19) immediately before use and 100 µl were dispensed into each well. The plates were incubated in the dark at room temperature, and after 20 min 5 µl of the stopping solution (Appendix 2.1) were added to all wells in the same order as the substrate had been added.

The optical density of the reaction was read by a Titertek Multiskan MCC^{R} Plate Reader at 490nm, with the standard based on a control well.

2.2.1.9 Preparation of Non-immune and Immune Donor Sera for Transfer to Naive Recipients.

The respective non-immune and immune donor sera for transfer were each pooled in 3 L sterile stainless steel pressure reservoirs (Amicon Corporation). From these pools, 450 ml aliquots were dispensed into each of 5 gamma-irradiated 500 ml Vaxipacs (Pitman-Moore N.Z. Ltd) by pressurizing the reservoir with nitrogen. The Vaxipacs were then sealed with sterile rubber stoppers and crimped aluminium caps. Packs of sterile saline were prepared in the same way.

2.2.1.10 Injection of Sera and Saline into Recipients.

The 15 recipient lambs were divided into 3 groups of 5, and a 15 ml samples of blood were collected from the jugular veins into anticoagulant-free vacutainers. The blood was held at 37°C for one hr and then at 4°C onvernight. The clot was removed and the serum centrifuged at 1100 x g for 25 min. The sera were dispensed into 2 ml aliquots and stored at -20°C until analysed on Western blots or ELISAs.

Each lamb in each group was to receive 450 ml of either pooled non-immune serum, pooled immune serum or saline prior to being challenged with *T. hydatigena* eggs. Saline was administered aseptically from the Vaxipacs into the jugular veins of the lambs of one group without any adverse reaction.

The first 2 lambs to receive non-immune serum and the first 2 lambs to receive immune serum, developed severe respiratory distress for 10 to 15 min after receiving approximately half of the serum intravenously. Because of this, the remainder of the serum for these 4 lambs, and all of the serum for the remaining 6 lambs, was administered intraperitoneally without apparent distress to the recipients. Twenty-four hr after the transfer, a 15 ml sample of blood was taken from each lamb to provide serum to determine by Western Blot if transferred antibody was present.

2.2.1.11 Challenge Infection of Recipient and Donor Lambs.

Twenty-four hr after receiving either non-immune serum, immune serum or saline, each lamb was challenged with approximately 50 activatable oncospheres in 500 µl of saline administered as described for the experimental infection of the serum donors, and a blood sample was taken.

Five of the original 10 serum donor lambs were similarly challenged to determine if the first infection had indeed rendered the donors immune.

2.2.1.12 Necropsy of Lambs.

The 15 recipients and the 5 challenged donor sheep were necropsied 28 days after challenge. Five donor lambs which were not challenged were also necropsied 35 days after the initial infection to establish whether or not cysticerci had indeed developed.

Necropsy was performed as follows: each sheep was stunned with a captive bolt pistol and killed by exsanguination and cervical dislocation. The entire abdominal viscera were removed and placed in a bucket containing 5 L of saline in order to wash off any cysts which had not attached to the peritoneal membranes. The viscera were inspected closely for attached larvae and fibrotic lesions. The walls of the peritoneal cavity and the abdominal surface of the diaphragm were also carefully examined.

All cysts present were counted. Those with an intact, fluid-filled bladder were recorded as living and those whose bladder contained no fluid or were in various stages of degeneration, as dead. Opaque, calcified or caseous lesions were incised with a scalpel to examine the state of any larvae contained within. The surface of the liver was examined and the number of fibrous or calcified tracks was counted; all tracks or lesions were examined by dissection for the presence of degenerating larvae.

2.2.2 Experiment 2. The Transfer of Serum from Lambs Given 3 Oral Doses of *T.hydatigena* Eggs to Naive Recipients.

2.2.2.1 Experimental Animals.

Twenty 2- to 3-month-old weaned wether lambs, destined to be the serum donors, were run at pasture. Fifteen wethers taken from the same flock, destined to be the serum recipients, were run on separate pastures to prevent their accidental infection.

2.2.2.2 Monitoring the Antibody Levels of the Donors.

Prior to the infections, 15 ml blood samples were collected to check by Western blot, that the lambs had not been previously exposed to *T.hydatigena*, or did not have detectable anti-*T.hydatigena* antibodies from their dams. The serum was harvested as described in Section 2.2.1.10. Further samples were taken on days 21, 42 and 47 to monitor the antibody levels resulting from the infections.

2.2.2.3 Experimental Infection of Serum Donors.

Ten of the donor lambs were infected orally with eggs. The numbers of eggs in the infecting doses were determined before each infection using the procedure described previously and are shown in Table 2.1. The remaining 10 lambs served as controls.

Table 2.1 The infection procedure used for serum donors in Experiment 2.

	day 0	day 14	day 42	day 56
10 Infected lambs	20	10^3 (activatable oncospheres)	10^3	Bled out
10 Control lambs	0	0	0	Bled out

2.2.2.4 Collection of Serum from Donors.

On day 56, which was two weeks after the third infection, 5 lambs from each group of 10 were kept to be given a challenge infection with the recipients, and the

remaining 5 were aseptically bled out in the following manner. They were anaesthetised with 7-10ml of 5% thiopentone sodium (Intraval sodium, Pitman-Moore Ltd) intravenously before being suspended by their back legs. Their necks, which had previously been shorn, were cleaned with 70% ethanol/30% Savlon (chlorohexidine digluconate 1.5% w/v and cetrimide B.P. 15% w/v (ICI)) prior to the major vessels of one side of the neck being severed with a sterile scalpel. The blood was collected in sterile 1 L containers, covered, and left to clot at room temperature for approximately 6 hr. The containers were then held at 4°C overnight and the clot removed. Erythrocytes were removed from the serum by centrifugation in sterile, capped centrifuge tubes at 1100 x g for 25 min at 4°C. The serum from each group was pooled in a sterile pressure reservoir and dispensed into gamma-irradiated 500ml Vaxipacs as described above. These were then sealed and stored at 4°C overnight.

The carcasses and peritoneal contents of these donor sheep were examined for the presence of *T.hydatigena* metacestodes.

2.2.2.5 The Transfer of Sera and Saline into Naive Recipients.

Prior to the transfer, 15ml blood samples were taken from each recipient lamb for analysis on Western blots to check that they had not been previously exposed to *T.hydatigena.*

The 15 recipient lambs were divided into 3 groups of 5. Each lamb was weighed and approximately 30 ml/kg of either immune serum, control serum, or saline was administered. Initially, intravenous injection was attempted but the first recipient developed respiratory distress after receiving 100 ml of serum. The remaining serum was therefore given intraperitoneally. All other lambs received the serum or saline intraperitoneally.

2.2.2.6 Post-Transfer Procedure.

Twenty-four hr after the transfer of serum into the recipients, a 10 ml blood sample was taken from each animal to determine on Western blot if transferred antibody was present in the serum of those lambs which received immune serum and not in those receiving either non-immune serum or saline.

These 15 sheep, together with the remaining 10 donor lambs, were challenged orally with 100 activatable oncospheres. One week later a second post-transfer blood

sample was taken to monitor, on Western blots, any change in the levels of the passively transferred antibody.

All 25 lambs were necropsied 28 days after challenge.

2.2.3 Experiment 3. The Transfer of Serum from Lambs Given 3 Immunizations of Solubilized Oncospheres into Naive Recipients.

2.2.3.1 Experimental Animals.

Fifteen 2-month-old wethers were taken from the same flock as in Experiment 2. Ten were destined to be immunized with solubilized *T.hydatigena* oncospheres and 5 to be the recipients of serum from these immunized donors.

This experiment was conducted at the same time as Experiment 2 and the same non-immune serum donor animals, the same 5 recipients of non-immune serum, and the same 5 recipients of saline were used as controls for both Experiments 2 and 3.

2.2.3.2 Monitoring the Antibody Levels of the Donors.

Prior to the immunizations, blood samples were collected and analysed as described in Section 2.2.2.2 to check that the lambs had not been previously exposed to *T.hydatigena*.

2.2.3.3 Preparation of FTS Oncosphere Antigen for Immunization.

The method for preparing FTS antigen is described in Section 2.2.1.7, except that, before being frozen overnight, the hatched embryos were concentrated to approximately 2000/ml by centrifugation for the first immunization and approximately $5x10^4$/ml for the second and third immunizations. For immunization, this antigen preparation was homogenized with an equal volume of a water-and-oil emulsion (STM, Bokhout *et al.*, 1981; Appendix 2.1).

2.2.3.4 Immunization of Serum Donors.

The 10 donor lambs were immunized with FTS oncosphere antigen-STM adjuvant prepared as above. The materials for injection were prepared fresh on each of the 3 occasions. Immunization injections were divided into 2 equal parts, one of which

was administered intramuscularly and one subcutaneously at each immunization (Table 2.2). Successive injections were given on alternate sides of the animal.

Table 2.2. The immunization procedure used for serum donors in Experiment 3.

	day 0	day 14	day 42	day 56
10 Immunized lambs	1.5×10^3	10^5 (oncosphere equivalents)	10^5	Bled out
10 Control lambs	0	0	0	Bled out

2.2.3.5 Collection of Serum from Donors.

Five of the immunized donor lambs were aseptically bled out, as described in Experiment 2, 56 days after the immunization procedure began.

2.2.3.6 The Transfer of Serum into Naive Recipients and the Post-Transfer Procedure.

The serum collected from the immunized donors was transfused intraperitoneally into 5 recipient sheep at a rate of approximately 30 ml/kg. The collection of the post-transfer blood samples was carried out as described for Experiment 2. The challenge infection of 100 activatable oncospheres was given at the same time, and from the same preparation of eggs, as Experiment 2. All lambs were necropsied 28 days after challenge.

2.2.4 Statistical Analysis.

Statistical analysis of the count-data described in this and later chapters, presented some difficulties. These arose in part from small numbers of replicates in some experiments, but mainly from the heterogeneous group variances coupled with the need to use procedures appropriate for discrete data.

A number of approaches were investigated including using log-linear methods, analysis of variance of square-root transformed data or non-parametric procedures.

In some experiments, the heterogeneity of the group variances was so extreme that log-linear methods proved unworkable. On the other hand, square-root transformation of the data substantially reduced and stabilized the group variances to the extent that conventional analysis of variance could be applied. For these reasons, in all experiments, the analyses of the count-data were performed using conventional analysis of variance procedures followed by Tukey pairwise comparisons on the square-root transformed data (Mead and Curnow, 1983; Zar, 1984) and by the Mann-Whitney and Kruskal-Wallis non-parametric procedures (Zar, 1984). Since these parametric and non-parametric analyses yielded virtually identical indications of significance, only the results of the parametric analysis of the transformed data will be presented.

Unless otherwise indicated, cyst numbers are presented as means ± standard errors.

The statistical procedures used in the analysis of other data are described in the appropriate chapters. All analyses were carried out using Statistix 3.0 (Analytical Software, St Paul, MN 55113) or SAS (SAS Institute Inc. N.Y.).

The analysis of variance table and the Tukey pairwise comparisons for the experiments desribed in this chapter are shown in Appendices 2.2 - 2.8.

2.3 RESULTS.

It proved impossible to distinguish with any certainty, between liver lesions caused by initial and challenge infection. It was also impossible to relate the number or extent of lesions with the numbers of cysts recovered from the peritoneal cavity. For these reasons, liver damage is reported only in general terms. Nevertheless, it was possible to distinguish older larvae from 28 day larvae with complete certainty. Larvae resulting from the challenge infection (28 days) were not only much smaller (between 5 and 9 mm long) than larvae from the initial infections (approximately 20mm), but they had a much less developed scolex which, and upon microscopic examination, contained hooks which were not fully developed. Differentiation of the two rows of hooks into large and small, has not occurred by the 28th day of the development of the larva. By day 42, large hooks reach their maximum length of 170 - 220 μm (Verster, 1969).

2.3.1 Experiment 1. The Transfer of Serum From Lambs Given a Single Oral Dose of *T.hydatigena* Eggs, to Naive Lambs.

2.3.1.1 SDS-PAGE Analysis of Donor Serum Samples.

Western blots of the serum samples from the donor lambs before infection showed a low level of reactivity with *T.hydatigena* oncosphere antigen (Figure 2.1). The bands of antigen which did react with the preinfection sera were evidently not associated with resistance to infection and probably represented cross-reacting antibodies as similar bands were observed in sera from other uninfected lambs in other experiments. Samples taken 28 days after infection reacted with an array of bands of *T.hydatigena* oncosphere antigen consistent with the normal response to infection. Twenty-four hr after transfer of this serum to the recipients, antibodies were detectable in their sera but evidently at low levels (Figure 2.2, lane 4).

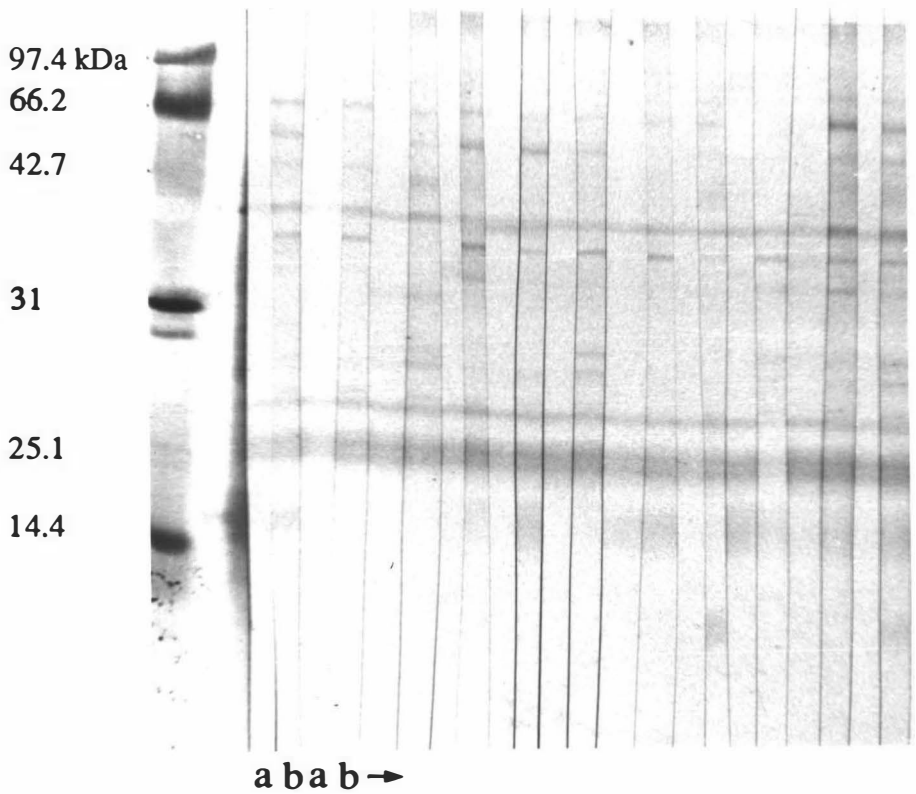
2.3.1.2 Cyst Numbers found at Necropsy.

Necropsy of the donor lambs showed that the 5 unchallenged lambs were all infected, having a total cyst number of 34.4 ± 11.4. The 5 challenged donors showed no evidence of development of the challenge infection, but the mean number of cysts from the first infection was 49.4 ± 11.21. The cyst numbers present from the first infection in these two groups of donor lambs are not significantly different ($p > 0.05$) (Appendix 2.2)

While the donors of the serum were completely immune to challenge infection after a single dose of 50 activatable oncospheres, the recipients of the sera from these immune animals were not protected against the challenge infection. The mean numbers of metacestodes found in the peritoneal cavities of the 5 recipient lambs for each group are shown in Table 2.3. The livers of these lambs were all damaged to a similar degree with fibrotic lesions and scarring (Figure 2.3).

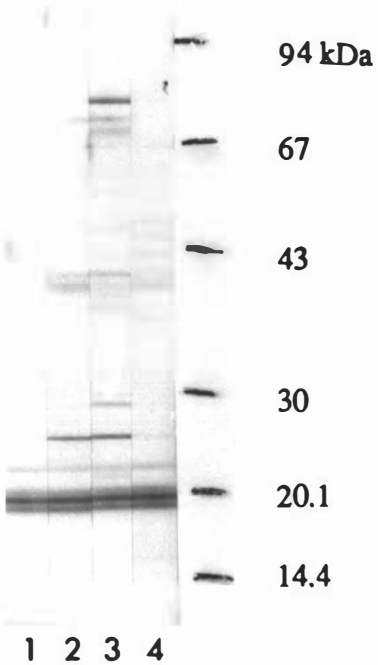
These data are illustrated in Figure 2.12a and show no significant differences in the cyst numbers between any of the groups with respect to either live, dead, or total cysts ($p > 0.05$ for live, dead, and total cysts) (Appendices 2.3, 2.4 and 2.5, respectively).

Figure 2.1 **Western blot of *T.hydatigena* oncosphere antigen probed with sera from each of the donor lambs of Experiment 1 (a) before and (b) after they were infected.**



a = Pre-infection serum for each of the 10 donor lambs
b = Post-infection serum for each of the 10 donor lambs

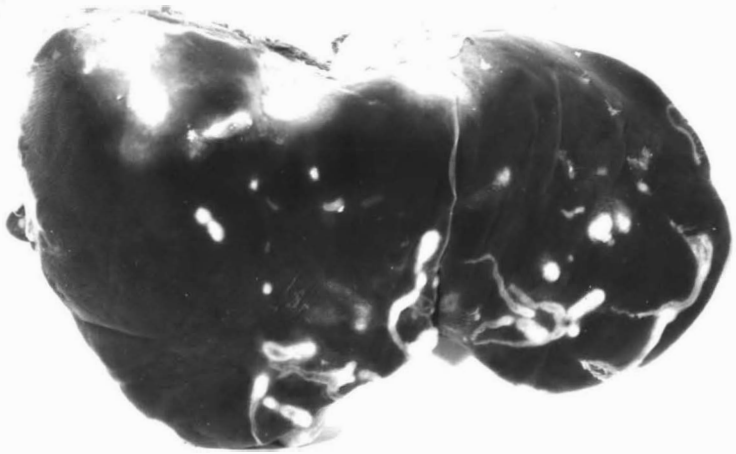
Figure 2.2 **Western blot of *T.hydatigena* oncopshere antigen probed with serum from the recipients 24 hr after the transfer. Experiment 2.**



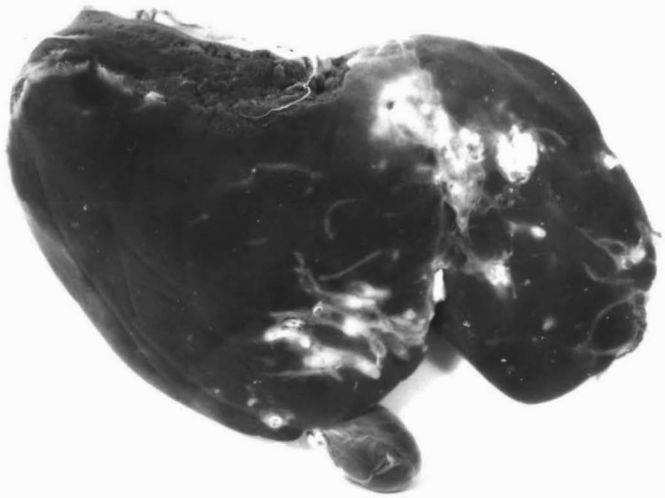
Lanes	Serum samples
1	Donated Non-Immune
2	Donated Immune
3	Immune donor, 24 hr post-transfer
4	Immune recipient, 24 hr post-transfer

Figure 2.3 **Livers with lesions typical of each group of recipient lambs 28 days after challenge.**

Recipient of Saline



Recipient of Control serum



Recipient of Immune serum

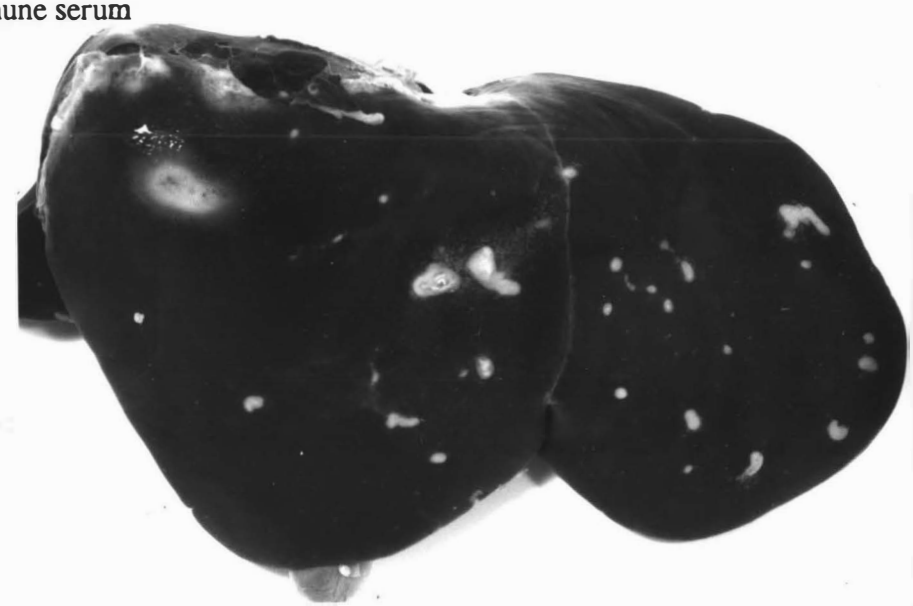


Table 2.3. Cyst Numbers found at Necropsy.

Recipients of:	Metacestodes in the peritoneal cavity. (Mean ± S.E.) Live	Dead	Total
Saline	12.4 ± 3.19	9 ± 2.74	21.4 ± 1.81
Control Serum	22.6 ± 5.67	15 ± 5.39	37.6 ± 9.44
Immune Serum	10.6 ± 6.79	21 ± 6.14	31.6 ± 5.53
Donors of serum	No evidence of new infection.		

2.3.2 Experiment 2. The Transfer of Serum From Lambs Given 3 Oral Doses of *T.hydatigena* Eggs, to Naive Lambs.

2.3.2.1 SDS-PAGE Analysis of Serum Samples.

Examples of the Western blots from serum samples taken from donor lambs prior to and during the infection schedule are shown in Figure 2.4. The increase in anti-*T.hydatigena* antibody level from pre-infection to 5 days after the 3rd infection (day 47), is apparent.

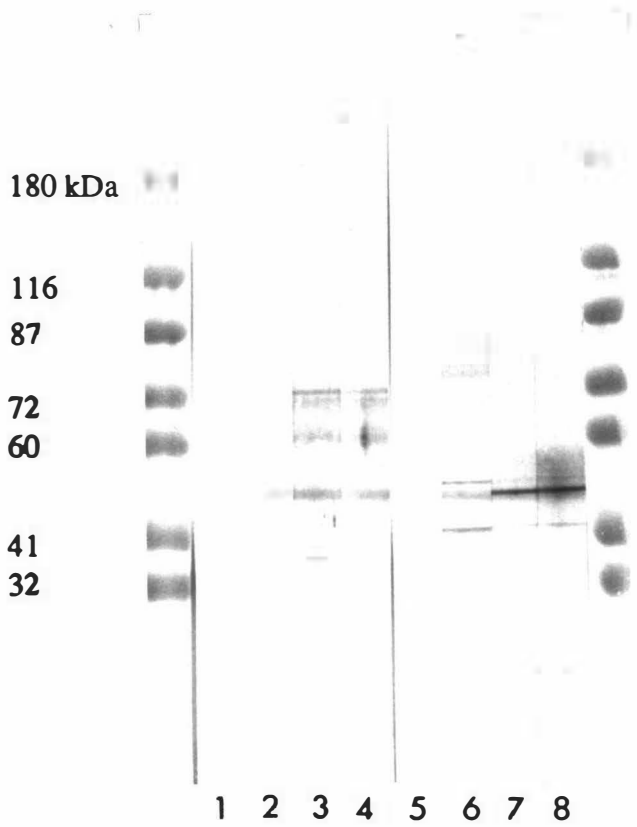
Antibody was monitored 24 hr after transfer in the serum of the recipients and again 7 days later (Figure 2.5). The blotting profiles were similar on both occasions.

2.3.2.2 Cyst Numbers Found at Necropsy.

The unchallenged control donors contained no cysts.
The mean number of live cysts in the 5 infected, unchallenged donors was 6.8 ± 3.7. These were all mature cysts.

The other 5, which were the challenged donors, contained an average of 6 ± 8.3 mature living cysts (Total cysts = 45.8 ± 43.32). There were no new, small, living cysts in the peritoneal cavity from the challenge infection. The challenged controls, however, had an average of 58.2 ± 19.37 immature, live cysts (68.8 ± 22.96 total) in the peritoneal cavity.

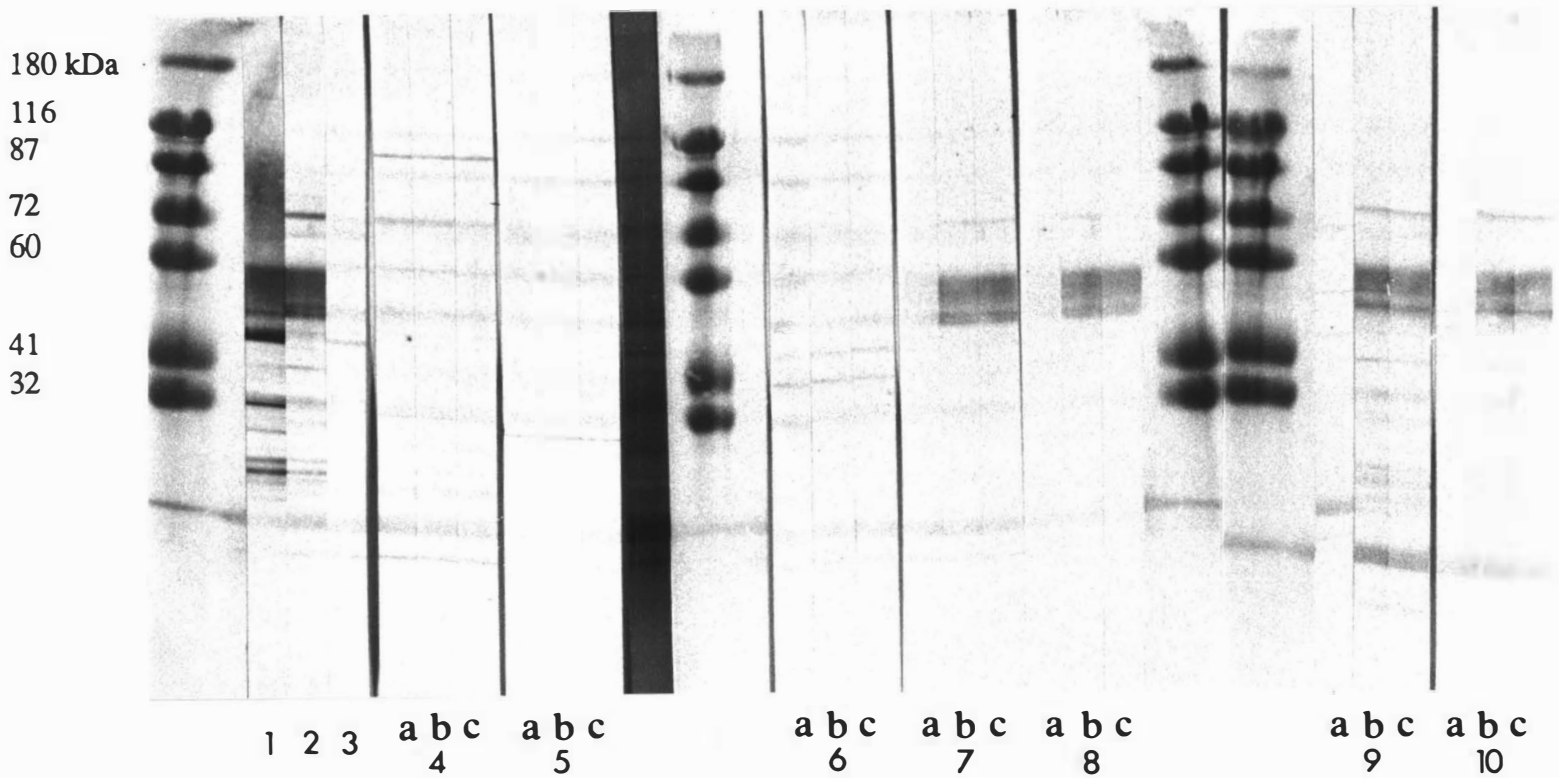
Figure 2.4 **Western blot of *T.hydatigena* oncosphere antigen probed with sera from 2 of the donors 0, 21, 42 and 47 days after the first infection in Experiment 2.**



Lane	Day of blood collection
	Lamb 527
1	0 - pre-infection
2	21 - 1st infection
3	42 - 2nd infection
4	47 - 3rd infection
	Lamb 523
5	0 - pre-infection
6	21 - 1st infection
7	42 - 2nd infection
8	47 - 3rd infection

Figure 2.5 **Western blot of *T.hydatigena* oncosphere antigen probed with sera collected from some of the recipients of Experiment 2, 24 hr and 7 days after transfer .**

Lane	Serum Samples
1	Pooled Immunized donor serum
2	Pooled Infected donor serum
3	Pooled Control donor serum
4	Saline recipient
5	Control serum recipient - e.g. 1:
6	Control serum recipient - e.g. 2:
7	Immune serum recipient - e.g. 1:
8	Immune serum recipient - e.g. 2:
9	Immune serum recipient - e.g. 3:
10	Immune serum recipient - e.g. 4:
a	Pre-transfer, day 0
b	24 hr post-transfer
c	7 days post-transfer


180 kDa
116
87
72
60
41
32
1 2 3
a b c
4
a b c
5
a b c
6
a b c
7
a b c
8
a b c
9
a b c
10

The numbers of metacestodes found in the recipients of the saline or serum are shown in Table 2.4, and in Figure 2.12b.

The numbers of dead cysts in the 3 recipient groups were not significantly different ($p > 0.05$) (Appendix 2.6), and there was no significant difference between the saline and control serum groups in either live or total cyst numbers (Appendices 2.7 and 2.8, respectively). However, significant differences in both live and total cyst numbers occurred between the saline and immune serum groups and between the control serum and immune serum groups ($p < 0.02$ for both live and total cyst numbers) (Appendices 2.7 and 2.8, respectively).

The recipients of serum from infected lambs were significantly protected against the challenge infection with a reduction in live cyst numbers of over 80% and of over 75% in total cysts from the combined mean of the cyst numbers in the recipients of saline and control serum (Table 2.4).

2.3.3 Experiment 3. The Transfer of Serum from Lambs Given 3 Immunizations, to Naive Lambs.

2.3.3.1 SDS-PAGE Analysis of Serum Samples.

The sera from the donor lambs collected at each successive immunization reacted with an increasing number of bands of antigen on a Western blot, and with a greater intensity of staining (Figure 2.6).

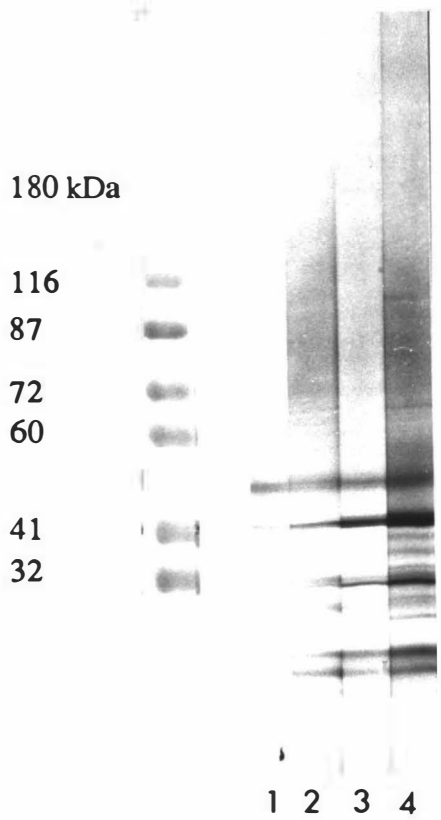
Twenty-four hr after the serum from the immunized donors was transferred, and again at 7 days, antibody was monitored in the serum of the recipients (Figure 2.7), and as was the case in Experiment 2, the blotting profiles were similar on both occasions.

2.3.3.2 Cyst Numbers Found at Necropsy.

At necropsy neither the 5 unchallenged nor the 5 challenged, immunized donors contained any cysts.

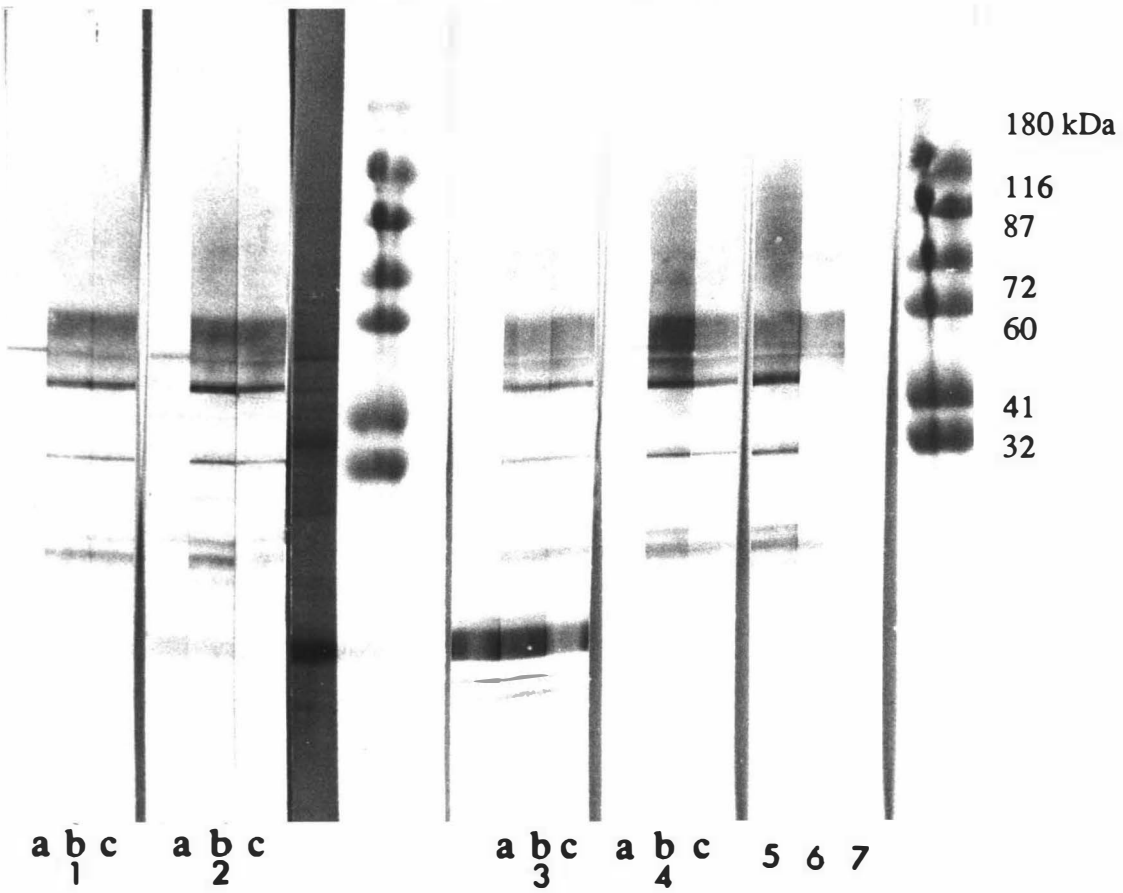
The numbers of metacestodes found in the recipients of the immune serum are shown in Table 2.5 and in Figure 2.12b along with the cyst numbers found in the recipients of non-immune serum and saline from Experiment 2.

Figure 2.6 **Western blot of *T.hydatigena* oncosphere antigen probed with sera from one of the donor lambs 0, 21, 42 and 47 days after the first immunization in Experiment 3.**



Lane	Day of sample collection.
1	Day 0 - pre-immunization
2	Day 21 - 1st immunization
3	Day 42 - 2nd immunization
4	Day 47 - 3rd immunization

Figure 2.7 **Western blot of *T.hydatigena* oncosphere antigen probed with serum from some of the recipients collected 24 hr and 7 days after the transfer in Experiment 3.**



Lane	Serum samples.
a	Day 0 - pre-transfer
b	Day 1 - post-transfer
c	Day 7 - post-transfer
1	Immune serum recipient 1
2	Immune serum recipient 2
3	Immune serum recipient 3
4	Immune serum recipient 4
5	Pooled sera from immunized donors
6	Pooled sera from infected donors
7	Pooled sera from control donors

The results of the sera from the controls for Experiment 3 are shown in Figure 2.5.

Table 2.4 The numbers of cysts found in the recipients of serum or saline.

Experiment 2

Mean ± SE

Recipients of:	Mean cyst number found in the peritoneal cavity.			% Reduction.	
	Live	Dead	Total	Live	Total
Saline	182.2 ± 48.2	15.2 ± 9.4	199.4 ± 40.9	0	0
Control serum	199.8 ± 35.4	9.0 ± 3.5	208.8 ± 34.2		
Immune serum	36.8 ± 13.9	9.2 ± 5.2	46.0 ± 13.4	80.8	77.0

The numbers of dead cysts in the recipients of immune serum did not differ significantly from those in recipients of saline or control serum ($p > 0.05$) (Appendix 2.6).

As noted earlier, the numbers of cysts in recipients of saline or control serum (Experiment 2) did not differ significantly. However, the numbers of live cysts and total cysts in recipients of immune serum (Experiment 3) were reduced by over 70% and 80%, respectively, compared with the combined mean number of cysts in the control serum and saline groups ($p < 0.02$ for both live and total cyst numbers) (Appendices 2.7 and 2.8, respectively).

2.3.3.3 **The Degree of Damage to the Recipients' Livers in Experiments 2 and 3.**

The damage to the livers of the lambs in each group appeared to be very similar (Figure 2.8) with all showing some fibrosis and scarring.

2.3.4 Comparison of Results From the Passive Transfer Experiments.

Samples of the sera transferred to the naive recipients in the 3 passive transfer experiments were compared on Western blots and ELISA.

From Figure 2.9 it is apparent that the donor immune serum from the first experiment (lane 5) contained less anti-*T.hydatigena* antibody than did either the sera from infected or immunized donor lambs in Experiments 2 and 3 (lanes 2, 3), respectively. This is supported by the ELISA results (Figure 2.10), which showed that the immune donor sera in Experiments 2 and 3 had absorbance values approximately 3 times greater than those of the immune serum in Experiment 1. Similarly the recipients of immune serum in Experiments 2 and 3 had ELISA absorbances which were 50% higher than those of the recipients in Experiment 1.

The differences in ELISA absorbances of the pooled sera are reflected in the density of banding in the Western blots (Figures 2.9 and 2.11) although the latter indicates somewhat higher levels in Experiment 3 than in Experiment 2. There was, however, no significant difference between cyst numbers in recipients of immune sera in Experiments 2 and 3 ($p > 0.05$).

Figure 2.8 **The livers of the recipients in Experiments 2 and 3, 28 days after challenge.**

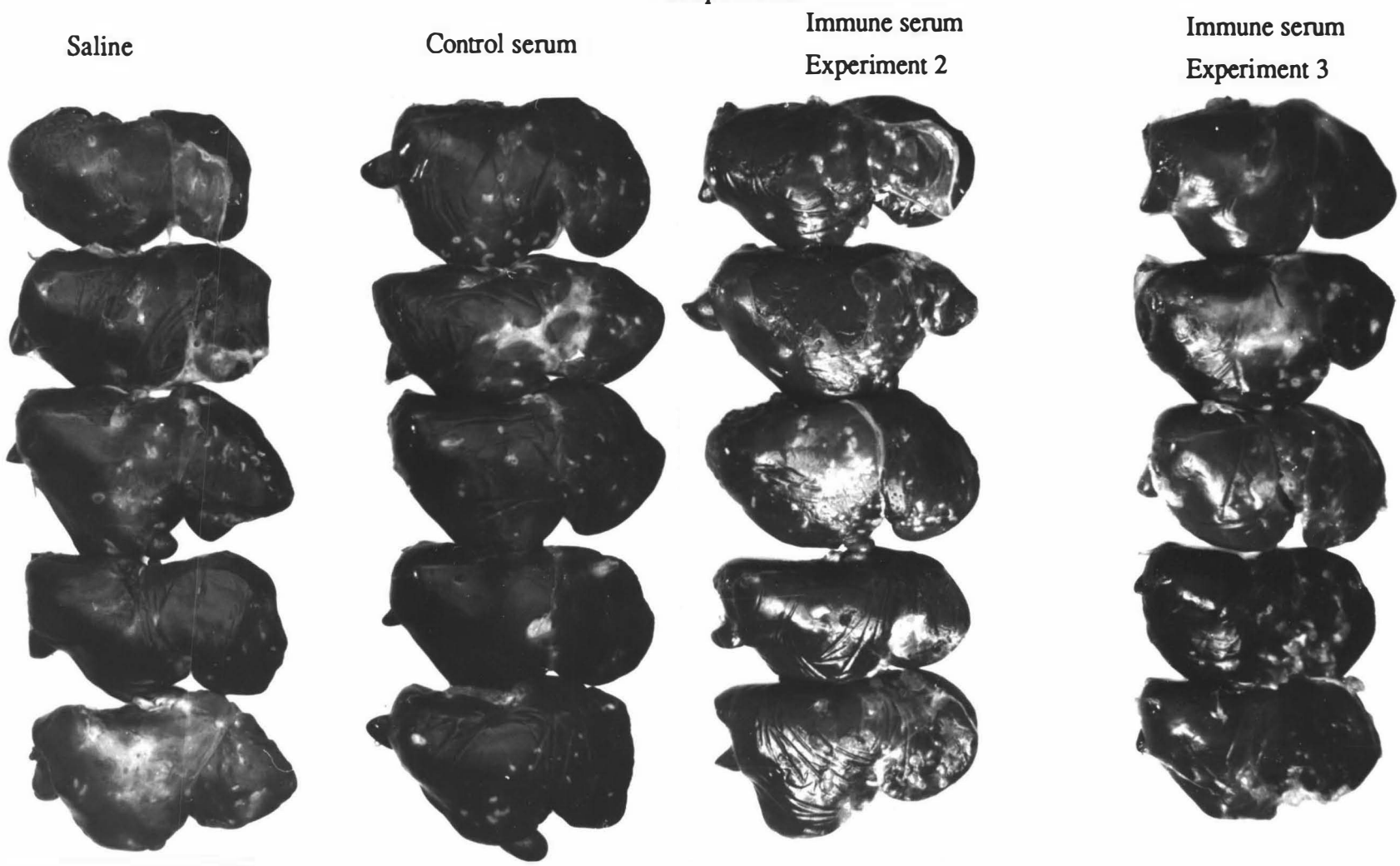
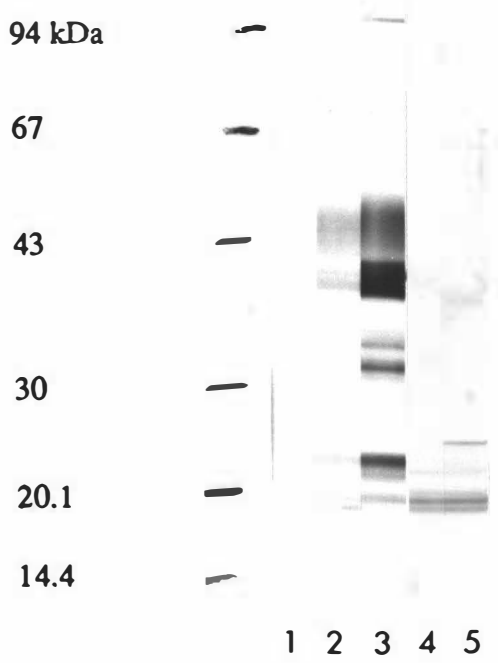


Figure 2.9 **Western blot of *T.hydatigena* oncosphere antigen probed with sera from the donors of Experiments 1, 2 and 3 after pooling the samples from each group.**



Lane	Serum samples
1	Pooled Control sera, Expts 2 and 3
2	Pooled Immune sera, Expt 2
3	Pooled Immune sera, Expt 3
4	Pooled Control sera, Expt 1
5	Pooled Immune sera, Expt 1

Figure 2.10 **The ELISA absorbances of the sera from donors and recipients of Experiments 1, 2 and 3 against *T.hydatigena* oncosphere antigen.**

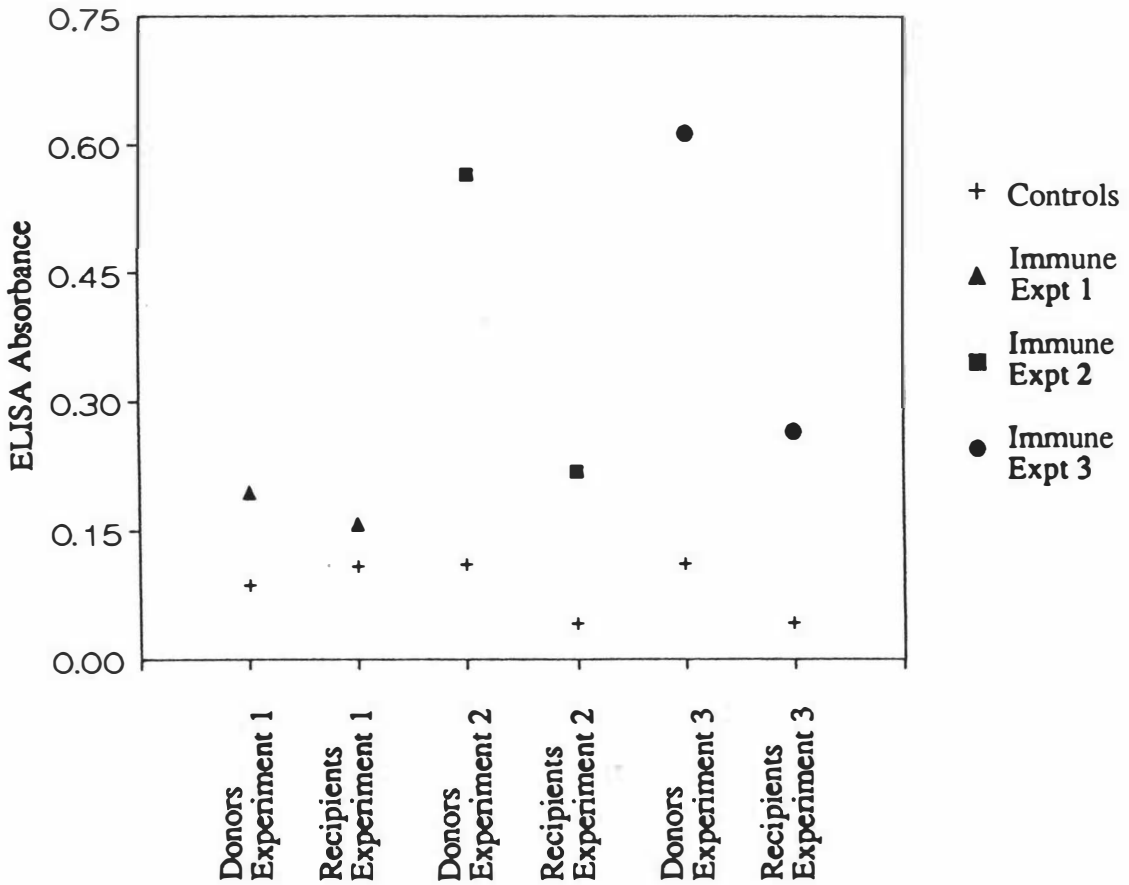
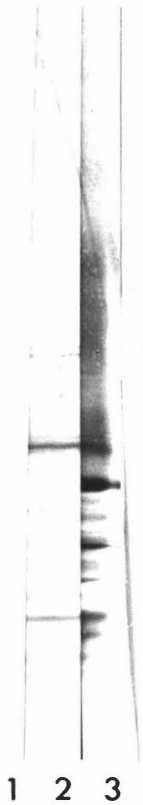


Figure 2.11 **Western blot of *T.hydatigena* oncosphere antigen probed with pooled sera from the donors of Experiments 2 and 3.**



Lane	Serum samples
1	Pooled Control donors
2	Pooled Immune donors, Expt 2
3	Pooled Immune donors, Expt 3

Table 2.5 Cysts found in the recipients of serum or saline.
Experiment 3
Mean ± SE

Recipients of:	Mean cyst number found in the peritoneal cavity. Live	Dead	Total	% Reduction. Live	Total
Saline	182.2 ± 48.2	15.2 ± 9.4	199.4 ± 40.9	0	0
Control serum	199.8 ± 35.4	9.0 ± 3.5	208.8 ± 34.2		
Immune serum	33.0 ± 10.4	19.2 ± 7.9	52.2 ± 15.7	82.0	74.0

The serum from the donor lambs in Experiment 1 was unable to protect the recipients from a challenge infection although the donors were totally immune to a challenge infection. However, serum from lambs which had received 3 infections or 3 immunizations was able to protect recipients of this serum.

2.4 DISCUSSION.

Initially there are two technical points which require comment.

Firstly, some Western blots of *T.hydatigena* oncosphere antigen showed predominant bands of antigen of approximately 14, 20, 22, 26, 39, 50 kDa, which were recognized by serum samples from control animals. ELISA results of these pooled samples showed very low absorbances (Figure 2.10) and, as mentioned in Section 2.2.1.1, only 1 animal out of all the sheep used in the experiments described in this thesis was found to harbour a *T.hydatigena* cyst from a natural infection. This indicates that these bands of antigen were recognized by cross-reacting antibodies in the control sera.

Secondly, the cysts which resulted from the challenge infection of Experiment 1 numbered between 20 and 40. These resulted from an oral dose of *T.hydatigena* eggs estimated to contain approximately 50 activatable oncospheres. In Experiments 2 and 3, the challenge dose was increased to contain an estimated 100 activatable oncospheres with the intention of increasing the numbers of larvae developing and enabling any subtle differences in the immunity induced in the recipients to be detected more readily. However, 28 days after the challenge, the control sheep in these experiments harboured approximately 200 metacestodes indicating that far more infective eggs, than indicated by the *in vitro* assessment, were present. It is clear from this that *in vitro* activation does not provide an accurate assessment of the number of metacestodes which can become established *in vivo*.

The results presented in this chapter clearly establish that a considerable level of resistance can be transferred in the sera from hyperinfected or immunized sheep. It is interesting to note that, in Experiment 1, the pooled sera from the donors, which had received a single oral infection of eggs estimated to contain only 50 activatable oncospheres, and which were completely resistant to a challenge infection, did not protect the recipients in the volumes administered (Figure 2.12a). The most obvious explanation for this is that the level of antibody present was insufficient and this is

Figure 2.12a **The numbers of cysts found in the recipients of saline, immune or non-immune serum in Experiment 1.**

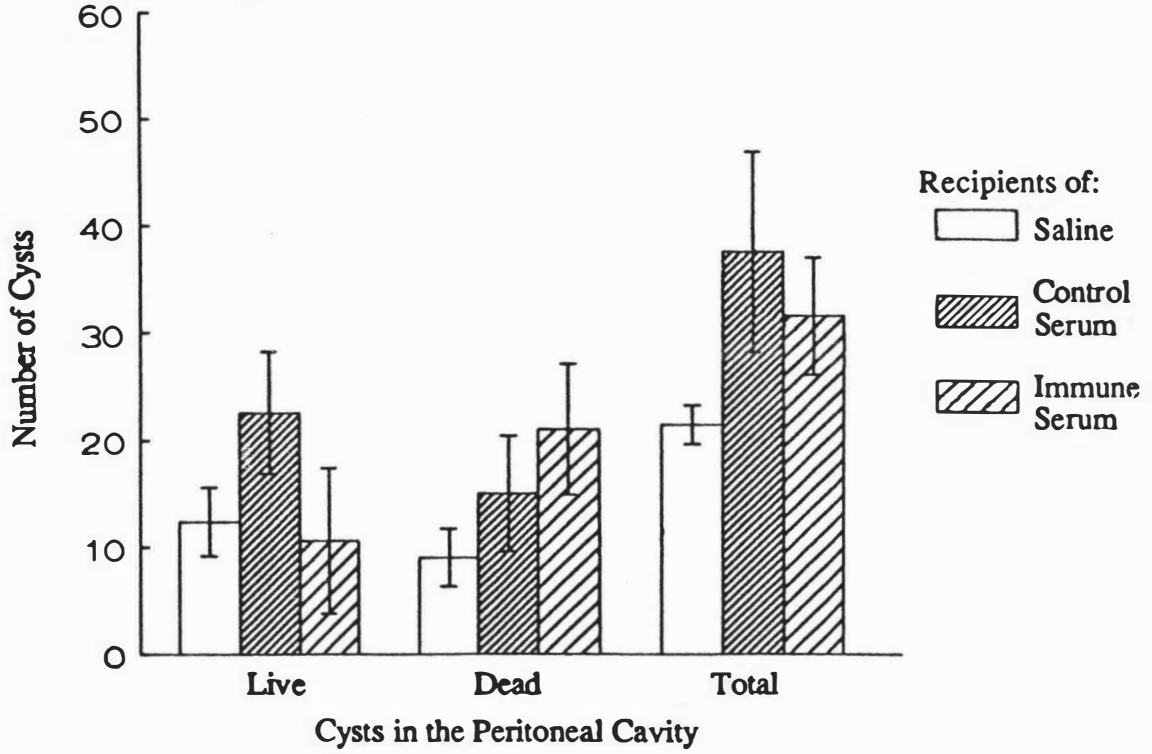
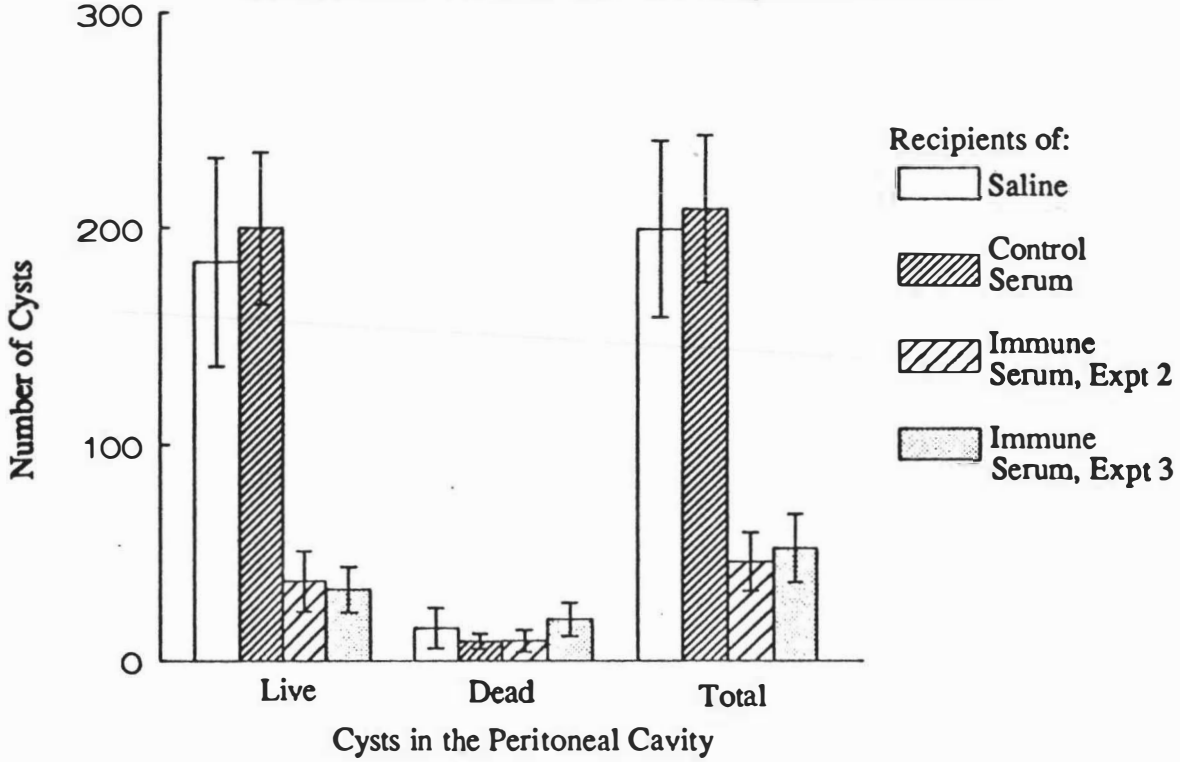


Figure 2.12.b **The numbers of cysts found in the recipients of saline, immune or non-immune serum in Experiments 2 and 3.**



borne out by the Western blot and ELISA results which indicated substantially higher anti-*T.hydatigena* antibody levels in the sera of Experiments 2 and 3, which induced 70-80% protection in its recipients (Figure 2.12b). Indeed, as judged by Western blots, the antibody level in the pooled serum used for the transfer in Experiment 1 was lower than in pooled sera collected from the serum donors only 24 hr after the last collection for transfer purposes. Sera for transfer were collected on 4 occasions and it is possible that earlier collections contained lower levels of antibody and so effectively diluted sera collected later.

The levels of protection achieved in Experiments 2 and 3 were substantial but total protection was not achieved. This leads one to question whether or not anti-*T.hydatigena* antibody alone, given in sufficient quantities, would be capable of inducing 100% protection in recipients. One approach to establishing this, and to determine whether or not there is a quantifiable relationship between antibody titers and the level of protection, would be to carry out further experiments of the type described here but involving larger numbers of animals and graded doses of antibody. This was not feasible in this study. Another approach to the question is through examination of the effects of passively transferred antibody in colostrum. This is described in Chapter 4.

Another matter of interest concerns the point at which the larvae are killed in an immune animal. Does it occur before grossly visible liver damage has been done, after this, or both? Clearly, if it were possible to distinguish reliably between liver lesions of different ages and to show a relationship between their nature and the numbers of live or dead larvae present in the peritoneal cavity, examination of the livers would shed some light on this. However, this did not prove practicable. The livers of the recipients were carefully examined but it was not possible to determine macroscopically which of the lesions in them were due to larvae which had emerged into the peritoneal cavity and which were caused by larvae that died before doing so. Furthermore, the degree of liver damage was similar in the livers of all 3 recipient groups of Experiment 1 and in the 4 recipient groups of Experiments 2 and 3 despite two of these groups being significantly protected. Not only was it impossible to distinguish between the lesions caused by escaped or dead larvae, even following a single infection, but it was also impossible to distinguish with any certainty between the lesions resulting from an initial immunizing infection and a subsequent challenge infection 28 days before necropsy. The livers of the infected donors which were challenged, therefore, also gave no indication of the point at which host resistance was effective. However, the livers of the FTS-immunized donors had not been subjected to an oral infection until the challenge infection was given. Not only

were no cysts found in the peritoneal cavity following this challenge but the livers of these animals showed only a few, barely visible, scars indicating that the larvae from the challenge infection had succumbed before they were able to cause gross liver damage (Figure 2.13).

Whether or not this is the case in partially protected animals remains to be established. It could be investigated by attempting to induce varying levels of immunity by using different antigen preparations and immunization regimes. Thus the livers would be free from any initial larval migration so that the liver damage due to the challenge infection alone could be assessed.

Figure 2.13 **The livers of the donors of the immune serum 28 days after challenge.**

CHAPTER 3

THE INFLUENCE OF IMMUNE SERUM, COMPLEMENT AND LEUKOCYTES ON ONCOSPHERE SURVIVAL *IN VITRO*.

3.1 INTRODUCTION.

The importance of factors in the serum of immune animals in protecting sheep against infection with *T.hydatigena* was described in the preceding chapter. It is clear that if enough antibody is present, highly significant protection against infection with *T.hydatigena* occurs *in vivo*. This chapter investigates the effect of immune serum and complement on the survival of *T.hydatigena* oncospheres *in vitro* and also examines the involvement of leukocytes from both immune and non-immune sheep in the killing of oncospheres *in vitro*. The question as to whether the failure to obtain complete protection in the experiments described in the previous chapter could be attributable to a need for leukocytes from actively immunized animals, will be discussed.

3.2 MATERIALS AND METHODS.

3.2.1 Experimental Animals and the Immunization Regime.

Six 6-month-old lambs were housed indoors in enclosed pens and fed sheep nuts, lucerne pellets and hay *ad libitum*. Three were immunized with oncosphere antigen prepared as described in Section 2.2.3.3. The sizes, timing and administration of the immunizing doses were as described in Section 2.2.3.4. Briefly, the regime was: FTS oncospheral antigen to be injected into each sheep on each occasion was divided into 2 equal parts, 1 part was injected intramuscularly and the other subcutaneously. On day 0, 1.5×10^3 oncosphere equivalents were given to each sheep, 1×10^5 on day 14 and 1×10^5 on day 42, with successive injections given on alternate sides of the animal. The remaining 3 sheep served as controls.

3.2.2 Preparation of the Sheep Leukocytes for use in Cultures.

On days 0, 3, and 6 of the culture period, blood was collected from each of the 3 immunized and 3 control sheep into sterile 15 ml vacutainers containing 500 µl of 15% potassium-EDTA solution (Becton-Dickinson). The vacutainers were centrifuged at 1000 x g for 15 min and the buffy coats transferred to sterile,

polycarbonate tubes. The cells from immunized and control sheep were pooled separately. The bloods remaining in the vacutainers were resuspended and centrifuged again and the second buffy coats recovered and combined with the first.

Ten ml of sterile 0.9% ammonium chloride was added to each combined cell harvest to lyse erythrocytes present and after approximately 3 min, when lysis was complete, the suspension was centrifuged at 800 x g for 15 min. The cells were washed once in Hank's balanced salt solution, pH 7.2 (without Mg^{++} or Ca^{+}) (GIBCO) containing 0.2% EDTA to prevent clumping, before being washed twice with McCoy's 5A (modified) medium (GIBCO). The cells were finally resuspended in 8 ml of McCoy's medium and the number of leukocytes/ml counted in a haemocytometer.

Differential counts of the first preparation of leukocytes used in the cultures were made to confirm that the preparation was representative of normal leukocyte percentages. These counts were made by mixing equal proportions of control sheep serum with the leukocyte preparation. Two 5 µl samples were taken and thin smears made on microscope slides. These were air dried, fixed for 10 min in absolute methanol and dried. After staining for 30 min in 10% (w/v) Giemsa (BDH) in buffered water (Appendix 2.1), the slides were dipped in distilled water to remove excess stain and air dried. One hundred cells on each slide were differentiated.

The viability of the leukocytes in each preparation was assessed by staining one drop of the cell suspension with one drop of 0.4% Trypan Blue. At least 45 cells were counted and the proportion of dead cells (i.e. those taking up the dye) recorded. The leukocytes were put into the cultures within 2 hr of blood collection.

3.2.3 **Preparation of Serum for Use in Cultures.**

Twenty ml of blood were collected from each of the 6 sheep into anticoagulant-free vacutainers on 3 occasions during the 10-day *in vitro* experiment. These were centrifuged at 1100 x g for 30 min at 4°C approximately 10 min after collection in order to preserve the complement. The serum was removed and the samples from each of the 3 sheep in each group were pooled. Half the serum of each group was stored on ice to retain complement activity while the other half was heated at 56°C for 30 min to remove complement activity. These sera were added to the cultures immediately after preparation.

3.2.4 Preparation of the Cell Line.

Preliminary culturing experiments (Heath, personal communication) had shown that the presence of Vero cells (African Green Monkey Kidney cells, Flow Laboratories) greatly enhances the health and survival of oncospheres in culture medium.

Vero cells, stored at -70°C with 10% dimethyl sulfoxide (DMSO, analytical grade) were thawed at 37°C and washed in 10 ml of McCoy's medium containing 20% foetal calf serum (FCS) at 37°C to remove the DMSO. Monolayers of Vero cells were established in 75 ml culture flasks in 10 ml of McCoy's medium containing 20% FCS at 37°C under 5% CO_2-in-air. When the cells became confluent they were subcultured. This was done by incubating the cells with 5-10 ml of a solution of saline, trypsin and versene (Appendix 2.1) at 37°C for approximately 2 min after the flask had been rinsed twice with sterile saline to remove FCS which inactivates trypsin. When the cells were rounded up and had lifted from the bottom of the flask, they were distributed into 10 sterile, 75 ml culture flasks. Approximately 8 ml of McCoy's medium containing 20% FCS were added to each flask which were then incubated as described above.

3.2.5 The Design of the *In vitro* Assay.

Table 3.1 shows the design of the experiment which examined the effects of antibody, complement and leukocytes on the survival of freshly-activated oncospheres.

Each of the assays was set up in triplicate in the wells of 6-well culture plates (Nunc) and to each well was added 2 ml of the appropriate serum, 100 µl nystatin (GIBCO, BRL, 10,000 U/ml in PBS, pH 7.2), approximately 2.5×10^6 of the leukocytes from either immune or control sheep, and approximately 60 µl of the Vero cell suspension in the saline trypsin-versene solution, harvested as described above. Approximately 450 oncospheres, which were hatched and activated in the manner described in Section 2.2.1.4, were added to each well and McCoy's medium added to a final volume of 10 ml. The plates were incubated at 37°C under 5% CO_2-in-air. On the third and sixth days of the 10 day culture period, half the supernatant from each well was removed and approximately 2.5×10^6 fresh leukocytes and 2 ml of serum were added. On each occasion fresh leukocytes and serum were prepared in the same manner as described above.

Table 3.1. Experimental design.

Serum and Complement		Leukocytes Absent	Leukocytes from Control Sheep	Leukocytes from Immunized Sheep
Control Serum	-C'			
	+C'			
Immune Serum	-C'			
	+C'			

Oncospheres and cells were observed and photographed during this 10 day culture using a Leitz Diaphot inverted microscope and Leitz Orthomat camera. The number of metacestodes surviving, their sizes, the formation of precipitates and the degree of cell-cover were recorded over this period.

3.2.6 **Statistical Analysis.**

The numbers of larvae surviving were compared by analysis of variance and Tukey's test for pairwise comparisons following square-root transformation of the data, as described in Section 2.2.4.

3.3 RESULTS.

3.3.1 **Analysis of Leukocyte Preparations.**

The differential counts performed on the first preparation of leukocytes for this experiment are shown in Table 3.2 where combined immune and control leukocyte preparations are compared with the normal range of leukocyte values of sheep (Blunt, 1975). The percentage of lymphocytes present slightly exceeded the normal range, and the percentage of monocytes was lower than expected. The percentages of other leukocyte types were within the normal range.

Table 3.2. Normal ovine blood leukocyte counts.

Cell Type	Normal % WBC (Blunt, 1975)	% WBC of Preparation ± SE
Lymphocytes	40 - 75	78.1 ± 4.6
Neutrophils	10 - 50	23.6 ± 3.7
Eosinophils	0 - 15	0.4 ± 0.2
Basophils	0 - 3	0.6 ± 0.2
Monocytes	1 - 6	0.5 ± 0.2

The percentage of viable leukocytes in the preparations made on days 0, 3 and 6 ranged from 88 - 93.7% (mean 91.6) and 84 - 94.3 (mean 86.7) in leukocytes from control and immunized animals, respectively.

3.3.2 Overall Effects of Immune Serum, Complement and Leukocytes on Larval Survival.

The numbers of larvae surviving after 10 days in culture are shown in Figure 3.1 and the data are shown in Appendix 3.1. The reference point for comparisons, i.e. the "controls", are cultures containing control serum but neither complement nor leukocytes.

The data showed highly significant ($p < 0.01$) effects of antibody, complement and leukocytes on survival of the oncospheres with highly significant interactions between antibody and complement and between antibody and leukocytes ($p < 0.01$). The interactions between complement and leukocytes, and between antibody, complement and leukocytes were not significant ($p > 0.4$). Exact p-values are shown in Appendix 3.2.

3.3.3 Tukey Pairwise Comparisons of Treatments.

The comparisons of individual pairs of treatments show where these significant effects arise (Figure 3.2). The rejection level used in the Tukey procedure was $p < 0.05$.

Figure 3.1 **Mean (± S.E.) number of larvae surviving after 10 days in culture.**

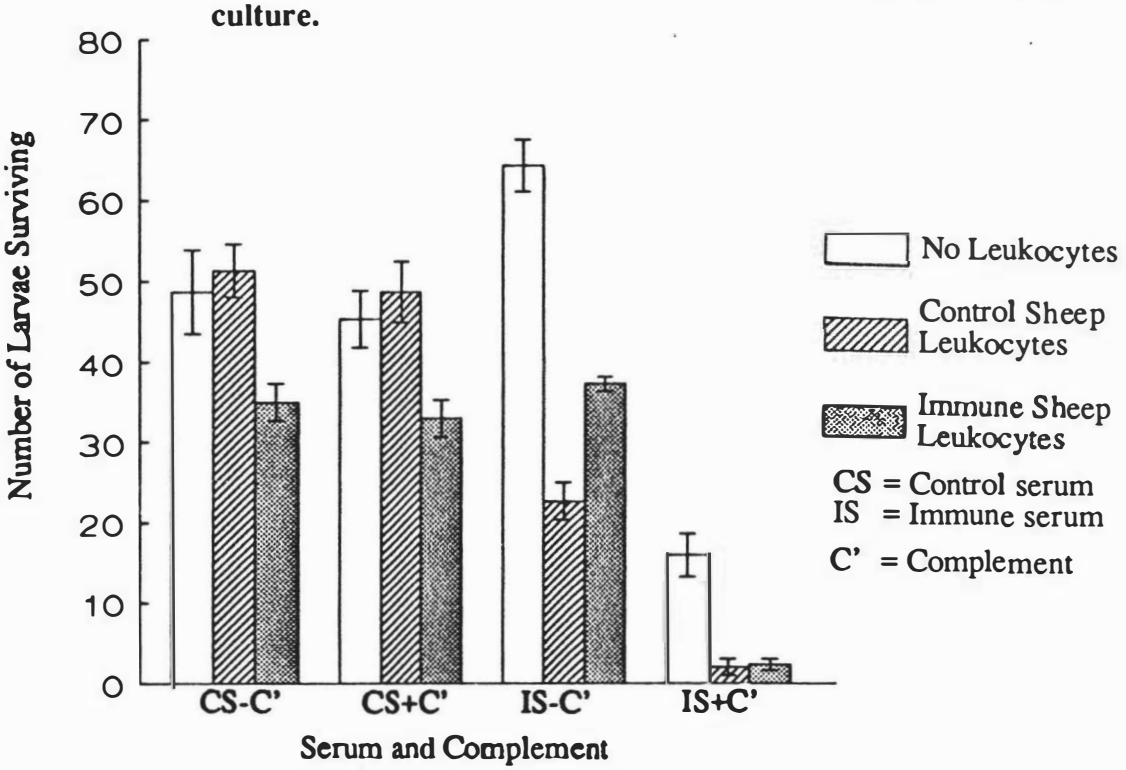
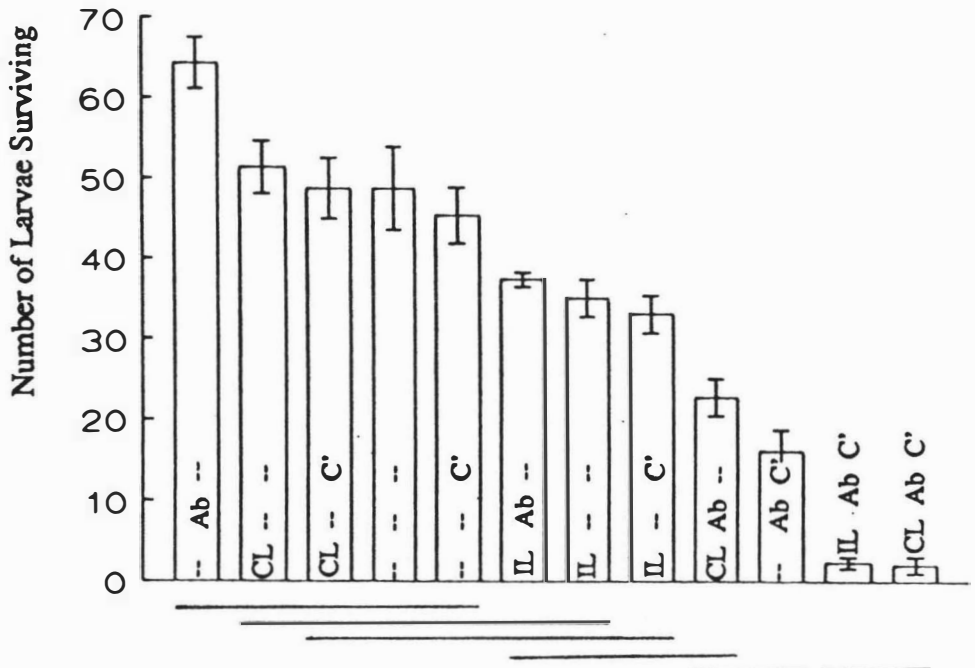


Figure 3.2 **Significant differences between numbers of surviving larvae.**

CL = Control Leukocytes Ab = Immune Serum C' = Complement

IL = Immune Leukocytes -- = Control Serum -- = No Complement

-- = No Leukocytes



Cultures linked with the same line are not significantly different ($p > 0.05$).

Effects of Complement.

In all groups containing immune serum, the presence of complement resulted in a significant decrease in the survival of the larvae compared with similar cultures without complement. However, in all cultures with control serum, the presence or absence of complement showed no significant effect.

Effect of Immune vs Control Serum.

In all groups containing complement, the presence of immune serum caused a significant decrease in survival when compared with serum from control sheep. In the absence of complement, a significant difference between the effects of immune serum and control serum was evident only in the groups containing leukocytes from control sheep, but not those containing leukocytes from immune animals or those with no leukocytes at all. In fact, in those groups without complement and leukocytes, the presence of immune serum apparently enhanced oncospheral survival, although the difference was not significant at the 5% level.

Effect of Leukocytes.

When complement was present with immune serum, the presence of either type of leukocyte resulted in a significant decrease in larval survival compared with the cultures in which no leukocytes were present, but there was no significant difference between the effects of the presence of the leukocytes from control sheep or immune sheep.

With complement absent from the immune serum, the presence of either type of leukocyte, again, significantly decreased larval survival compared with the absence of leukocytes from the cultures. However, in this case, the presence of leukocytes from control animals resulted in significantly fewer larvae surviving compared with the presence of leukocytes from immune animals.

In control serum, both with and without complement, there was no significant effect of the presence of either type of leukocyte compared with the absence of leukocytes.

3.2.4 Adherence of Leukocytes to Larvae *In Vitro.*

In the presence of antibody, leukocytes from both immune and control sheep rapidly adhered to the oncospheres and by day 4 had substantially covered them (Figure

3.3). On day 10 of culture, the surviving oncospheres were still heavily coated with leukocytes (Figure 3.4). In the absence of antibody, neither leukocytes from immune animals nor those from control animals rapidly adhered to the oncospheres (Figure 3.5) but after 10 days it appeared that a few leukocytes from both control and immune animals were adherent (Figure 3.6).

In the wells containing serum from immunized sheep, a precipitate around the larvae was clearly visible, but only in those wells which did not contain leukocytes (Figure 3.7). Where cells were present, the precipitate was not visible but whether it was absent or simply obscured by the leukocytes could not be determined.

3.2.5 **Degree of Larval Development.**

A difference in the size and development of the metacestodes was observed which appeared to be related to the presence of leukocytes and unrelated to the numbers of larvae surviving.

After 10 days in culture with leukocytes from either immune or non-immune sheep, all the surviving metacestodes had developed to the stage of becoming elongated. Even in those cultures where fewest survived, i.e. in the presence of immune serum, complement and leukocytes, those that did, were large and elongate. The average sizes of the elongate metacestodes ranged from approximately 135 x 45 μm to 200 x 135 μm.

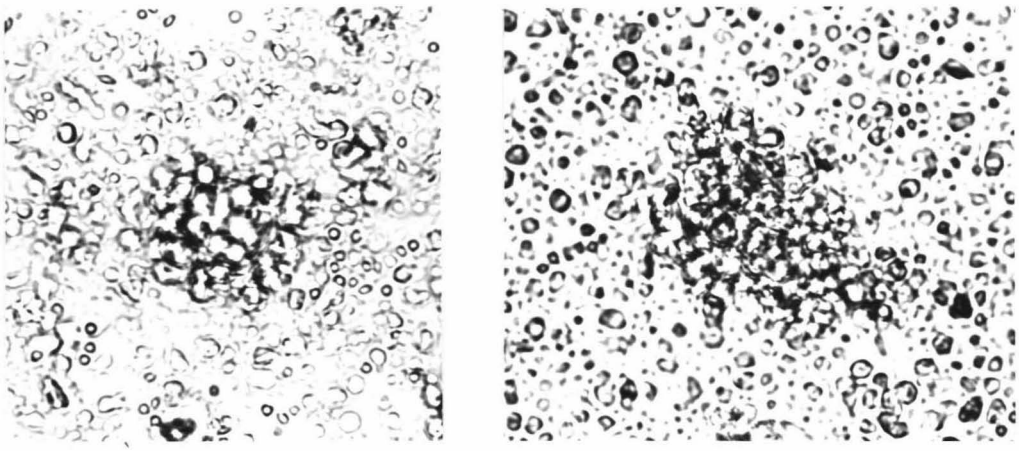
In the wells containing no leukocytes, but with immune serum and complement present, all of the surviving larvae remained spherical and approximately 45 μm in diameter. In all the other wells lacking leukocytes, approximately half of the surviving larvae became elongated and were of similar size to those described above, while the other half remained spherical (Figure 3.8).

3.4 DISCUSSION.

This chapter examines the interaction of antibody with complement and leukocytes in the killing of oncospheres *in vitro*. While the results, discussed below, add further to our understanding of the operation of host resistance against oncospheres, there are some technical matters which first require comment, in particular relating to the difficulty of distinguishing live from dead or moribund oncospheres during

Figure 3.3 **Larvae cultured in the presence of immune serum and leukocytes from (a) control sheep or (b) immune sheep; Day 4.**
X 350 approx.

(a)



(b)

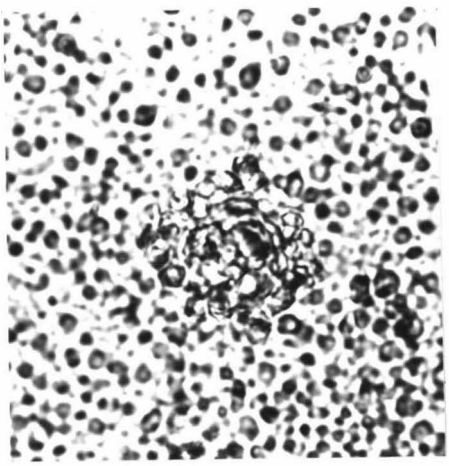
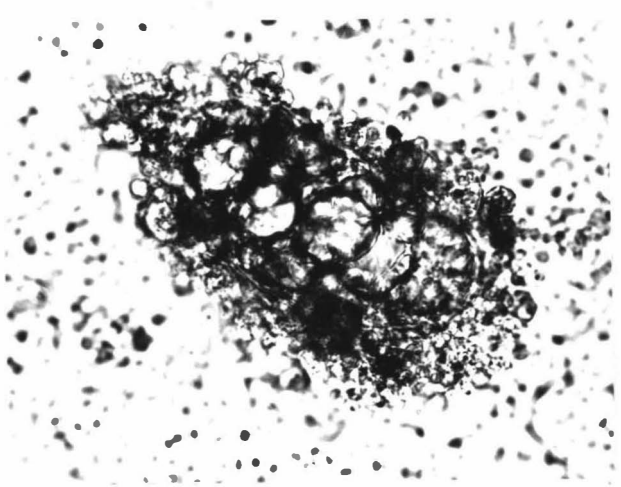


Figure 3.4 **Larvae cultured in the presence of immune serum and leukocytes from (a) control sheep or (b) immune sheep; Day 10.**

X 250 approx.

(a)



(b)

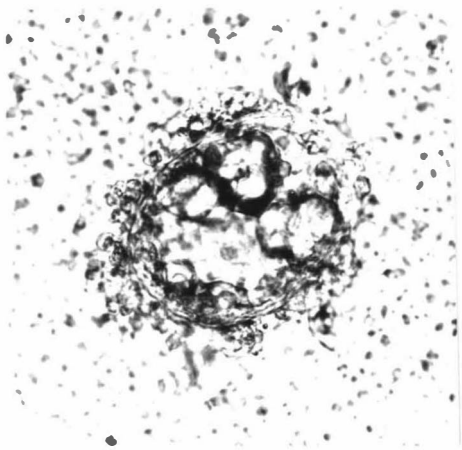
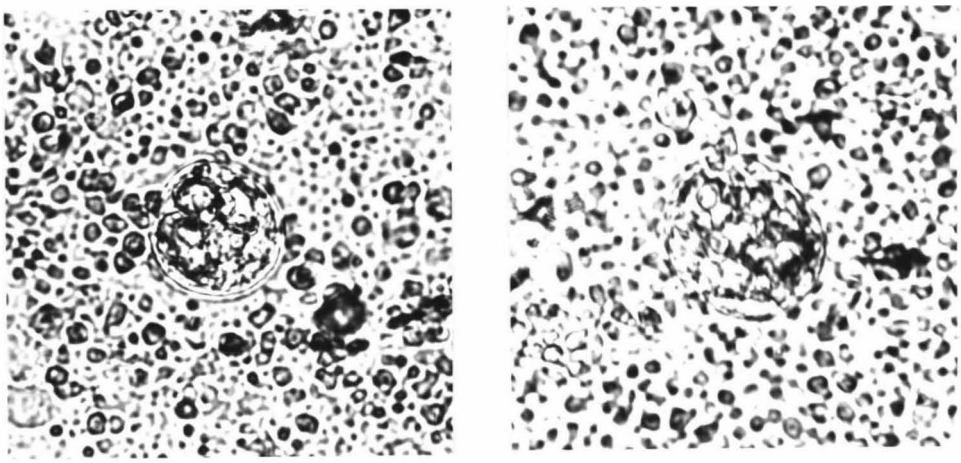


Figure 3.5 **Larvae cultured in the absence of antibody and in the presence of leukocytes from (a) control sheep or (b) immune sheep; Day 4.**
X 550 approx.

(a)



(b)

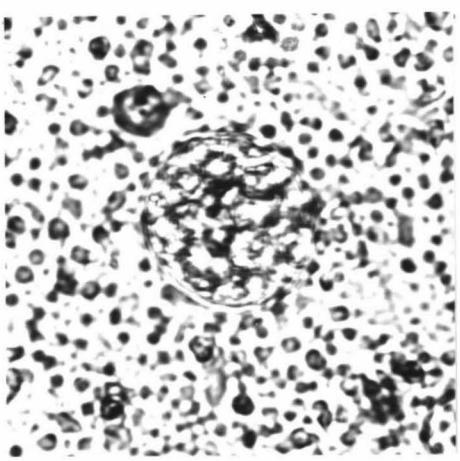
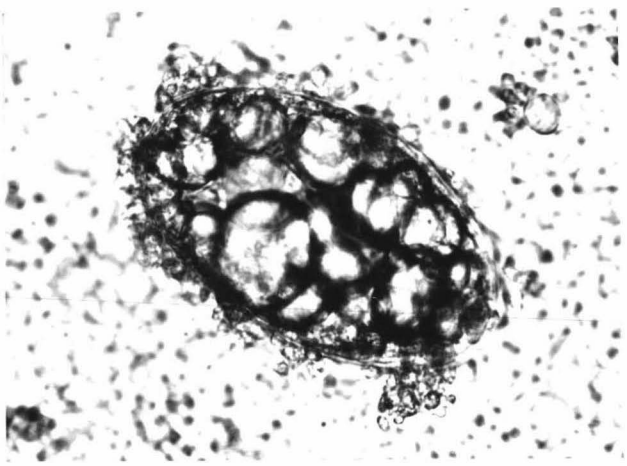


Figure 3.6 **Larvae cultured in the absence of antibody and in the presence of leukocytes from (a) control sheep or (b) immune sheep; Day 10.**

X 300 approx.

(a)



(b)

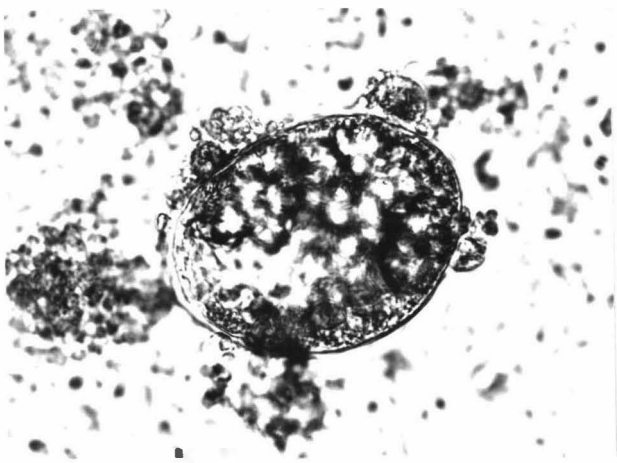


Figure 3.7 **Larvae cultured in the presence of immune serum and in the absence of leukocytes; Day 4.**

x 500 approx.

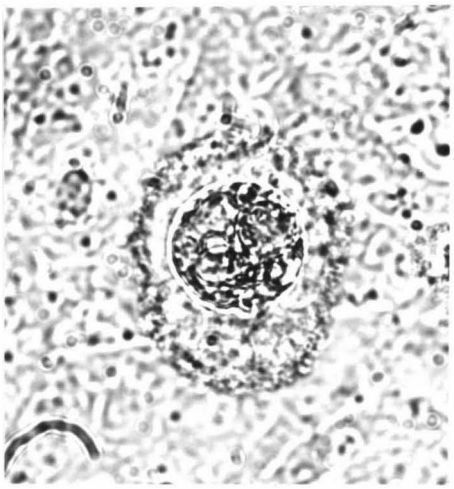
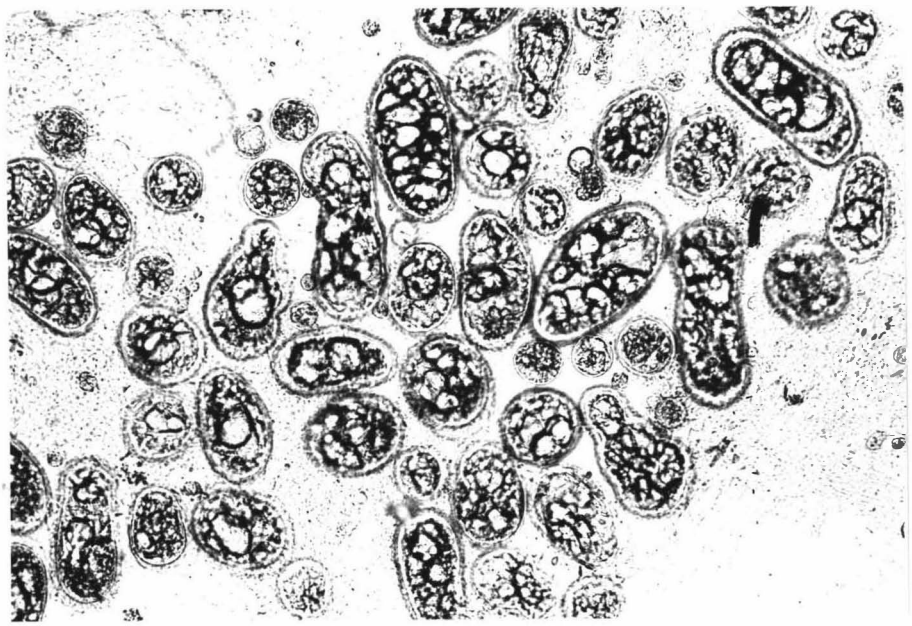


Figure 3.8 **Larvae cultured in the presence of immune serum and in the absence of leukocytes; Day 10.**

x 125 approx.



the culture process. The difficulty arose from the fact that in cultures which contained leukocytes and immune serum, the oncospheres became totally obscured by adherent cells making it impossible to see the oncospheres themselves. Extensive investigations were carried out in an attempt to develop a procedure which would distinguish between live and dead oncospheres after only 24 hr in culture and allow quantification of their death in culture at this time. The suitability of dimethyl-thiazolyl-diphenyl-tetrazolium bromide (MTT) and fluorescein diacetate (FDA) and the release of ^{51}Chromium were examined as indicators of oncosphere death in the following ways:

1. The conversion of MTT (yellow) to formazan (blue) by metabolising tissues or organisms can be quantified by measurement of the absorbance of the colour reaction (Mosmann, 1983; Green *et al.*, 1984; Denizot and Lang, 1986). However, even with considerably larger numbers of oncospheres than used in the culture experiments, colour changes were minimal and inconsistent, and unrelated to the numbers of activated oncospheres present.

2. The metabolism of cells or organisms converts FDA to fluorescein, the fluorescence of which can be measured (Rotman & Papermaster, 1966). This technique provides ready identification of individual, metabolising oncospheres but attempts to quantify the fluorescence in cultures using a fluorimeter were unsuccessful.

3. Measurement of the release of ^{51}Cr into the supernatants has been used to quantify the cytotoxicity of lymphocytes towards target cells (Brunner *et al.*, 1968, 1970; Neville, 1987; Ljunggren *et al.*, 1987), the death of, or damage to, schistosomules *in vitro* (Butterworth *et al.*, 1974, 1976, 1977; Attallah *et al.*, 1987) and *Mesocestoides corti* killing *in vitro* (Cook *et al.*, 1988). While labelling the *T.hydatigena* oncospheres with ^{51}Cr was successfully achieved, the radioisotope was not released into the supernatant in significant quantities when the oncospheres were damaged or killed by incubation in distilled water or in immune serum and complement, or by being frozen and thawed. Boiling the labelled oncospheres in SDS sample buffer for 5 min was the only treatment which resulted in a significant release (40%) of the total ^{51}Cr contained in the oncospheres before treatment.

Since none of these techniques worked satisfactorily, it was only possible to assess oncosphere survival at the end of the 10 day culture period when the larvae surviving had developed to the extent where they were clearly visible.

The presence of antibody together with complement greatly reduced the survival of the larvae ($p < 0.05$), and the additional presence of leukocytes, from either immune or control sheep, resulted in a further significant reduction. These results again indicate the importance of antibody in the expression of resistance to *T.hydatigena.* They also show that the effect is mediated by complement and via the classical pathway since neither immune serum in the absence of complement or complement in the absence of immune serum adversely affected larval survival. Such a marked effect of antibody appears inconsistent with the findings of Beardsell and Howell (1984) who found that, in leukocyte-free cultures, antibody had no adverse effect on larval survival. Given the evident dependence of antibody-mediated oncosphere death in the absence of leukocytes, on complement, the noted inconsistency could be accounted for by a difference in the amount of complement available in the two situations

In the results described in this chapter, the presence of heat-inactivated immune serum, in the absence of leukocytes, appeared to substantially increase the survival of the larvae over that achieved with control serum. The question as to how this might occur must be considered. One possibility is that it is a consequence of culturing the larvae in a restricted environment. The secretions of the penetration glands of activated oncospheres are antigenic and it has been suggested that they also include enzymes involved in host tissue lysis (Silverman & Maneely, 1955; Harris *et al.*, 1987; Heath, 1971). It is also known that antibody binds both to penetration gland secretions and the surface of the oncosphere (Harris *et al.*, 1987)(Figure 3.7). Enzymes, or some other factors present in the secretions, might be damaging to the developing oncosphere, but the effect of these would be avoided by migration of the developing larva or by inactivation or dilution of the enzymes *in vivo.* In culture such avoidance is not possible. If, however, these damaging factors are also antigenic, they could be rendered ineffective in the presence of antibody by becoming bound to it and thus being unable to attach to their target substrate(s). Thus in the absence of complement or leukocytes, antibody, in the confines of a culture well might, in fact, enhance oncosphere survival.

The significant overall effect of leukocytes indicated by the analysis of variance (Appendix 3.2) is substantially influenced by the enhanced survival of oncospheres in immune serum in the absence of leukocytes or complement and, at the other extreme, by the greatly reduced survival of larvae in the presence of either type of leukocyte together with immune serum and complement. If the two groups of cultures which include these two extremes (IS-C' and IS+C') are examined (Figure 3.1), it can be seen that there was a significant reduction in larval survival in the

presence of leukocytes whether complement is present or not. It also appears that the effect of leukocytes is superimposed on the combined effect of immune serum and complement.

However, the nature of the interrelationship between leukocytes and immune serum is not clear. In the absence of complement, the effect of leukocytes from immune sheep did not differ significantly between cultures containing control and immune serum, whereas leukocytes from control sheep had a significantly more deleterious effect than those from control animals when immune serum was present. If there were to be such a difference in the effects of leukocytes from the two sources, one might have expected leukocytes from immune animals to be the more deleterious since Beardsell & Howell (1984) have reported increased numbers of Fc-receptors on neutrophils from sheep immunized with *T.hydatigena*. Of course, the results could be anomalous and due to experimental error. If not, there is no apparent explanation for them.

The effect of the presence of antibody on the adherence of leukocytes to the surfaces of developing larvae is consistent with experiments on schistosomules (Capron, 1975; Butterworth *et al.*, 1977; David *et al.*, 1980). This adherence could result in epithelial damage and oncosphere destruction due to the release of oxygen free radicals from phagocytes, mast cell degranulation and damage due to eosinophils (see Section 1.3.2.6). The observation that larval survival is reduced in the presence of leukocytes, providing antibody is also present, is consistent with this and supports the findings of Beardsell and Howell (1984).

Another interesting effect of the presence of leukocytes was the relatively advanced development of the surviving larvae compared with those cultured in the absence of leukocytes. It is known that the presence of Vero cells improves the development of oncospheres in culture (Heath, personal communication), and perhaps sheep leukocytes also provide factors favouring oncospheral growth which are not supplied by Vero cells in sufficient quantities. It could be deduced that the factors which resulted in the reduction of the survival of the larvae were not related to the factors affecting the larval development. Also the healthy, well-developed nature of the few surviving larvae in the cultures containing immune serum, complement and leukocytes, suggests that they may only be active against oncospheres in the early stages of development. However, to confirm this would require some means of clearly identifying damage to, or death of, oncospheres or larvae at all stages of development.

The results thus indicate a possible role for leukocytes *in vitro* but how important this is *in vivo* is not clear. It would appear that their effect is secondary to that of antibody and complement and that leukocytes from an actively immunized animal are no more effective than those from naive animals. The failure to obtain complete protection in the passive transfer experiment cannot, therefore, be attributed to a need for leukocytes from an immune animal and was probably due to insufficient quantities of antibody being transferred.

CHAPTER 4.

THE TRANSFER OF IMMUNITY AGAINST *T.HYDATIGENA* FROM IMMUNE EWES TO LAMBS VIA COLOSTRUM.

4.1 INTRODUCTION.

In the preceding chapters, it has been shown that serum from sheep which are immune to *T.hydatigena* is effective in preventing development of the parasite, both *in vitro* and *in vivo*. In the case of *T.ovis*, protection of lambs against infection also occurs through the passive transfer of immunity from their ewes via colostrum (Rickard and Arundel, 1974; Rickard *et al.*, 1977b; Heath *et al.*, 1979c; Sutton, 1979). Previous experiments involving the transfer of protection from ewes naturally infected with *T.hydatigena*, to lambs via colostrum (Gemmell *et al.*, 1969; Heath, 1978) reported that low levels of immunity were transferred. Gemmell and Heath both suggested a possible correlation between the level of immunity of the ewe and the protection gained by the lamb. This chapter investigates the protection gained by lambs born to 2 groups of ewes given different levels of oral infection with *T.hydatigena*, against an homologous oral challenge.

4.2 MATERIALS AND METHODS.

4.2.1 **Experimental Animals.**

Thirty mated, uninfected Romney ewes were taken from pasture and housed indoors in pens 3 weeks before the first lambs were due to be born.

Commercial sheep nuts, lucerne pellets and hay were fed *ad libitum* to both ewes and lambs. Throughout the whole experiment the lambs remained with their dams and were allowed to suckle freely. A further 9 lambs from uninfected ewes were reared naturally on pastures free from taeniid eggs as controls for some of the groups of lambs suckling infected ewes (see Section 4.2.3).

4.2.2 **Pre-challenge Procedure for Ewes and Lambs.**

The 30 housed ewes were randomly allocated into 3 groups, 2 of which were to be orally infected with *T.hydatigena* eggs. Not more than 24 hr before each administration of eggs to the ewes, the percentage of oncospheres that could be

activated *in vitro* (Section 2.2.1.4) was estimated and the ewes were infected according to the schedule in Table 4.1. The dilution necessary to provide 200 activatable oncospheres/ml was made for the first infection of the High Infection ewes and for all infections for the Low Infection ewes, and dilutions of 5000 activatable oncospheres/ml were prepared for the second and third infections for the High Infection ewes.

Table 4.1. **The infection of housed ewes with *T.hydatigena* eggs.** The figure given is the estimated number of activatable oncospheres.

			Week		
Group	Ewes	n	0	2	3
1	Controls	5	0	0	0
2	Low Infection	5	150	150	150
3	High Infection	20	100	10 000	10 000

The different groups of ewes were penned separately to prevent cross-suckling by lambs.

Starting on day 0, blood samples were taken weekly from all ewes to follow the development of serum antibody levels to *T.hydatigena* by an ELISA.

One ewe from the Low Infection group (Group 2) died before parturition on day 23, apparently from liver damage associated with the infection. A ewe from the High Infection group (Group 3) was removed from the experiment before it lambed because of recurring uterine prolapse. Three other ewes from this group produced no lambs and so were also excluded from the experiment. A further 3 ewes in Group 3 lambed before the infection schedule had been completed. These ewes and their lambs were retained in the experiment, the latter to act as sentinels for the presence of free *T.hydatigena* eggs in the environment. Only lambs born at least 3 days after completion of the immunization schedule of the ewes were subsequently challenged with eggs. The animals to be challenged comprised:

Group 1: 5 housed ewes and their 6 lambs (controls),
Group 2: 4 housed ewes and their 5 lambs (Low Infection),
Group 3: 13 housed ewes and their 14 lambs (High Infection); (one lamb died before necropsy from white muscle disease) and
Group 4: 9 ewes at pasture and their 9 lambs (pasture controls).

Twenty ml samples of colostrum or milk, collected by hand-milking, and 15 ml blood samples, were collected from the housed ewes at weekly intervals, starting within 24 hr of parturition and continuing until necropsy 4 weeks after challenge. The housed lambs were bled at weekly intervals from 1 week after birth until necropsy 4 weeks after challenge, in order to measure anti-*T.hydatigena* antibody levels. Ewes at pasture were not experimentally infected prior to parturition and a blood sample was taken from each before challenge to provide serum for testing by Western blot for evidence of previous infection. Serum samples were not collected from the pasture lambs since these lambs were included only as controls for assessing the viability of eggs.

4.2.3 **Challenge of Lambs and Ewes.**

Groups of penned lambs were challenged with a single dose of 50 activatable eggs at either 1, 3, or 5 weeks of age (Table 4.2). The lambs of Group 3 were divided into 3 subgroups in order of birth (i.e. the first lamb to be born was allocated to Group 3a, the second to Group 3b, the third to Group 3c, the fourth to Group 3a and so on.), and challenged at different times after birth to determine the duration of any passively aquired immunity. The lambs reared on pasture were selected so that their birth dates corresponded with those of the lambs in Groups 3b and 3c and were challenged at the same times in order to provide controls for the viability of the challenge eggs. As noted above, each lamb was necropsied 4 weeks after challenge and the numbers of live and dead cysts and the nature and extent of liver lesions recorded.

The ewe corresponding to each lamb was challenged with 500 activatable eggs on the same day as its lamb. In the one case where a ewe had twins which were to be challenged at different times, the ewe was challenged when the second lamb was challenged. This was to avoid any effect on the susceptibility of the second lamb to the challenge infection, due to a possible increase in milk antibody level.

Table 4.2 **Days after birth when lambs were challenged with 50 activatable oncospheres.**

Group	Designation	Number of lambs	Time of Challenge (weeks after birth)
1	controls	6	1
2	Low infection	5	1
3a	High infection	5	1
3b	High infection	4	3
	Pasture controls	5	3
3c	High infection	4	5
	Pasture controls	4	5

Four weeks after challenge, each ewe was slaughtered and examined for newly-developing cysts to determine the effectiveness of the infection schedule in inducing immunity in the ewes. Cysts resulting from the challenge infection were distinguishable from the more mature cysts by size. In addition, cyst maturity could be determined by examining the scoleces of the cysts by light microscopy and measuring the length of the larger hooks present (see Section 2.3).

All challenge doses were made up from the same batch of eggs which had been stored at 4°C in saline with antibiotics and Tween 20 (Section 2.2.1.2). The final concentrations used were 200 and 2000 activatable oncospheres/ml for lambs and ewes, respectively.

The length of time from the first challenge infection to the last was 46 days due to the spread of the dates of birth of the lambs. During this time the percentage activation of the oncospheres was calculated on 2 occasions by hatching and activating samples of these eggs (Section 2.2.1.4).

4.2.4 Detection of Immunoglobulin in Lamb Sera Using the Sodium Sulphite Turbidity Test (SSTT).

Sera were prepared from the blood samples as described in Section 2.2.1.10 and stored at -20°C.

Samples of the sera from the lambs at 1 week post-partum were tested for the presence of immunoglobulins by the sodium sulphite turbidity test (Stone and Gitter, 1969). Briefly, 100 µl samples of test serum were added to 1.9 ml aliquots of 36% (w/v) $Na_2SO_3.7H_2O$. The tubes were inverted 3 times and the precipitates which resulted were graded according to differences in the translucency of the suspension, 0 indicating minimum precipitate and therefore no immunoglobulin present, to +4 indicating maximum precipitate and high levels of immunoglobulin present. The samples allocated to each grade were compared and cross-compared with the other groups to ensure uniformity in the grades.

4.2.5 Detection of Anti-*T.hydatigena* Antibody in Lamb Sera by Western Blot.

Western blots of SDS-PAGE-fractionated oncosphere antigens and ELISAs using FTS antigen were used to detect anti-*T.hydatigena* antibody in the lamb sera. One batch of SDS oncosphere antigen was prepared for the SDS-PAGE; the separation methods used are described in Section 2.2.1.7.

4.2.6 Detection of Anti-*T.hydatigena* Antibody in Lamb Sera by ELISA.

FTS antigen was prepared as described in Section 2.2.1.8 with one batch prepared for all tests. The method used is also described in Section 2.2.1.8.

4.2.7 Detection of Anti-*T.hydatigena* Antibody in Ewe Sera and Whey.

The sera were collected as described in Section 2.2.1.10. The colostrum and milk samples were each diluted with 4 volumes of saline and the whey separated as described by Fu (1988). Briefly, the samples were adjusted to pH 4.6 with 0.1 M HCl and centrifuged at 3000 x g for 10 min. The lipid layer was removed and the whey collected. The pH of the whey was adjusted to its original value (approximately pH 7.3) with NaOH and aliquots of 5 ml were stored at -20°C until use.

4.2.8 Statistical Analysis.

Group means and standard errors were calculated for the numbers of cysts found in the lambs, the ELISA absorbances of lamb sera at one week after birth and at the time of challenge and of ewe sera and ewe whey on the day of parturition. The significance of differences in cyst numbers between groups was examined using

analysis of variance following square-root transformation of the data; the Tukey pairwise comparison procedure was used to indicate significant differences between groups at the 5% level unless otherwise stated (Appendices 4.2, 4.3 and 4.4). The statistical relationships between the antibody levels in the lambs' sera and that of their respective dams were examined by regression analysis. The relationship between cyst numbers in the lambs and the absorbance levels of sera samples taken on the day of challenge were also examined.

4.3 RESULTS.

4.3.1 Analysis of Ewe Serum Samples.

There was no serological evidence of infection in any of the housed ewes before those in Groups 2 and 3 were infected. The Western blots shown in Figure 4.1 show that the sera from both the Low and High Infection groups (Groups 2 and 3) reacted with various components of the *T.hydatigena* oncosphere antigen 3 weeks after the infection schedule began, whereas the sera from the control animals cross-reacted with only 3 bands, a result which is often given by control serum preparations. A Western blot probed with the sera from the ewes in Group 1 and those at pasture also showed that they were uninfected at the time of challenge.

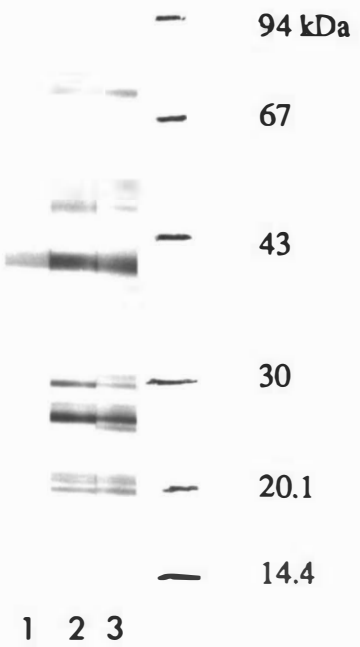
4.3.2 Immunoglobulin Levels in Lamb Sera Detected by the Sodium Sulphite Turbidity Test (SSTT).

The SSTT grades of the lamb sera collected 1 week after birth are shown in Appendix 4.1. A grade of +1 was recorded for the serum from lamb 3008 indicating that this lamb absorbed minimal amounts of antibody from its dam's colostrum. For this reason the data concerning cyst numbers for this lamb were not included in the statistical analysis (see Table 4.3).

4.3.3 Viability of the Challenge Eggs.

There was no decline from the initial 40% *in vitro* activation of eggs from samples of the stock suspension over the 46 days during which the challenge infections were administered. The consistency of the viability of the challenge doses was confirmed by the numbers of cysts found in the peritoneal cavities of the control lambs challenged over this period (Table 4.3); these numbers were not significantly different (Appendices 4.2b, 4.3b and 4.4b and c).

Figure 4.1 **Western blot of *T.hydatigena* oncosphere antigen probed with the pooled sera collected from the ewes 21 days after the first infection.**



Lane	Serum samples
1	Control ewes
2	Low Infection ewes
3	High Infection ewes

Table 4.3. Total cysts in lambs.

(Mean ± S.E.)

Group	Treatment of Ewes	Time of Challenge (after birth) 1 week	3 weeks	5 weeks
1	Controls	22, 16, 14, 17, 14, 15 (16.3 ± 1.23)	7, 13, 15, 13, 13 (12.2 ± 1.36)	15, 0, 18, 39 (18 ± 8.03)
2	Low Infection	2, 0, 0, 3, 0 (1 ± 0.63)		
3	High Infection	25*, 0, 0, 0, 14 (3.5 ± 3.5)	9, 6, 8, 0 (5.75 ± 2.02)	23, 4, 6, 13 (11.5 ± 4.3)

* The serum sample from this lamb (3008) collected at 7 days after birth gave a sodium sulphite turbidity test grade of +1 (Table 1), indicating that it had failed to absorb a significant quantity of colostral immunoglobulin. The data from this lamb were excluded from statistics when comparing the mean of Group 3a with other groups, but when correlating the level of antibody present in each lamb's serum sample with the numbers of cysts present at necropsy, the data were included.

4.3.4 Cyst Numbers Present in Ewes and Lambs at Necropsy.

The necropsy procedure has been described earlier (Section 2.2.1.12). All ewes of Groups 2 and 3 (Low and High Infections) were found to be immune to the challenge infection, containing only mature cysts, while all control ewes showed evidence of the challenge infection only. The variability of the numbers of cysts in the control ewes was greater than expected (Appendix 4.1). The sentinel lambs were found to be free of *T.hydatigena* infection indicating that there were no eggs contaminating the pens in which the animals were housed.

Table 4.3 shows the total numbers of live and dead cysts found in the peritoneal cavities of the lambs. Liver lesions were excluded from the counts because it was not possible to determine whether they represented larvae that had already emerged from the lesions or which were degenerating within the lesion.

The results illustrated in Figure 4.2 indicate that immunoglobulins transferred from either group of immune ewes to their lambs fully protected 6 out of 9 lambs against a challenge infection in their first week of life. Two of the other lambs had far fewer cysts than did the controls. Tukey's pairwise comparisons of the numbers of cysts in the 2 groups (i.e. Groups 2 and 3a) showed that both were significantly different from the controls. The livers of fully protected lambs showed only small and barely visible scars.

The level of protection attained by the lambs which were challenged in the third week after birth (Group 3b) was lower than that of the lambs in Groups 2 and 3a. After challenge, only one lamb in Group 3b had no cysts in the peritoneal cavity and the total numbers of cysts for Group 3b were not significantly different from those of the controls (Figure 4.3, Appendices 4.4b and 4.4c). The liver of the fully protected lamb in this group contained only a single, large fibrotic track while the other livers had lesions and scars similar to those on the livers of the controls.

Cyst numbers present in the lambs challenged at week 5 were not significantly different from those of any of the control groups; the lesions and scarring of the livers were also similar.

Figure 4.2 **The numbers of cysts found in the lambs challenged 1 week after birth.** (Mean ± S.E.)

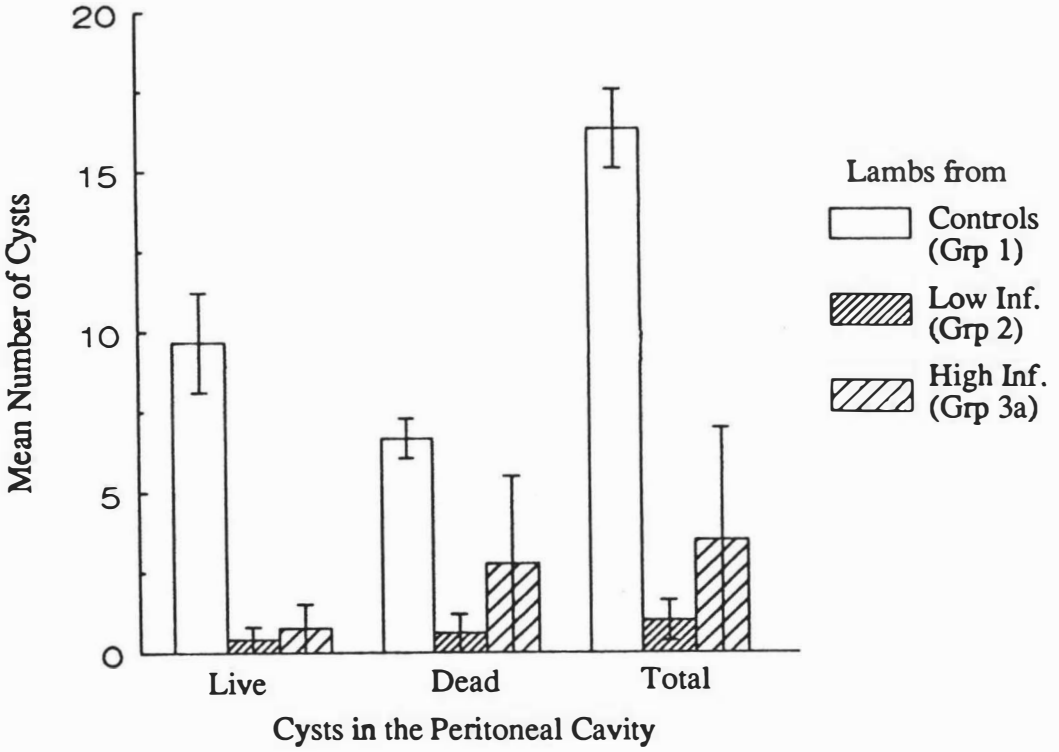
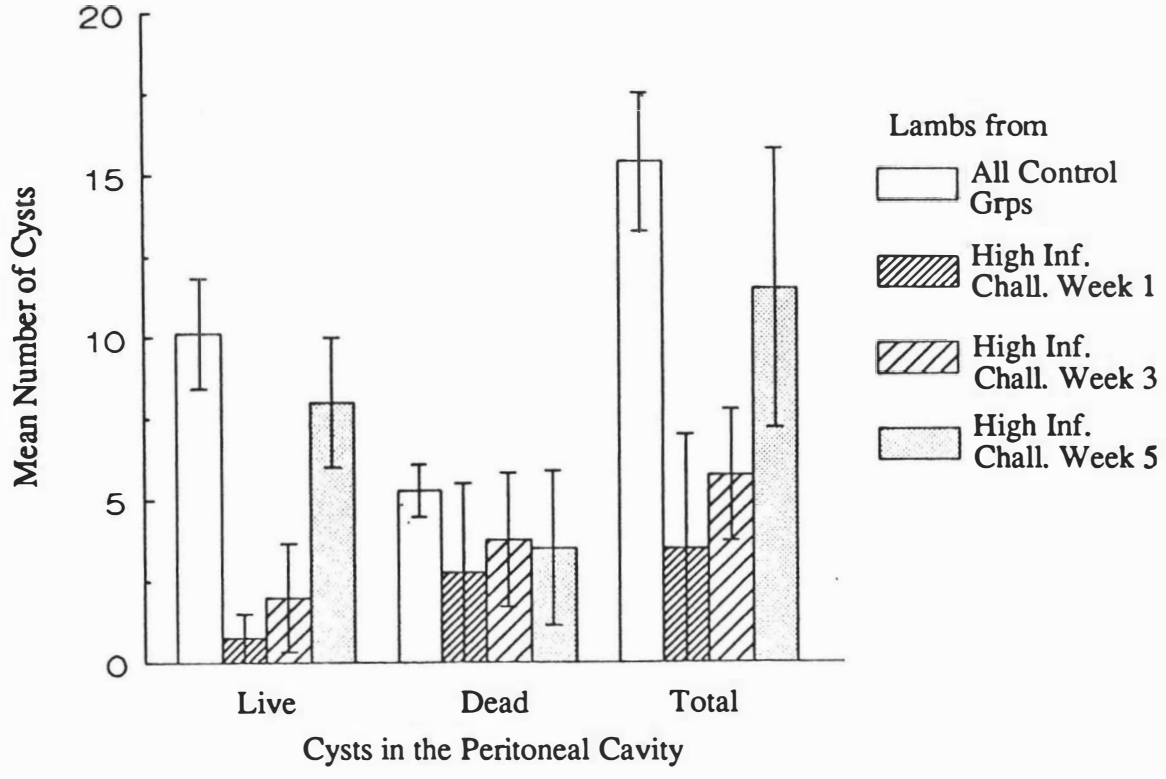


Figure 4.3 **The numbers of cysts in the lambs from High Infection ewes.** (Mean ± S.E.)



Due to the variability in the cyst numbers the differences between the total numbers of cysts found in the lambs from all of the groups of infected ewes (Groups 2, 3a, 3b and 3c) failed to reach statistical significance at the 5% level (Appendix 4.4b). However, the total numbers of cysts in lambs from High Infection ewes challenged at week 5 (Group 3c) were significantly greater than those in lambs from Low Infection ewes (Group 2) at a 6% level of probability but not from the other groups (Appendix 4.4c). Furthermore, the numbers of live cysts in the lambs born of infected ewes and challenged at week 1 (Groups 2 and 3a), were significantly lower than in lambs challenged at week 5 (Group 3c) and all lambs from control ewes ($p < 0.05$) (Appendix 4.3b).

The numbers of live, dead or total cysts in the three control groups of lambs which were challenged at different times after birth, did not differ significantly.

4.3.5 Analysis of Serum and Whey Samples by Western Blot.

The sera collected one week after birth from all lambs born to ewes which had been infected before parturition, reacted with a variety of *T.hydatigena* oncosphere antigens on Western blot while those from control ewes did not (Figure 4.4). The reactions of sera from lambs in Groups 2 and 3a are shown in more detail in Figure 4.5. The weak or negative reactions of sera from lambs which were not protected, or only partially protected, can be seen. The virtual absence of anti-*T.hydatigena* antibody, as detected on Western blots, in the unprotected or only partially protected animals in these groups, gives no indication as to which bands could be eliminated as being unrelated to protection. Indeed, the reactions of sera from protected lambs were extremely variable with respect to both the intensity of reaction and the presence or absence of particular bands (Figure 4.5).

4.3.6 Analysis of Serum and Whey Samples by ELISA.

The considerably lower level of reactivity of sera from unprotected lambs compared with those from protected ones (Figure 4.5) suggested that the level of antibody might be particularly important. Sera were therefore tested for anti-*T.hydatigena* antibody using an ELISA.

Figure 4.4 **Western blot of *T.hydatigena* oncosphere antigen probed with sera collected from all the housed lambs 1 week after birth.**

Lanes	Serum samples
1 - 5	Lambs from High Infection ewes Challenged 1 week of age
6 - 9	Lambs from High Infection ewes Challenged 3 weeks of age
10 - 13	Lambs from High Infection ewes Challenged 5 weeks of age
14	Immunized sheep (Section 3.2.)
15	Control sheep (Section 3.2.)
16 - 20	Lambs from Low Infection ewes Challenged 1 week of age
21 - 26	Lambs from control ewes Challenged 1 week of age
*	Lambs in which no cysts were found in the peritoneal cavity.

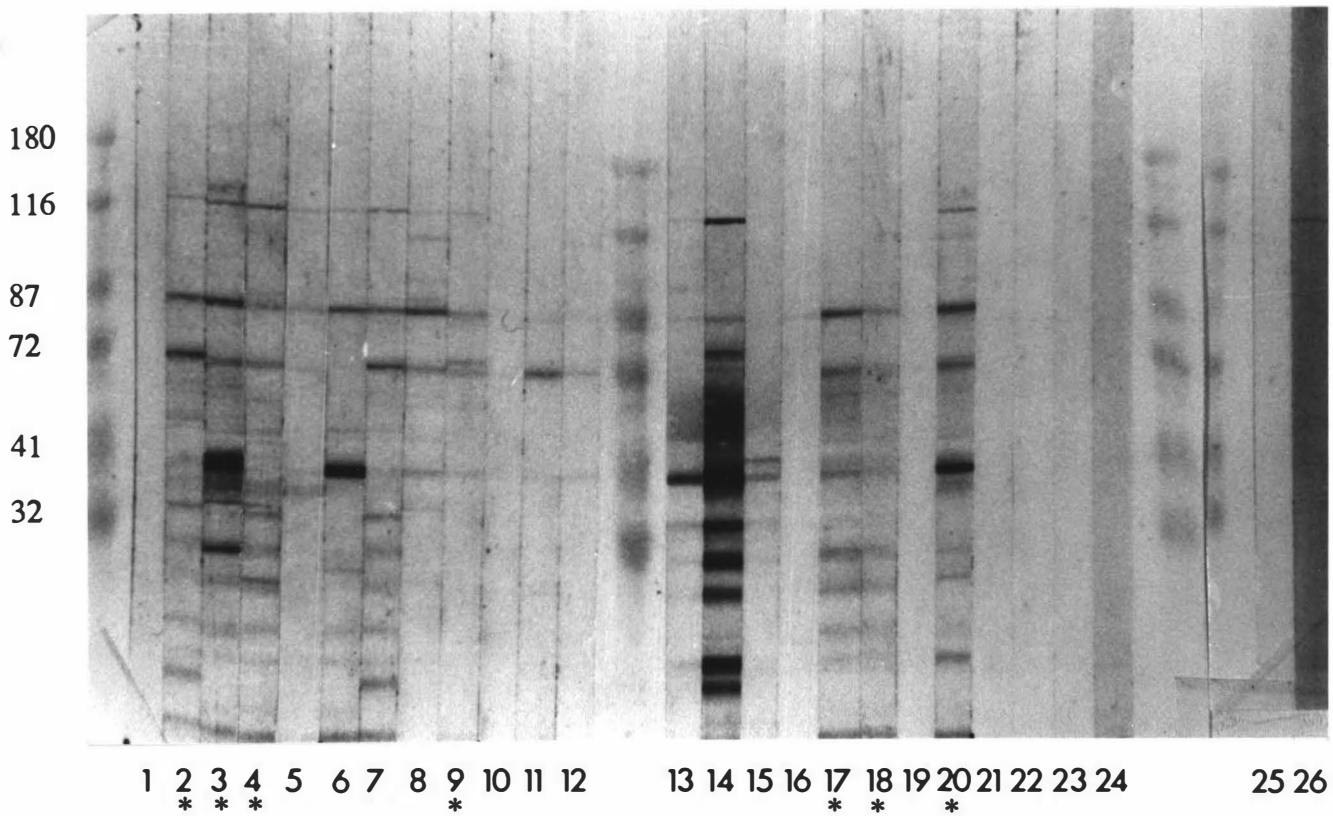
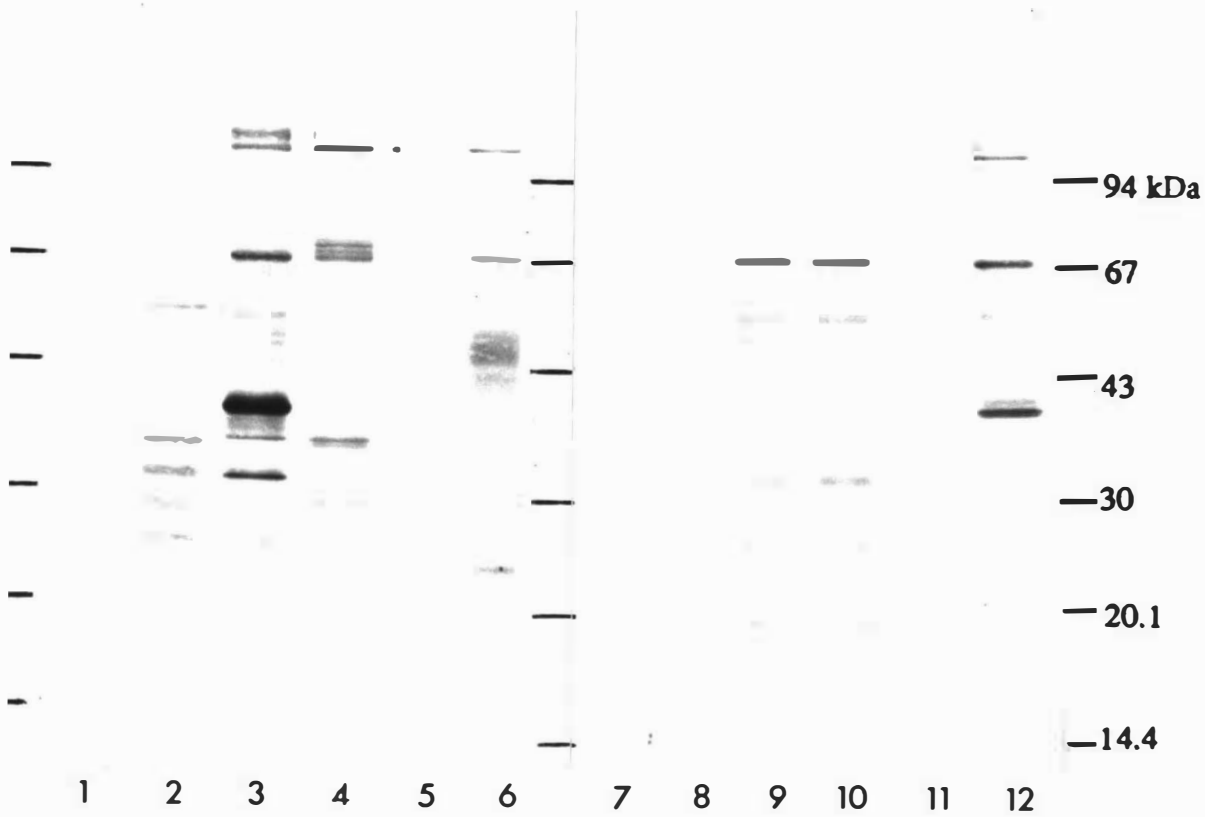

180
116
87
72
41
32
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
* * * * * * *

Figure 4.5 **Western blot of *T.hydatigena* oncosphere antigen probed with sera collected from the lambs in Groups 2 and 3a, 1 week after birth.**



Lanes	Serum samples
1 - 5	Lambs from High Infection ewes Challenged at 1 week of age
6	Immunized sheep (Section 3.2.)
7	Control sheep (Section 3.2.)
8 - 12	Lambs from Low In fection ewes Challenged at 1 week of age.

The ELISA absorbance values for the weekly samples from all lambs (other than the 3 week and 5 week controls reared outdoors) are shown in Figure 4.6. The relationship between total cyst numbers in the lambs and the ELISA absorbances of their sera at the time of challenge (Figure 4.7) shows there was a highly significant ($p < 0.001$) negative regression of cyst numbers on lamb ELISA values at the time of challenge. The data also show that, with the exception of one animal, all those which were totally protected had ELISA absorbance levels above 0.28. The one exception (Appendix 4.1, lamb 3006) had a level of 0.356 and was partially protected with only 2 cysticerci detected at post-mortem. ELISA absorbance levels in lambs from uninfected control ewes were less than 0.120 at challenge.

The ELISA absorbance levels of anti-*T.hydatigena* antibody in the ewes' sera and whey on the day of parturition have a highly significant ($p = 0.0001$) positive relationship, as indicated by the proximity of the coefficient of determination ($r^2 = 0.5324$) to unity (Figure 4.8). This is also the case for the ELISA values of the lambs sera at 1 week of age with both ewe whey and serum levels on day 0 ($p = 0.0005$, $r^2 = 0.4484$, and $p = 0.0000$, $r^2 = 0.6496$, respectively) (Figures 4.9 and 4.10).

Consistent with the highly significant relationship between lamb antibody levels at week 1 and ewe antibody levels, as shown above, there is also a significant, though weaker, negative regression of lamb cyst numbers on ewe ELISA values from whey or sera at day 0 ($p = 0.030$, $r^2 = 0.2048$ and $p = 0.0005$, $r^2 = 0.4301$, respectively) (Figures 4.11 and 4.12). Although some of the infected ewes appeared to have developed less anti-*T.hydatigena* antibody than others, and their lambs were not fully protected, all of these ewes were immune to an oral challenge infection, having no evidence of the challenge infection present at necropsy.

4.4 DISCUSSION.

The doses of eggs given to the two groups of ewes in the immunizing procedure were given at different levels in an attempt to obtain two levels of protection in the lambs. An estimated 150 activatable oncospheres were given in each of 3 doses to the Low Infection ewes. This number was given because of the failure of a single dose of 50 activatable oncospheres to induce any transferable humoral immunity as described in Chapter 2. In hindsight, it would have been interesting to include an additional group of ewes which were given even lower repeated doses of eggs which might simulate field infections more closely. Such an experiment could have

Figure 4.6 **The levels of anti-*T.hydatigena* antibody in sera collected weekly from lambs after birth.** The cross on the graph for each group indicates the point at which the absorbance is 0.28 and the time of challenge. Lambs with ELISA absorbance levels above this point at the time of challenge were resistant to infection. Those lambs which were resistant are indicated by *.

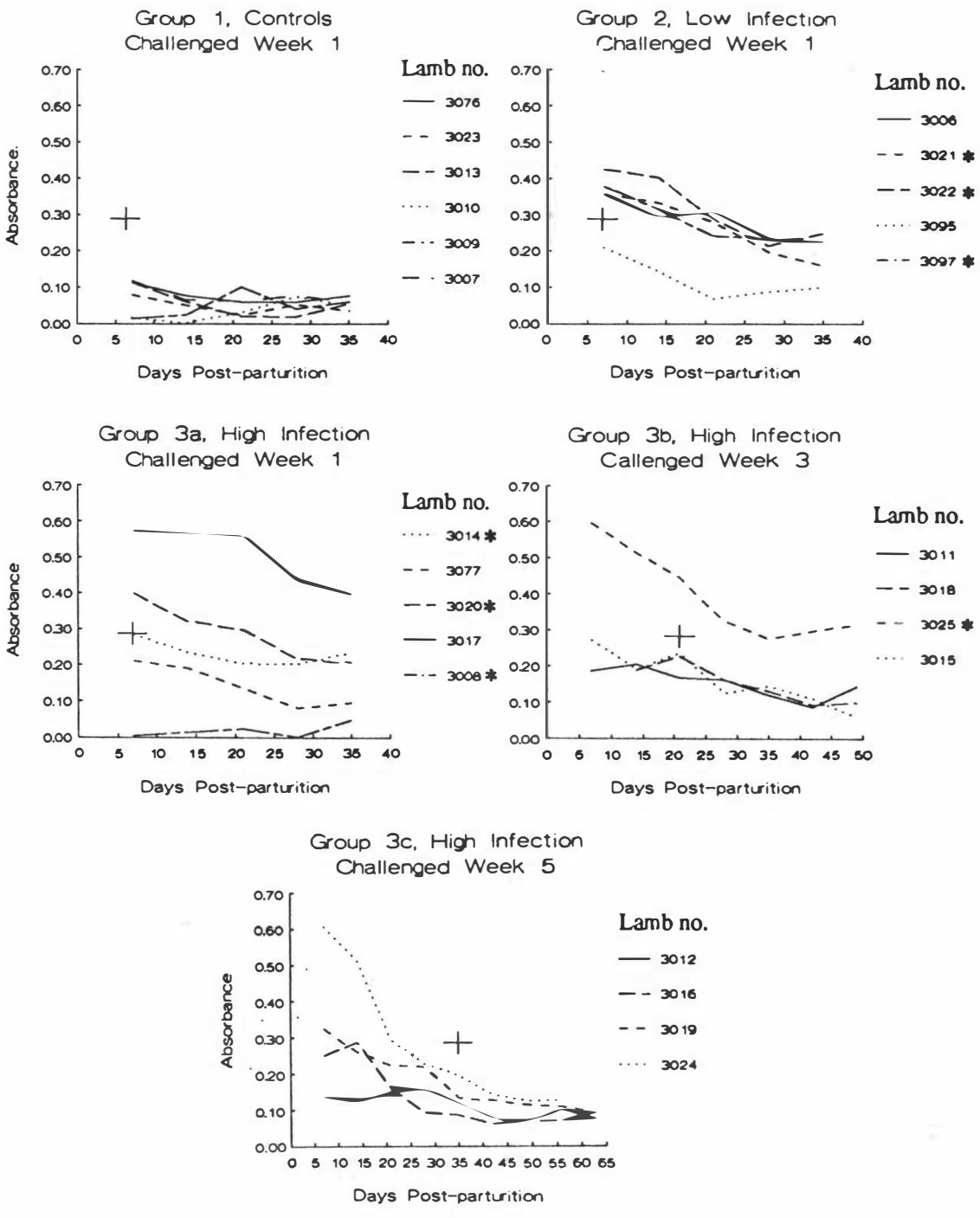


Figure 4.7 **The relationship between the level of anti-*T.hydatigena* antibody at the time of challenge and the number of cysts present at necropsy.** $r^2 = 0.6285$, $p = 0.0000$.

Number of cysts = 18.13 - 42.56 x Absorbance at the time of challenge

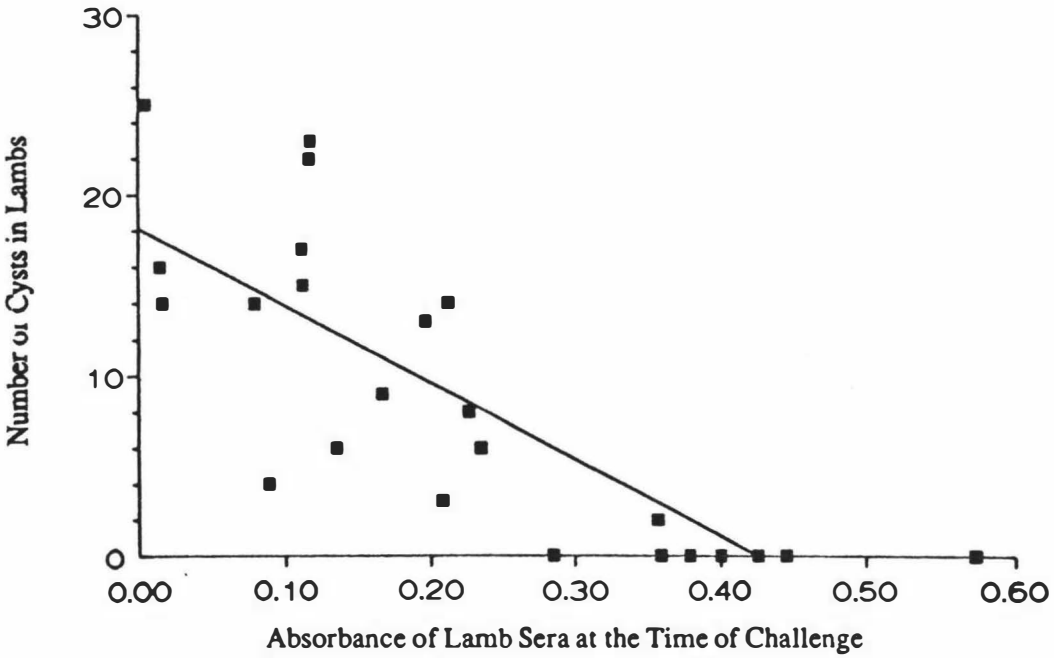


Figure 4.8 **The relationship between the level of anti-*T.hydatigena* antibody in the ewes' whey and sera on the day of parturition.** $r^2 = 0.5324$, $p = 0.0001$.

Absorbance of whey (day 0) = 0.30 + 1.78 x Absorbance of ewe sera (day 0)

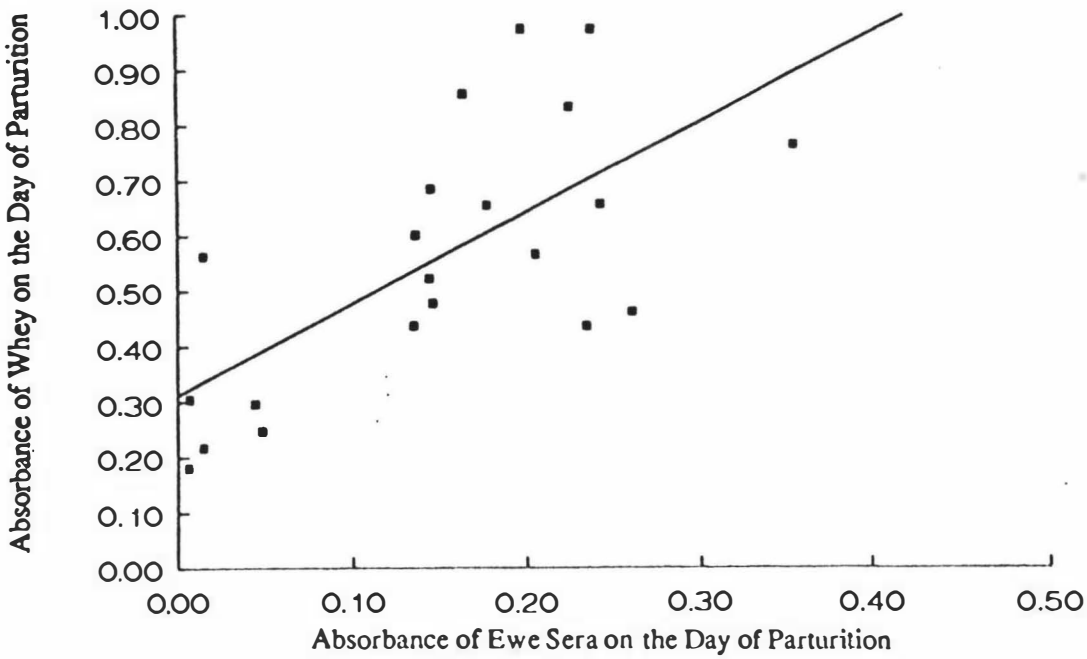


Figure 4.9 **The relationship between the level of anti-*T.hydatigena* antibody in the lamb sera 1 week after birth and in the ewe whey on the day of parturition. $r^2 = 0.4484$, $p = 0.0005$.**

Absorbance of lamb sera (week 1) = -0.025 + 0.52 x Absorbance of whey (day 0)

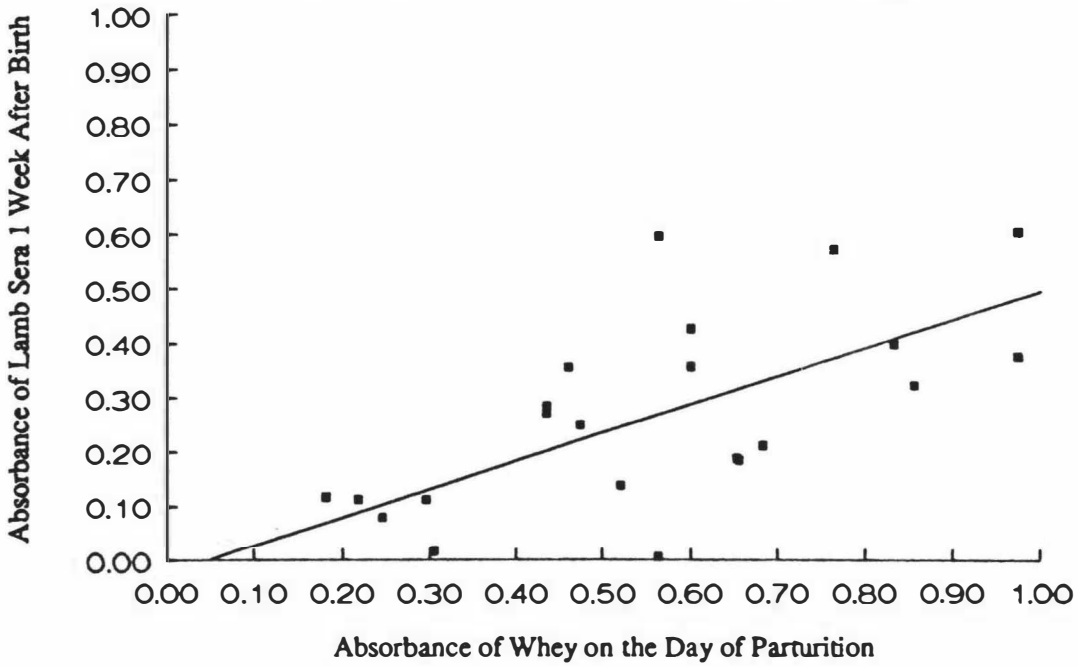


Figure 4.10 **The relationship between the level of anti-*T.hydatigena* antibody in the lamb sera 1 week after birth and in the ewe sera on the day of parturition. $r^2 = 0.6496$, $p = 0.0000$.**

Absorbance of lamb sera (week 1) = 0.046 + 1.54 x Absorbance of ewe sera (day 0)

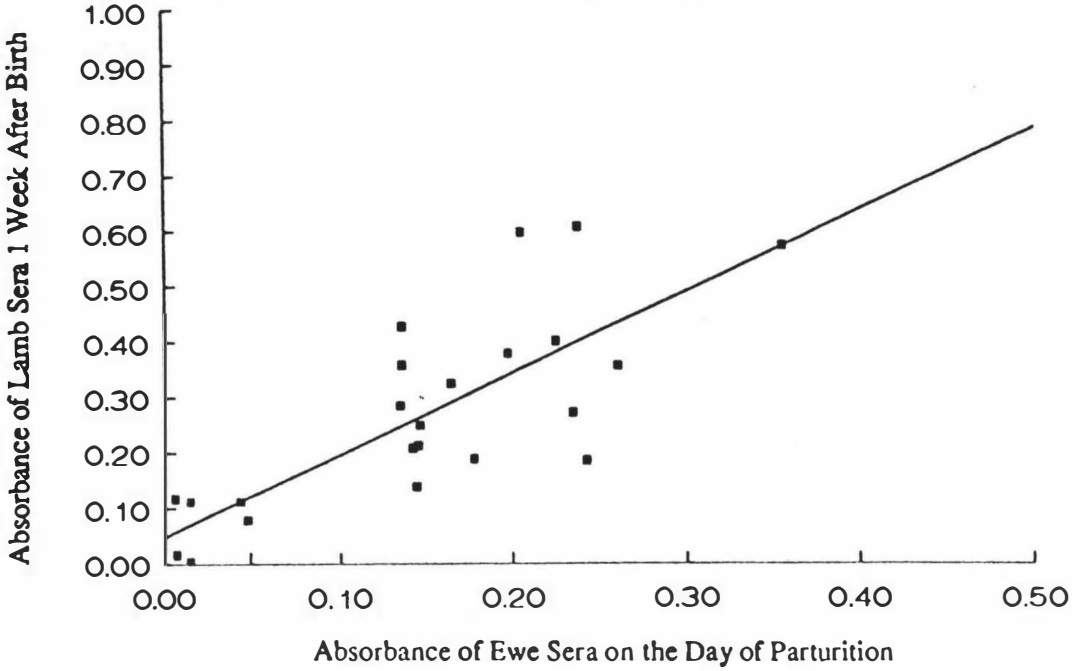


Figure 4.11 **The relationship between the number of cysts in the lambs and the level of anti-*T.hydatigena* antibody in the ewe whey on the day of parturition.** $r^2 = 0.2048$, $p = 0.0302$.

Number of cysts in lambs = 17.9 - 16.19 x Absorbance of the ewe whey (day 0)

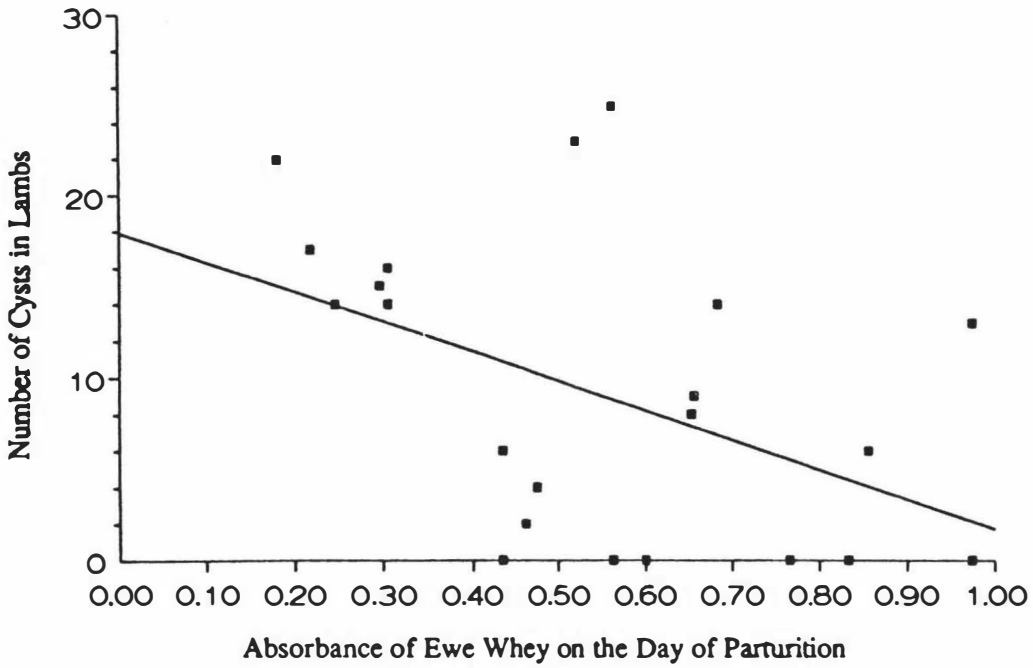
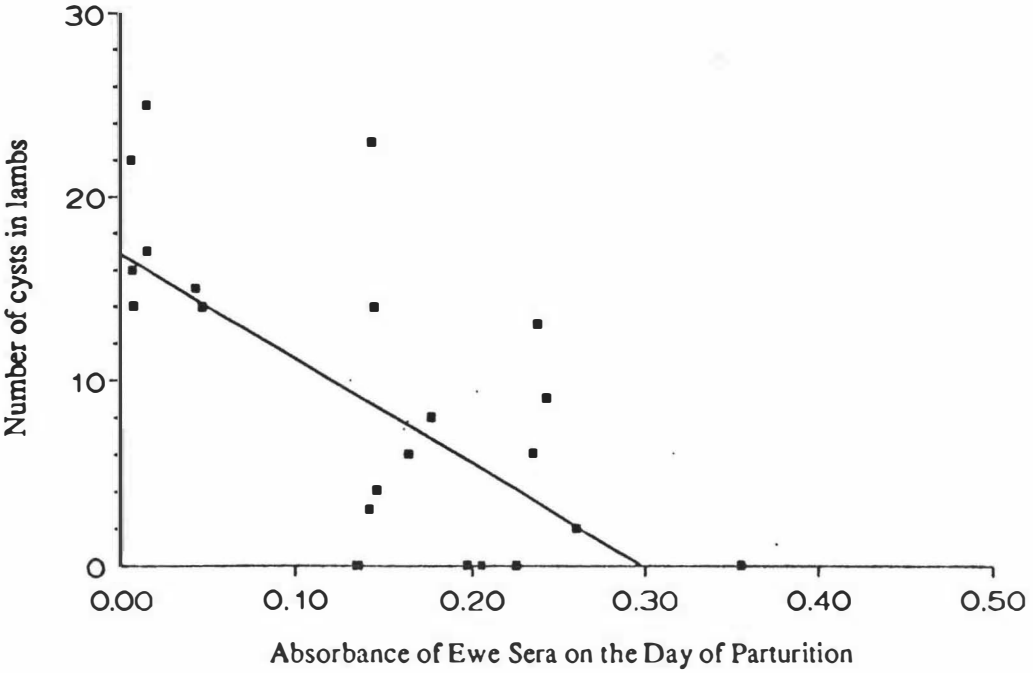


Figure 4.12 **The relationship between the number of cysts in the lambs and the level of anti-*T.hydatigena* antibody in the ewe sera on the day of parturition.** $r^2 = 0.4301$, $p = 0.0005$.

Number of cysts in lambs = 16.81 - 57.83 x Absorbance of the ewe sera (day 0)



provided more data on correlations between lower antibody titers and cyst numbers. This relationship is to be discussed later.

Fifty activatable oncospheres were used to challenge each lamb since it was thought that a larger number of larvae migrating through the liver of a 1-week-old lamb may well have proved fatal. However, damage to the livers of the control lambs was not extensive although obvious scarring and lesions were present.

The complete protection of the infected ewes from the large challenge dose of 500 activatable oncospheres showed that the immunity induced by the immunization procedure was effective. The large variation in the numbers of cysts in the control ewes raises the possibility that those harbouring lower numbers of cysts may have been infected early in their lives with the cysts having died and disintegrated. The very low ELISA absorbance values of their sera and those of their lambs, show that they had not been infected recently and had not passed significant amounts of antibody to the lambs.

The short duration of the immunity in the lambs which received colostrum from immune ewes is consistent with previous reports (see Section 1.3.2.4). The significant difference in the numbers of live cysts, but not in the number of dead cysts, supports the claim of Gemmell *et al.* (1969) that immunity transferred via colostrum acts on the larvae before they become established as cysts in the peritoneal cavity. However, this is contrary to a more recent report by Gemmell *et al.* (1990) which claimed that the short acting colostral immunity to *T.hydatigena* is directed towrds the cysts after they have become estalished.

The ELISA results of these experiments show, not surprisingly perhaps, that the antibody level in a ewe's serum is reflected in that of the corresponding colostral whey (Figure 4.8). There is also a strong relationship between the levels of antibody in a given lamb's serum and the whey collected from its dam on the day of parturition (Figure 4.9). Variations in the amount of whey ingested and the ability of each lamb to absorb proteins in the first 24 - 48 hr would account for much of the variation in the lamb serum antibody levels which is not accounted for by the levels in the whey. Given the variability in the amount of antibody concentrated in the udder (Halliday, 1976; 1978), the volume of colostrum ingested and the ability of each lamb to absorb the ingested protein (Simpson-Morgan and Smeaton, 1972; Larson *et al.*, 1974; Halliday, 1976; Sawyer, 1977; Logan and Irwin, 1977), one would expect that the relationship between the levels of antibody in a lamb's serum and the serum of its dam would be considerably weaker than that indicated by an r^2

value of 0.6496 (Figure 4.10). In the circumstances, the strength of this relationship is difficult to explain and may be a chance occurrence.

Only about 20% of the variation in the numbers of cysts becoming established in the lambs can be accounted for by the variation in antibody level in the whey (Figure 4.11) although the relationship is still significant ($p = 0.0302$). Variations in the number of cysts can also be attributed to variable amounts of antibody ingested and absorbed, as mentioned above, and also variations in the amount of decay of antibody during the delay from the ingestion of the colostrum to the time of challenge. A further variable factor influencing the relationship between the number of cysts and the levels of antibody in ewe serum (Figure 4.12), is the amount of antibody transferred from the serum of each ewe to her colostrum. One would expect that the additional variation associated with this (Sawyer, 1977) would result in the numbers of cysts in lambs having a weaker relationship with the antibody levels in ewe serum, than with the antibody levels in ewe whey. However, this was not the case in this experiment - a result which is difficult to explain.

The strength of the relationship between the numbers of cysts and the antibody levels in the lambs serum at the time of challenge is further evidence for the importance of antibody in resistance to *T.hydatigena* (Figure 4.7). It would also appear (see Figures 4.6 and 4.7) that a critical level of antibody is required at the time of challenge for the lambs to be totally protected.

CHAPTER 5

THE EFFECT OF COMPLEMENT AND IMMUNOGLOBULIN FRACTIONS ENRICHED WITH IgG_1 OR IgG_2 ON THE SURVIVAL OF *T.HYDATIGENA* LARVAE *IN VITRO*.

5.1 INTRODUCTION.

The short duration of the colostral immunity to *T.hydatigena* demonstrated in Chapter 4 implies that the class of the antibody which is most important in the protection against infection might be IgG_2 since very little IgG_2 is passed to the lamb via colostrum and there is no placental transfer of immunoglobulin in sheep (see Section 1.3.2.4 for references). The experiments described in this chapter aimed to investigate the roles of IgG_1 and IgG_2 in killing oncospheres *in vitro*. The material is presented in 2 parts: 1) the isolation of immunoglobulins from the serum or colostrum of immune sheep and the preparation, from these isolates, of isoelectrically focussed fractions which were then pooled into groups according to their content of IgG_1 and IgG_2; and 2) the use of these pooled groups of immunoglobulins in the culture of oncospheres, both with and without complement present, to examine any differences in the potency of IgG_1 and IgG_2 for oncospheres *in vitro*.

5.2 MATERIALS AND METHODS.

5.2.1 Part 1: Preparation of Pooled Groups of Immune Serum- or Immune Colostral-Immunoglobulin Containing IgG_1 and IgG_2.

The preparation of IgG_1- and IgG_2-enriched fractions entailed the separation of immunogloblulins from serum and colostrum by caprylic acid and ammonium sulphate precipitation, followed by the isoelectric focussing (IEF) of these immunoglobulins into 20 fractions of equal volume. For each serum or colostrum sample prepared, the IEF fractions were then pooled into 5 groups as described in section 5.2.1.6.

5.2.1.1 Experimental Animals and the Immunization Regime.

Six 6-month-old lambs were housed in enclosed pens and fed sheep nuts, lucerne pellets and hay *ad libitum*. Three served as controls and the remaining 3 were immunized as described in Section 2.2.3.4 with FTS oncosphere antigen (Section 2.2.3.3).

5.2.1.2 Isolation of Immunoglobulin from Serum and Colostrum.

Immunoglobulins were isolated from both immune and control sera and also from colostral whey samples.

Serum preparation: One hundred and twenty ml of blood were collected from the jugular veins of each of the 3 immunized and the 3 control lambs. The blood was held at 4°C overnight, the clot removed and the serum clarified by centrifugation at 1100 x g for 30 min. The sera from the control and immune animals were pooled separately.

Whey preparation: Pooled samples of colostral whey were produced from each of the control, Low Infection and High Infection groups of ewes used in the colostral transfer experiment (See Chapter 4; Section 4.2.7), by combining equal volumes of whey from each of the ewes in the groups.

Immunoglobulin isolation: One hundred ml of 0.06 M acetate buffer, pH 4.0 (Appendix 2.1) were added to 50 ml of serum or colostral whey. After adjusting the pH to 4.8 with NaOH, 3.3 ml of 6.86 M caprylic acid (Sigma) were added in a dropwise manner to each sample with continuous stirring at room temperature (Steinbuch and Audran, 1969). The stirring was continued for 30 min and the precipitate which formed was then removed by centrifugation at 10 000 x g for 30 min at 4°C. The supernatant of each sample was dialysed at 4°C against 50 volumes of PBS, pH 7.4 (Appendix 2.1) until a pH of 7 was reached. At this point, the dialysed samples were each added to equal volumes of saturated ammonium sulphate and stirred gently for 30 min at 4°C. Each sample was centrifuged again at 10 000 x g for 20 min at 4°C and each pellet resuspended in a small volume of PBS and dialysed against 2 changes of 50 volumes of 10 mM NaCl over 24 hr. Insoluble material was removed by centrifugation at 8000 x g for 15 min and the supernatant was made up to 50 ml with 10 mM NaCl.

5.2.1.3 Isoelectric Focussing of Immune Serum and Low Infection Whey Immunoglobulin Preparations.

Of the control and immune serum, and the control, Low Infection and High Infection colostral whey, from which immunoglobulins were precipitated, only the immunoglobulins prepared from the immune serum and the Low Infection whey were isoelectrically focussed. The Low Infection whey was used rather than the High Infection whey since the former group had resulted in a somewhat higher degree of protection, though not significantly so, in the colostral transfer experiment described in Chapter 4.

The preparations were separated into 20 fractions according to their pH using a Rotofor^{R} preparative isoelectrofocussing cell (Bio-Rad) and standard procedures for the equipment. To twenty-five ml of the immunoglobulin preparation were added solutions of ampholytes (pH 3.3 - 9.8), urea and 10 mM NaCl to give a final volume of 30 ml containing 1% ampholytes and 1M urea. This mixture was loaded into the Rotofor cell and electrophoresis carried out for 4 hr before the separated fractions were harvested. The pH of each of the 20 fractions for each immunoglobulin preparation was measured and recorded before they were dialysed overnight against PBS, pH 8 (Appendix 2.1).

5.2.1.4 Immunoglobulin Class Analysis by ELISA of Isoelectrically-Focussed (IEF) Fractions.

The levels of the sub-classes of IgG contained in each of the 20 fractions were initially determined by ELISA. The antigens used to coat the plates were the IEF-fractions from the immune serum and Low Infection colostrum immunoglobulin preparations. The serum IEF-fractions were diluted 1:200 and the colostrum IEF-fractions 1:80 with coating buffer (Appendix 2.1). One hundred µl of each immunoglobulin fraction were placed in each of 3 wells of 2 plates. After overnight incubation at 37°C in a humidified chamber and 4 washes in 0.05% Tween-20 in distilled water, the plates were incubated for 30 min with blocking buffer before being washed 3 times as before.

One of the 3 wells coated with each immunoglobulin fraction was then incubated for 1 hr with 100 µl of mouse anti-sheep IgG_1 monoclonal antibody and another of the 3 wells with mouse anti-sheep IgG_2 monoclonal antibody (both monoclonal antibody preparations were kindly provided by Dr M.W. Lightowlers, University of Melbourne, Veterinary Clinical Centre, Werribee, Australia). The monoclonal

preparations were diluted 1:1000 with 0.5% BSA in PBS, pH 7.4, with 0.05% Tween 20 for use and had been tested by ELISA previously to determine that they were in fact specific for IgG_1 or IgG_2. The third well for each fraction was incubated with 100 µl of this diluent to serve as a control to detect any non-specific binding of the conjugated immunoglobulins to the fractionated immunoglobulins coating the plates. HRP-conjugated goat anti-mouse antiserum (Immuno-Chemical Products Ltd (ICP) N.Z.) diluted 1:1000 in 0.5% BSA in PBS, pH 7.4, with 0.05% Tween 20, was used to detect the anti-IgG class antibodies and the plates were developed in the usual way.

5.2.1.5 **Immunoelectrophoresis (IEP) of the Immunoglobulin Fractions.**

The immunoglobulin classes contained in each of the isoelectrically focussed fractions from immune serum and from colostrum were also examined by immunoelectrophoresis.

Agarose-coated polyester GelBond^R film (LKB Pharmacia)(0.2mm thick) was cut to fit 8.5 cm x 9 cm glass plates and placed hydrophilic side up on the leveled plates. Sixteen ml of 0.9% agarose in sodium barbitone buffer, pH 8.6, (Appendix 2.1) at 55-60°C were pipetted onto the Gelond^R. After the gels were set, troughs and wells were cut using a template held in position in a tray (LKB 9390 6386 tray).

The plates were arranged, 2 at a time, on an LKB Multiphor^R electrophoresis system. The top 2 wells on each plate received 10 µl of sheep IgG_1 and IgG_2, respectively (kindly provided by Dr G.B.L. Harrison, Pitman-Moore NZ). Each of the other wells received 10 µl of one of the IEF-fractions of serum immunoglobulin or colostral immunoglobulin. The gels were run at 80-100 V for 45-60 min until the Bromophenol Blue marker added near one of the wells at the start of the separation procedure had traversed the length of the gel.

After removing the plates from the electrophoresis unit, the troughs were filled with approximately 150 µl of rabbit anti-sheep immunoglobulin antiserum and the plates were placed in humidified containers for 3 days for the immunoprecipitation arcs to develop.

The gels were pressed, washed overnight in 0.91% NaCl, dried at 56°C for 2-3 hr and stained for 20 min in Brilliant Crocein Scarlet stain (Appendix 2.1) according to the method described by Wallenborg and Andersson (1978). Excess stain was removed under running tap water and the gels were destained in 0.3% acetic acid for

approximately 5 min until the background was clear. The gels were air dried and photographed.

5.2.1.6 Isoelectrically Focussed Fractions Pooled in Preparation for use *In Vitro*.

In order to determine the relative effectiveness of IgG_1 and IgG_2 in killing oncospheres *in vitro* (see Section 5.2.2), pools of the IEF-fractions were made so that one pooled group contained high levels of IgG_1 subclass and low levels of IgG_2 and another pooled group contained high levels of IgG_2 and low levels of IgG_1. These pooled groups of the serum IEF-fractions were formed by combining equal volumes of those fractions containing similar proportions of either IgG_1 or IgG_2, as identified by the ELISAs (Figures 5.1 and 5.2). The composition of the pooled groups is shown in Table 5.1.

Table 5.1. The immune serum IEF-fractions pooled into groups.

Group numbers	IEF-fraction	pH Ranges.
S1	1-4	2.05 - 4.55
S2	5-8	4.8 - 5.80
S3	9-12	6.0 - 6.85
S4	13-15	7.2 - 7.85
S5	16-19	8.2 - 11.7

IEF-fractions 9 - 12 (pH 6 - 6.85) of the immune serum preparation were pooled (Group S3) since they contained relatively high levels of IgG_1 activity while excluding the highest levels of IgG_2. Fractions 16 - 19 (pH 8.2 - 11.7), containing the peak of the IgG_2 activity with relatively low levels of IgG_1, were pooled (Group S5). Fractions 1 - 8 were divided into 2 groups of 4 fractions each (Groups S1 and S2) and fractions 13 - 15 were pooled into 1 group (Group S4). Fraction 20 contained very little immunoglobulin and was excluded (Figure 5.1).

The division of the colostral IEF-fractions was arranged to cover similar pH ranges (Table 5.2). Fractions 19 and 20 contained very little immunoglobulin of any class and were excluded (Figure 5.2).

Table 5.2. The immune colostrum IEF-fractions pooled into groups.

Group numbers	IEF-fraction	pH Ranges
C1	1-3	3.6 - 4.4
C2	4-6	4.8 - 5.5
C3	7-9	6.0 - 6.8
C4	10-12	7.2 - 7.75
C5	13-18	8.2 - 12.6

5.2.1.7 Levels of anti-*T.hydatigena* IgG_1 and IgG_2 in Each of the Pooled Groups Determined by ELISA.

The levels of total and anti-*T.hydatigena* oncosphere-specific IgG_1 and IgG_2 in each of the pooled groups were determined by ELISA. To determine the total levels present, the pooled immunoglobulin preparations were diluted and used to coat the plates as described for the individual fractions in Section 5.2.1.4. Three wells were allocated to each preparation. A group of wells was also included which contained equal volumes of each of the 5 pooled groups for both the serum and colostral preparations. The plates were washed, blocked, and washed again in the manner described. In each set of 3 wells, the first was probed with 100 µl of a 1:100 dilution of the anti-IgG_2 monoclonal in 0.5% BSA in PBS, pH 7.2 with 0.05% Tween 20, the second with a 1:1000 dilution of anti-IgG_1 monoclonal in the same diluent, and the third with 100 µl of the diluent. The conjugate used was HRP-conjugated goat anti-mouse antiserum (ICP) and the plates were developed in the usual way.

To determine the levels of anti-*T.hydatigena* oncosphere IgG_1 and IgG_2 contained in each pooled group, plates were prepared as described in Section 2.2.1.8 with a 1:90 dilution of *T.hydatigena* FTS oncosphere antigen in coating buffer. After washing, blocking, and washing again, a 1:20 dilution of the pooled groups of immunoglobulin fractions with 0.5% BSA in PBS with 0.05% Tween 20, was used to probe the wells. Following the washing procedure, the plates were probed with the anti-IgG_1 and anti-IgG_2 monoclonal antibodies, then probed with the HRP-conjugated antiserum and developed as usual.

5.2.1.8 SDS-PAGE of FTS Oncosphere Antigen and Western Blot Probed with Group 5 of the Serum Preparation.

The results of the *in vitro* experiments (described in Section 5.2.3) indicated that Group 5 of the serum immunoglobulin preparations had the greatest capacity to cause oncosphere death. In order to identify the oncosphere antigens recognized by antibodies in this group, FTS antigen was fractionated in an SDS-PAGE gel and the antigens from half of the gel were transferred onto nitrocellulose paper as described in Section 2.2.1.7. Four strips were cut from the nitrocellulose and the remaining nitrocellulose containing antigen and the molecular weight markers was stained with Amido Black (Appendix 2.1). Of the 4 strips containing antigen, one was probed with a 1:200 dilution of Group 5 of the serum preparation for 4 hr, a 1:300 dilution of the anti-IgG_2 monoclonal preparation overnight, and a 1:1000 dilution of the HRP-conjugated goat anti-mouse antiserum (ICP) for 4 hr before being developed. Another was also probed with Group 5 of the serum preparation but was then probed with HRP-conjugated rabbit ant-sheep antiserum (Cappel) for 4 hr, before being developed. The third and fourth strips were probed for 4 hr with a 1:100 dilution of serum from either a sheep which had received 3 immunizations of FTS oncosphere antigen as described in Section 2.2.3.4, or an uninfected control sheep, before being probed with HRP-conjugated rabbit anti-sheep antiserum (Cappel) as above. All strips were developed concurrently and for the same length of time. Due to a shortage of the separated fractions of the anti-*T.hydatigena* serum, and of the ant-IgG_1 and -IgG_2 monoclonal antiserum, a more comprehensive study of the antigens recognized by these preparations was not possible.

The other half of the SDS-PAGE gel was stained with Silver stain (See Section 5.2.1.9).

5.2.1.9 Silver Staining the SDS-Polyacrylamide Gel Containing *T.hydatigena* Oncosphere Antigen.

The gel containing separated *T.hydatigena* oncosphere antigen was silver-stained (Blum *et al.*, 1987) using the reagents described in Appendix 2.1. All steps were carried out with the solutions being continually mixed by means of a shaker table. The gel was soaked in the fixing solution for at least 1 hr, then washed 3 times for 20 min on each occasion in 50% methanol. Following this the gel was placed in the pretreatment solution for 60 sec and then washed in 3 changes of double-distilled water, each for 20 sec, before being soaked in the impregnation solution for 20 min.

After a further two washes of 20 sec in double-distilled water, the gel was developed by soaking in the developing solution for up to 10 min. Just prior to the optimal stage of staining, the gel was removed and washed in 2 or 3 washes of double-distilled water for 30 sec each before being placed into the stopping solution. At this stage the gel was photographed. Two more washes in distilled water were followed by soaking for 10 min in 50% methanol before the gel was soaked in a solution of 30% methanol and 3% glycerol for at least 2 hr and dried under vacuum at 80°C for 3 hr in a Bio-Rad slab gel drier.

5.2.2 Part 2a : Culture of Oncospheres in the Presence of IgG_1 and IgG_2 from Immune Serum With or Without Complement.

A preliminary culture experiment was done to determine if the survival of oncospheres *in vitro* was affected when cultured in the presence of IgG_1- or IgG_2-enriched fractions from immune serum, with or without complement.

5.2.2.1 Preparation of IgG_1- and IgG_2-Enriched Serum Samples and Complement.

Groups S3 (containing high levels of IgG_1 and low levels of IgG_2) and S5 (containing high levels of IgG_2 and low levels of IgG_1 of the serum fractions) were sterilized by passage through a 0.2 µm filter. Approximately 15 ml of blood were collected from the jugular veins of each of 3 uninfected, control lambs. The serum was harvested and half of it was treated to inactivate the complement while the other half was stored on ice to preserve the complement as described in Section 3.2.3. Sera were also collected from the 3 immunized lambs (Section 5.2.1.1), from which the immunoglobulin preparations and IEF-fractions had been prepared, and were heat-inactivated in the same way.

5.2.2.2 Design of the *In Vitro* Assay.

Four hundred µl of either the immune or control heat-inactivated serum or of the pooled Groups S3 or S5 were placed in each of 4 replicate wells of a sterile, 24-well culture plate. Into 2 wells of each of the replicates, were added 500 µl of control sheep serum stored on ice to provide complement, and to the remaining two, 500 µl of the heat-inactivated control serum. Approximately 200 activated oncospheres in 1.5 ml Medium NCTC-135 (GIBCO) were dispensed into each of the 24 wells. (Medium NCTC-135 is a complex medium of similar composition to McCoy's 5A

modified medium which was used in the experiments described in Chapter 3. McCoy's medium was not available for the experiments described here.)

After 24 hr incubation at 37°C in 5% CO_2-in-air, 50 oncospheres in each well were examined and the percentage which were dead was calculated. Swollen oncospheres with their hooks protruding from the plasma membrane were judged to be dead (Herd, 1976) whereas living oncospheres could be seen to be developing.

5.2.3 Part 2b: Culture of Oncospheres in the Presence of Complement With Either Immune Serum or Colostral Whey Immunoglobulins, or Fractions of These.

Following the preliminary experiment, the effect on the survival of oncospheres *in vitro* of all 5 groups of the pooled, IEF-fractions from both immune serum and immune colostrum immunoglobulin preparations, was determined.

5.2.3.1 Preparation of the Immunoglobulin Samples and Complement.

Each group of pooled fractions were sterilized by passage through 0.2 µm filters. Serum from control sheep which had been prepared as a source of complement (Section 5.2.2.1) and stored in liquid nitrogen was thawed, and stored on ice until needed.

5.2.3.2 Design of the *In Vitro* Assay.

Five hundred µl of each of the 5 pooled IEF-fraction groups from the serum preparation, were placed into 2 wells each of a 24-well culture plate, and 500 µl of each of the 5 pooled IEF-fraction groups from the colostral whey preparation, placed into 2 wells each of another plate. Samples of the immunoglobulin preparations of serum from immunized or control sheep, or of colostrum from Low Infection or control sheep (Section 4.2.2), which had not been isoelectrically focussed, were also dispensed in 500 µl aliquots into duplicate wells of both 24-well plates.

All wells received 500 µl of control sheep serum stored on ice and 1.5 ml of Medium NCTC-135 containing approximately 200 activated oncospheres. After 24 hr incubation at 37°C in 5% CO_2-in-air, the plates were examined using a Leitz Diaphot inverted microscope and at least 20 oncospheres were examined from each well and designated dead or alive according to the characteristics described above. The percentage of living oncospheres was calculated.

5.2.4 Statistical Analysis.

As it is not appropriate to analyse percentages or proportions by analysis of variance without transformation of the data (Mead and Curnow, 1983; Zar, 1984), the percentages of oncosphere survival in the second culture experiment were transformed to their arcsines before subjecting them to analysis of variance followed by the Tukey test for pairwise comparisons with a rejection level of 5%. The analysis of variance table is shown in Appendix 5.2, and the Tukey pairwise comparison in Appendix 5.3.

Regression analysis of the percentage survival of oncospheres and the individual absorbance levels of anti-*T.hydatigena* IgG_1 and IgG_2 in the pooled groups, was performed.

Since both IgG_1 and IgG_2 were present in all cultures in varying amounts, a multiple regression analysis was also performed to determine the relative effectiveness of the two IgG subclasses on the percentage of oncosphere survival.

5.3 RESULTS.

5.3.1 Part 1: Determination of the Levels of IgG_1 or IgG_2 in the Isoelectrically Focussed Fractions.

5.3.1.1 IgG Sub-Class Analysis of All Fractions by ELISA.

Figures 5.1 and 5.2 show the absorbance level of the IgG_1 and IgG_2 contained in each of the 20 IEF-fractions for the serum and colostrum preparations. Also shown in these figures is the pH of each of the isoelectrically focussed fractions. It should be noted that a quantitative comparison between the levels of IgG_1 and IgG_2 cannot be made since the anti-IgG_1 and -IgG_2 monoclonal preparations used to determine the levels of antibody on ELISA were entirely separate preparations.

IgG_1 levels did not vary greatly with pH but tended towards a maximum in fractions 9 - 15 (between pH 6 and 8 approximately), in both the serum and colostrum preparations. IgG_2 levels, on the other hand, showed a sharp peak in fractions 14 - 19 (between pH 7 and 12) in the serum preparation but were present not significant in the colostrum preparation (Figures 5.1 and 5.2).

Figure 5.1 Total IgG_1 and IgG_2 levels, and the pH of each of the fractions of the immune serum preparation.

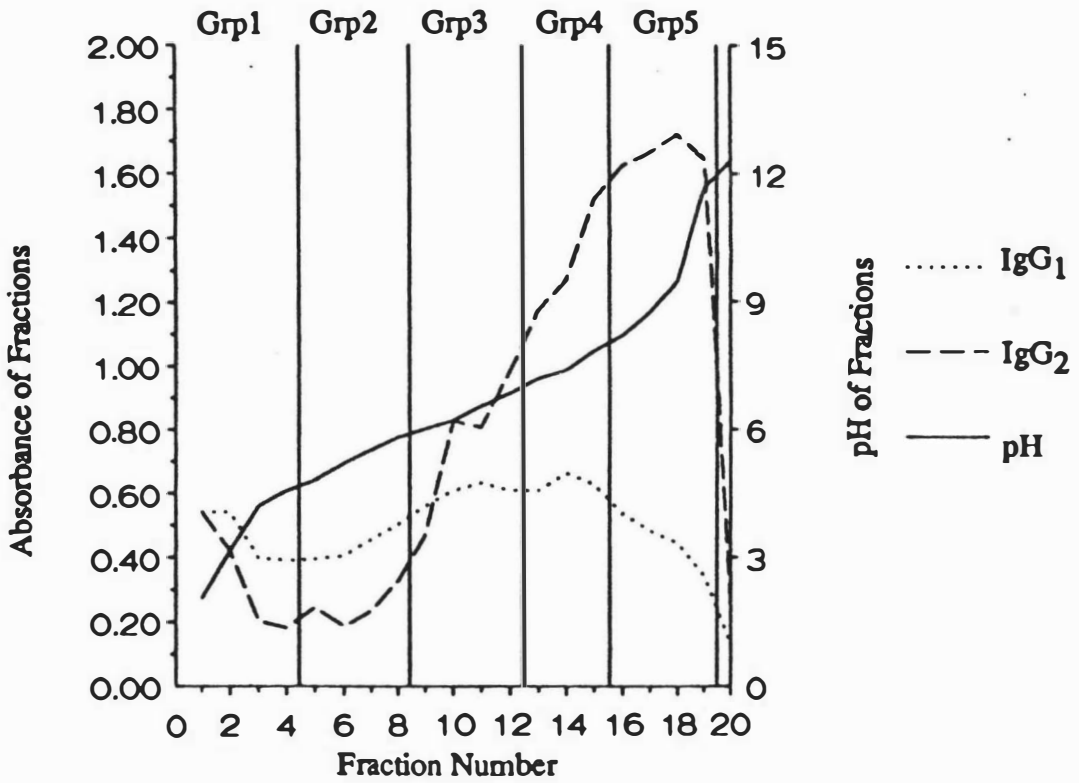
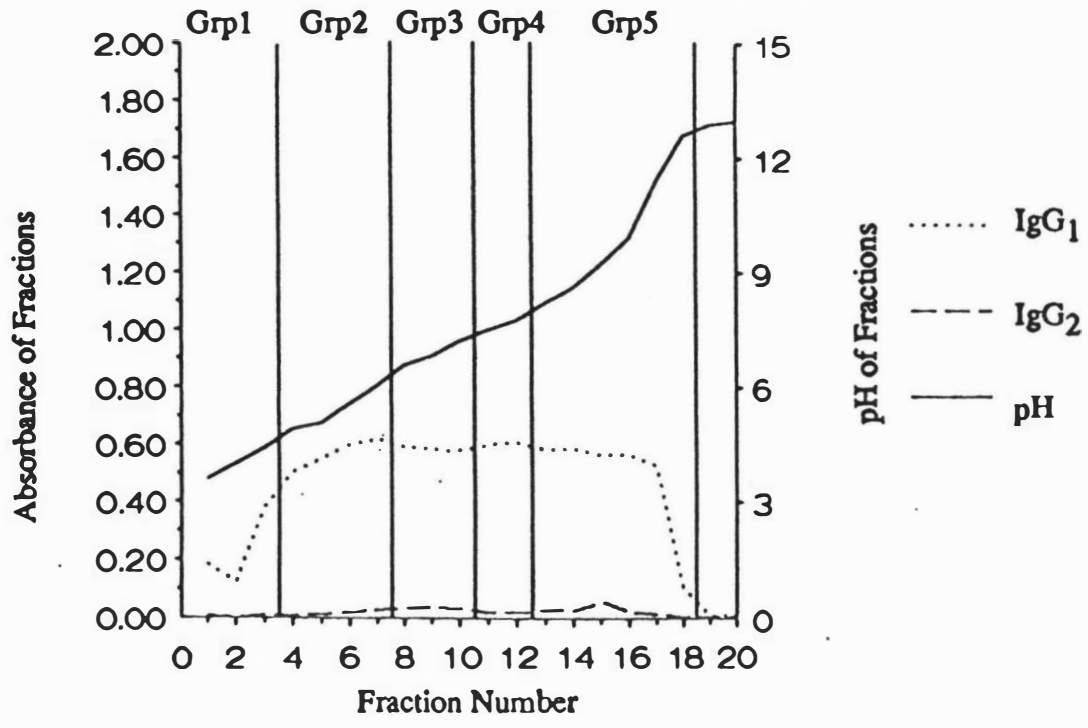


Figure 5.2 Total IgG_1 and IgG_2 levels, and the pH of each of the Fractions of the Immune Colostral Whey Preparation.



5.3.1.2 IgG Sub-Class Analysis of all Fractions by IEP.

The patterns of precipitin arcs for each fraction of both the serum preparation and the colostral whey preparation are shown in Figure 5.3. IgG_1 arcs were only visible from pH 4.8 - 6 in the colostral fractions, and were difficult to distinguish from other precipitation arcs associated with the serum fractions. IgG_2, however, was clearly absent from the colostral fractions and present in the serum fractions at higher pH levels.

5.3.1.3 Levels of Anti-*T.hydatigena* IgG_1 and IgG_2 in the Pooled Groups of Fractions as Determined by ELISA.

The means of the duplicate absorbance levels for each pooled immunoglobulin group are shown in Figure 5.4 and in Appendix 5.4. It should again be stressed that quantitative comparisons can only be made within each subclass.

5.3.1.4 Analysis of Antigens on Western Blot.

The results of the Western blot are shown in Figure 5.5. All of the antigens recognized by both IgG subclasses in Group 5 of the serum preparation (Lane 4) appeared to be recognized by IgG_2 antibodies alone (Lane 5). The major bands of antigen recognized by immune serum (Lane 3) did not correspond to protein antigens of similar molecular weights shown on nitrocellulose stained by Coomassie blue (Lane 1) or in the Silver stained gel (Figure 5.6).

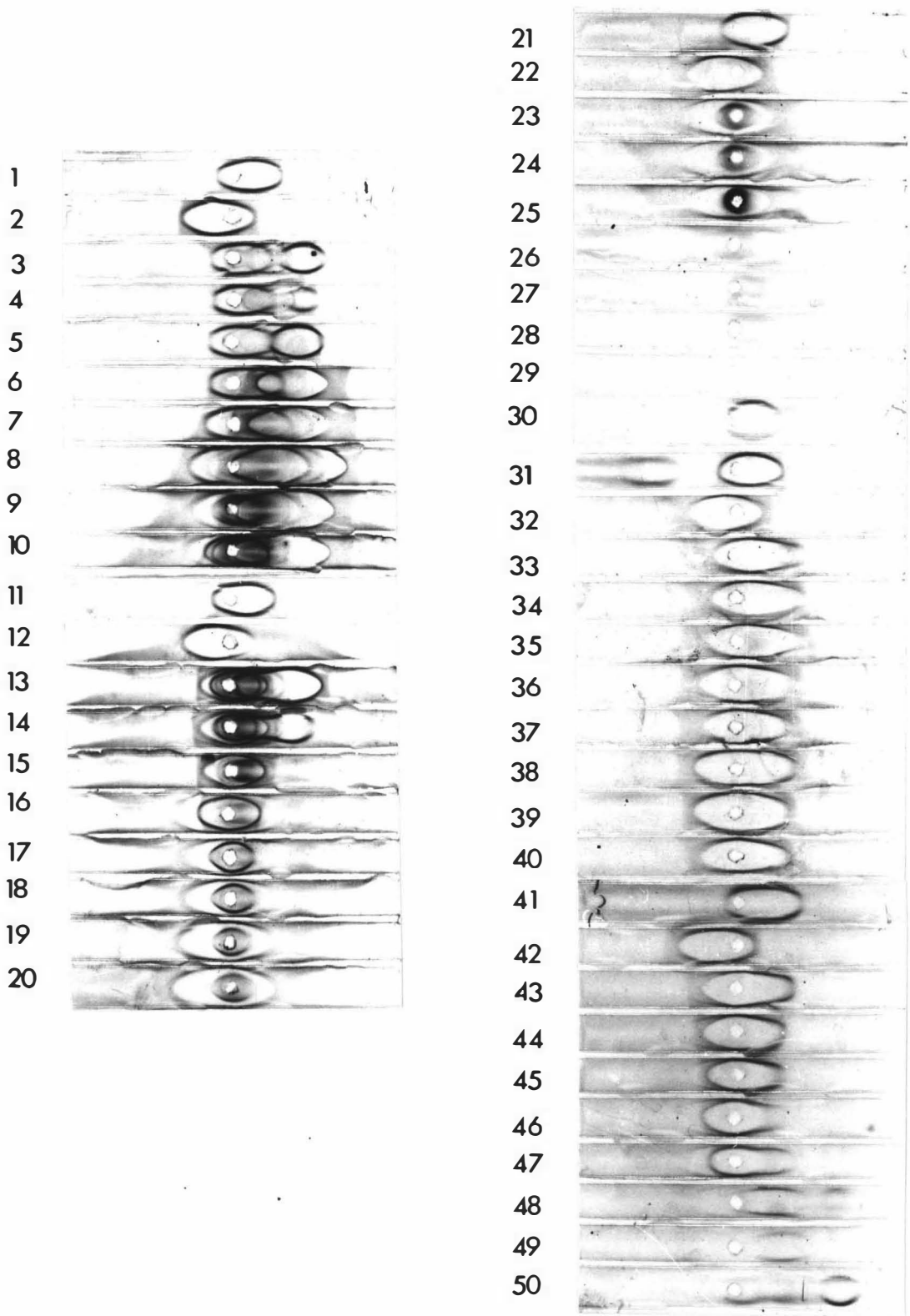
5.3.2 Part 2a: The Effect of IgG_1 and IgG_2 from Immune Serum on the Survival of Oncospheres *In Vitro* With or Without Complement.

The level of oncosphere survival was not reduced when complement was absent. Survival was lowest in wells containing either unfractionated immune sheep serum or Group S5 (the pooled group of immune serum IEF-fractions containing high levels of IgG_2 and low levels of IgG_1). Group S3 (containing high levels of IgG_1 and low levels of IgG_2) resulted in a percentage of survival intermediate between that of the unfractionated immune serum and control serum immunoglobulin prepartions. Table 5.3 gives the percentage of oncospheres surviving in each of the duplicate wells.

Immunoglobulin No.	Sample	pH
1	IgG_1	
2	IgG_2	
3	Serum 1	2.05
4	Serum 2	3.15
5	Serum 3	4.2
6	Serum 4	4.55
7	Serum 5	4.8
8	Serum 6	5.2
9	Serum 7	5.5
10	Serum 8	5.8
11	IgG_1	
12	IgG_2	
13	Serum 9	6.0
14	Serum 10	6.2
15	Serum 11	6.55
16	Serum 12	6.85
17	Serum 13	7.2
18	Serum 14	7.4
19	Serum 15	7.85
20	Serum 16	8.2

Immunoglobulin No.	Sample	pH
21	IgG_1	
22	IgG_2	
23	Serum 17	8.75
24	Serum 18	9.45
25	Serum 19	11.7
26	Serum 20	12.3
27	Colostrum 1	3.6
28	Colostrum 2	4.0
29	Colostrum 3	4.4
30	Colostrum 4	4.8
31	IgG_1	
32	IgG_2	
33	Colostrum 5	5.05
34	Colostrum 6	5.5
35	Colostrum 7	6.0
36	Colostrum 8	6.55
37	Colostrum 9	6.8
38	Colostrum 10	7.2
39	Colostrum 11	7.5
40	Colostrum 12	7.75
41	IgG_1	
42	IgG_2	
43	Colostrum 13	8.2
44	Colostrum 14	8.6
45	Colostrum 15	9.2
46	Colostrum 16	9.95
47	Colostrum 17	11.4
48	Colostrum 18	12.6
49	Colostrum 19	12.85
50	Colostrum 20	12.95

Figure 5.3 **Immunoelectrophoresis of the isoelectrically focussed fractions of immune serum and colostrum immunoglobulin preparations, precipitated with rabbit anti-sheep-immunoglobulin antiserum.**



Ctrl.S = Control serum immunoglobulin preparation.

Imm.S = Immune serum immunoglobulin preparation.

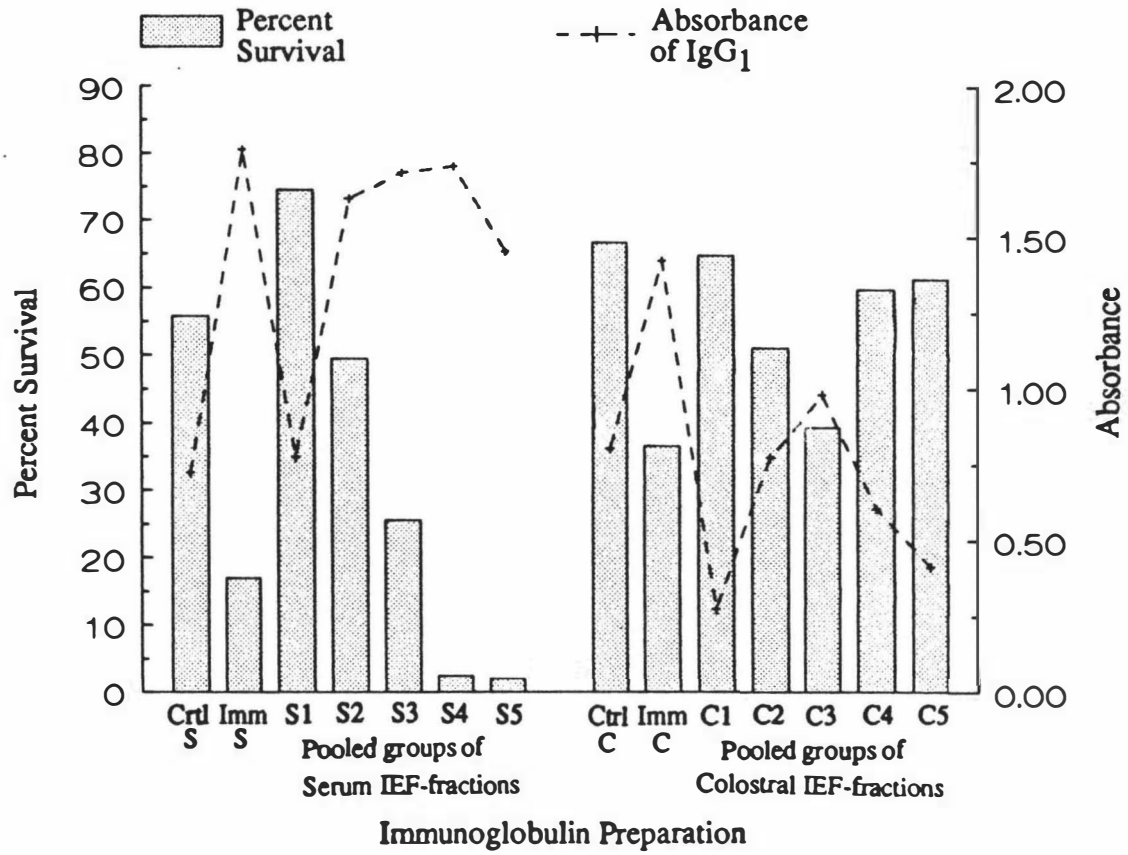
S1
S2
S3 = The pooled groups of the IEF-fractions from the immune serum immunoglobulin preparation.
S4
S5

Ctrl.C = Control colostrum immunoglobulin preparation.

Imm.C = Immune colostrum immunoglobulin preparation.

C1
C2
C3 = The pooled groups of the IEF-fractions from the immune serum immunoglobulin preparation.
C4
C5

Figure 5.4 **Survival of oncospheres when cultured with immunoglobulin preparations and complement.**



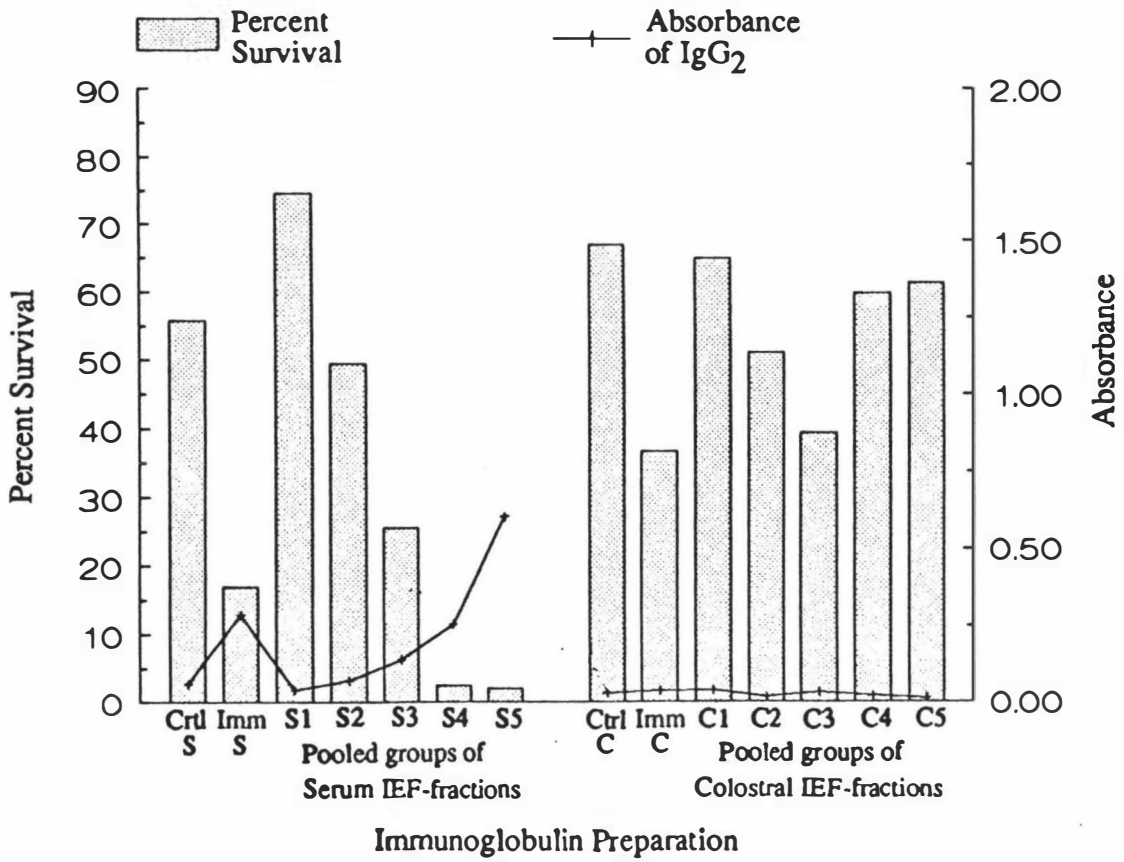
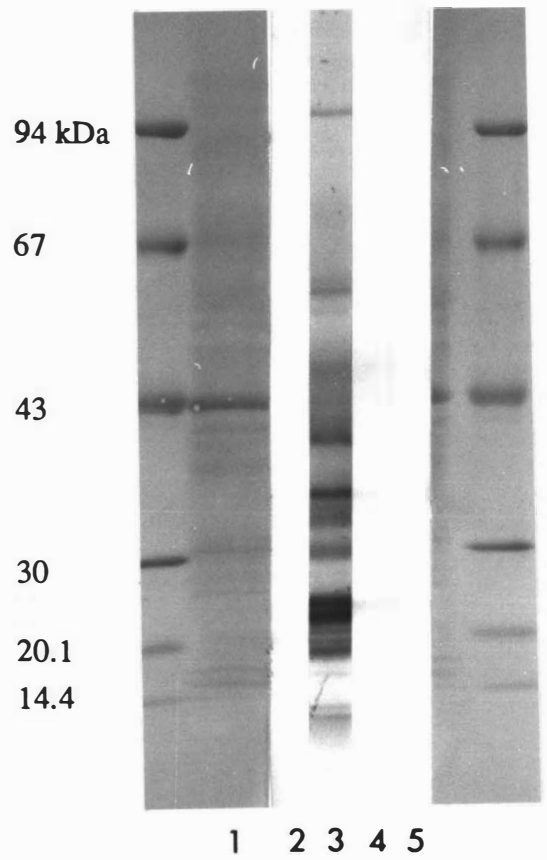


Figure 5.5 **Western blot of *T.hydatigena* oncosphere antigen probed with the pooled Group 5 of the IEF-fractions of immune serum immunoglobulin preparation compared with whole immune serum.** (see Section 3.2.1)



Lane	Serum sample
1	(Amido black stain of antigen)
2	Control sheep serum (see Section 3.2.1)
3	Immune sheep serum (see Section 3.2.1)
4	Pooled Group 5 of IEF-fractions of serum probed with anti-IgG serum.
5	Pooled Group 5 of IEF-fractions of serum probed with anti-IgG_2 monoclonal.

Figure 5.6 **A silver stain of *T.hydatigena* oncosphere antigen separated on SDS-PAGE.**

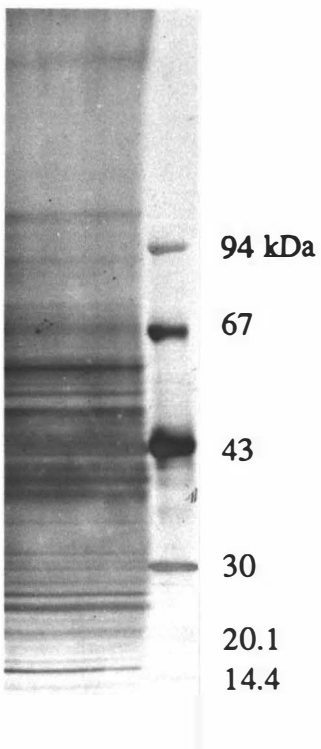


Table 5.3. **The percentage of freshly activated oncospheres surviving 24 hours culture in the presence of high levels of IgG_1 with low levels of IgG_2, or vice versa.**

Immunoglobulin preparation.	Without Complement	With Complement
Group S3 (High IgG_1)	48, 46	30, 23.5
Group S5 (High IgG_2)	40, 42	16, 18
Control serum (Whole serum)	48, 50	42, 38.5
Immune serum (Whole serum)	40, 31.4	16, 21.6

5.3.3 Part 2b: The Effect of Serum and Colostrum Immunoglobulin Preparations and Fractions of These in the Presence of Complement on the Survival of Oncospheres *In Vitro*.

The percentages of larvae surviving after 24 hr in culture with immunoglobulin preparations and complement are shown in Figure 5.4 (and Appendix 5.1). The ELISA absorbance values of IgG_1 and IgG_2 in the pooled groups, the proportion of living incospheres in the cultures containing these groups and the arcsine transformed data of the proportion of living oncospheres, are shown in Appendix 5.4. While the small numbers of replicate cultures containing the pooled IEF-fractions made it difficult to show significant differences between the effects of many of the groups, some significant effects were apparent (see Appendix 5.3). Survival in cultures containing the unfractionated control serum preparation (Ctrl.S), the unfractionated control whey preparation (Ctrl.C) or the unfractionated Low Infection whey preparation (Imm.C) did not differ significantly. The unfractionated immune serum preparation (Imm.S), however, caused a significant decrease in the percentage of oncosphere survival when compared with the control serum preparation.

The presence of serum Groups S4 and S5 had the most marked effect by reducing oncosphere survival significantly more than all other preparations, including Imm.S. At the other extreme, the highest percentage survival occurred in the presence of serum Groups S1 and S2 and Ctrl.S, which were not significantly different from each other. The presence of serum Group S3 resulted in an intermediate result, significantly reducing the oncosphere survival compared with Ctrl.S and S1, but not in comparison with S2 or Imm.S. Survival in the presence of Imm.S was also significantly less than with S1 and S2.

Of the colostral whey preparations, the presence of the Group C3 and Imm.C preparations resulted in the lowest survival but the results were not significantly different from those of the other colostral whey groups. However, survival in the presence of C3 or Imm.C was not significantly different from that with S3 or Imm.S (See Appendix 5.3).

Pairwise comparisons of the levels of anti-*T.hydatigena* IgG_1 or IgG_2 in each of the groups of fractions determined by ELISA showed even fewer significant differences between the groups because there were only duplicate absorbance readings. Simple regression analysis revealed highly significant negative relationships between the ELISA absorbance for each of the IgG subclasses and the percentage of the oncospheres surviving. These are shown in Figures 5.7 and 5.8. In order to compare the relationships of these IgG_1 and IgG_2 absorbances with the survival of oncospheres, linear equations were fitted to each set of data. Analysis of the slopes of these lines indicated that the relationships were significantly different ($p < 0.001$ see Appendix 5.5). The multiple regression analysis showed that the effects on percentage oncosphere survival of the two subclasses of anti-*T.hydatigena* antibody, were very similar and highly significnat ($F = 18.17$, $p = 0.0001$ for IgG_2 with IgG_1 held constant, and $F = 25.64$, $p = 0.0000$ for IgG_1 with IgG_2 held constant).

5.4 DISCUSSION

The results of the preliminary experiment confirm the antibody mediated, cell-independent death of oncospheres *in vitro* to be dependent on complement. This was also shown in the results of the *in vitro* experiments of Chapter 3.

The ELISA tests clearly defined the levels of the two subclasses in each fraction and the pooled groups of fractions. The clarity of the differentiation between each subclass as shown by IEP, however, was variable, perhaps because the rabbit anti-

Figure 5.7 **The linear relationship between the level of IgG_1 and the percentage of oncosphere survival *in vitro*.**

r = 0.7628

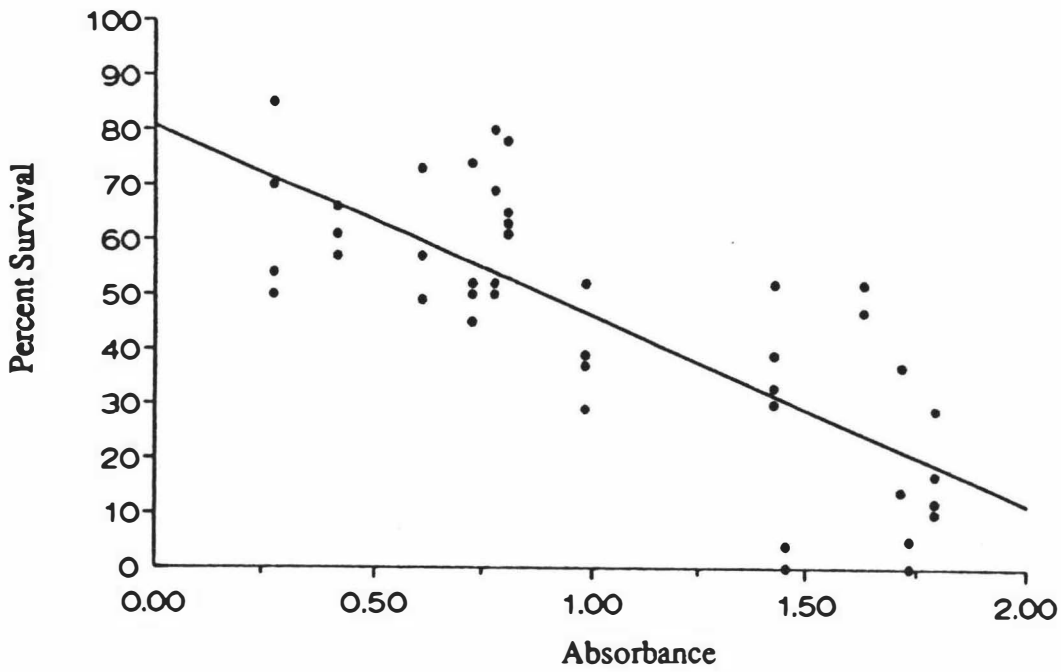
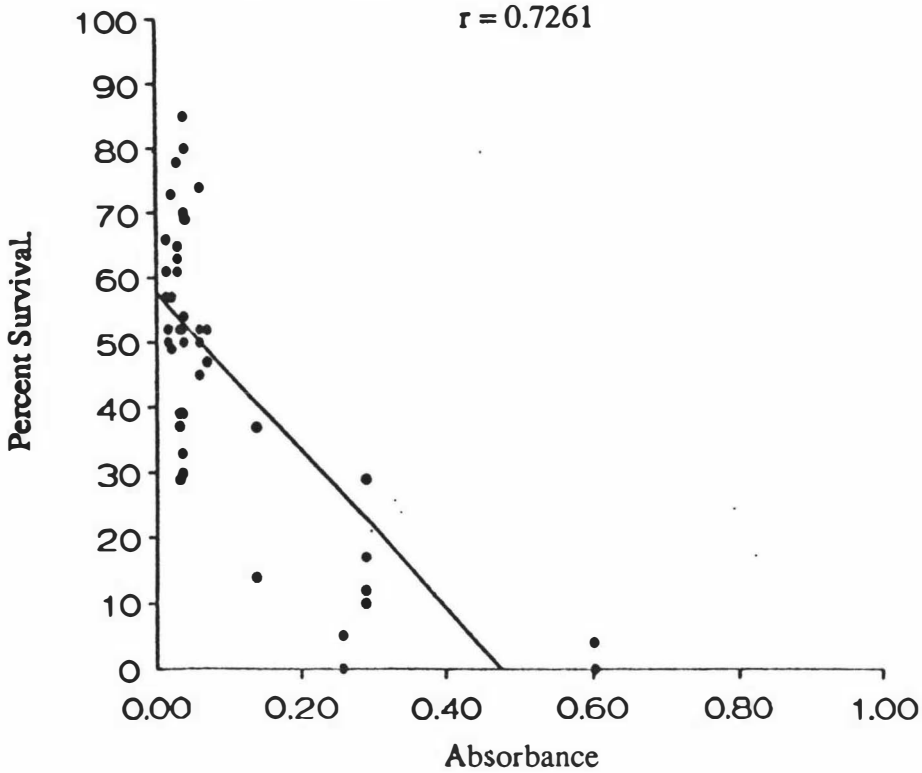


Figure 5.8 **The linear relationship between the level of IgG_2 and the percentage of oncosphere survival *in vitro*.**

r = 0.7261



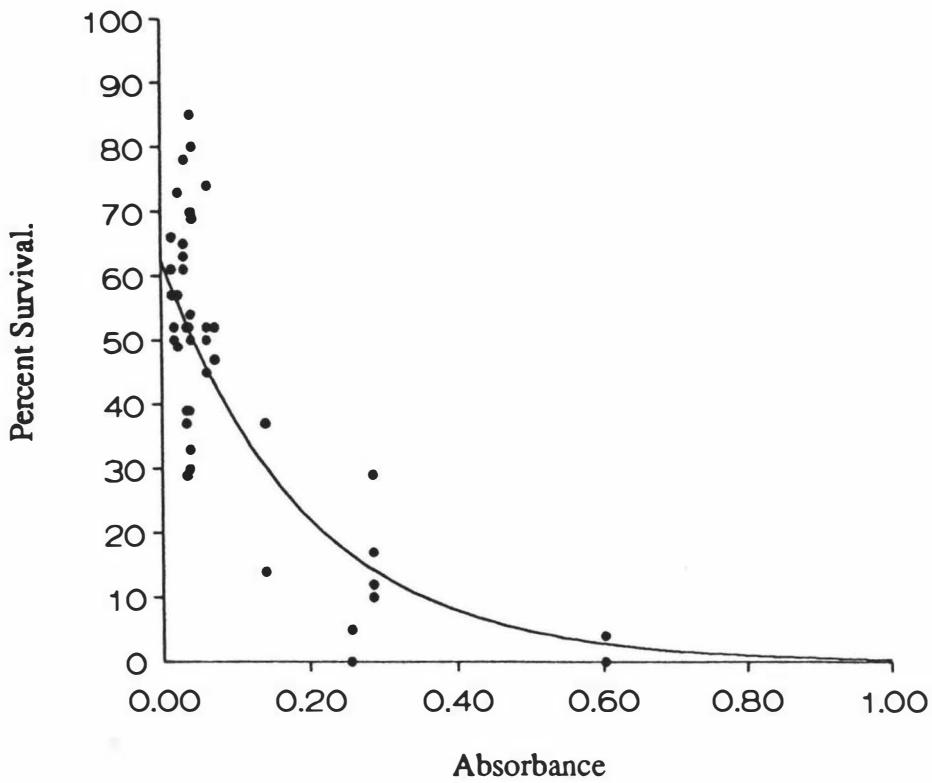
sheep immunoglobulin antiserum used in the IEPs to precipitate the sheep immunoglobulins, contained antibodies to other sheep serum proteins. It was revealed by both tests that IgG_1 was present in most fractions. This could be due to at least two types of IgG_1 being present (Aalund *et al.*, 1965), each possibly having a different isoelectric pH. This, almost ubiquitous, presence of IgG_1 made the differentiation of the effects of the 2 subclasses in culture somewhat more difficult than had been hoped.

Another problem in differentiating the effects of IgG_1 and IgG_2, was the small numbers of replicate cultures. This made it difficult to assess the statistical significance of differences between the effects of the groups of immunoglobulin fractions. An attempt to rectify this problem, by repeating this experiment using larger numbers of replicates, failed. The experiment was carried out as described in Section 5.2.3 except that only 10% of the original volumes were used as the cultures were carried out in 96-well plates rather than 24-well plates. The complement used was shown, by a complement fixation test, to be active and present in excess. However, the immunoglobulin preparations appeared to have lost their ability to kill oncospheres after storage at -20°C for 11 months. These antibodies were still able, nevertheless, to bind to the oncosphere antigens after this period of storage as shown by the ELISA results (Figure 5.4) and the Western blot (Figure 5.5 Lanes 4 and 5). No explanation can be offered for this result.

In spite of the low numbers of replicates, the pairwise comparisons indicated that those groups containing the highest levels of IgG_2 resulted in the lowest survival of oncospheres. While the levels of IgG_1 were also relatively high in these groups, in other groups where IgG_1 was high and IgG_2 was low (Group 2 of the serum for example) the percentage of oncosphere survival was significantly higher. This suggests that IgG_2 might have a greater ability to lead to the death of oncospheres than IgG_1.

Regression analysis showed that the levels of both anti-*T.hydatigena* IgG_1 and IgG_2, had a highly significant effect on the percentage survival of oncospheres and that their relative contributions to the death of oncospheres in culture were very similar. However, the significant difference in slope of the two regression lines indicates a substantial difference in the effect of the increasing levels of the two sublasses. The slope of the linear regression line indicates a very rapid increase in effect with increasing levels of IgG_2. Indeed, the best fit for the IgG_2 data is an exponential regression line (Figure 5.9). With anti-*T.hydatigena* IgG_1, however, the relationship between antibody level and oncosphere survival is linear and the effect

Figure 5.9 **The exponential relationship between the level of IgG_2 and the percentage of oncosphere survival *in vitro*.**
r = 0.8548



of increasing levels is much less marked than with IgG_2. The results indicate that only at very low levels is there no effect of IgG_2 on oncospheres in culture.

These results indicate that, while IgG_2 may be more efficient than IgG_1 at killing oncospheres, the latter is also involved. However, its lower potency may be a significant factor in the apparent brevity of the immunity derived from colostrum. The level of specific IgG_1 in the colostrum clearly affects the level of protection transferred (see Chapter 4), and a relatively high level in the lamb's serum is required to be effective in protecting against a challenge infection. The short duration of colostral protection may, therefore, be due to the IgG_1 antibody levels falling below a critical level very quickly after birth.

The results of the Western blot indicate that not only do the effects of IgG_1 and IgG_2 seem to be directed towards similar oncospheral antigens, but that these antigens may contain a large proportion of carbohydrate moieties. This latter possibility may present difficulties in the identification of DNA clones of *T.hydatigena* oncospheral antigens when a genetically engineered vaccine is being developed.

CHAPTER 6

THE IMMUNIZATION OF SHEEP WITH FRACTIONS OF *T.HYDATIGENA* ANTIGENS SEPARATED BY SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS.

6.1 INTRODUCTION.

Having demonstrated the importance of antibody in the protection of sheep against *T.hydatigena*, it was decided to make a preliminary attempt to identify potentially protective antigens by the fractionation of *T.hydatigena* antigen separated on SDS-PAGE. The induction of protection in sheep following the injection of any of the fractions of these separated antigens, might indicate a range of molecular weights in which protective antigens could be found. This technique has been used successfully for the identification of protective antigens in the oncospheres of *T.ovis* (Johnson *et al.*, 1989).

As discussed in Chapter 4, the profiles of antigens recognized by immune and non-immune serum on Western blots did not clearly identify any antigens which may be involved in protection against *T.hydatigena*. In this chapter an experiment is described where a preparative SDS-PAGE gel containing *T.hydatigena* oncosphere antigen was divided into 3 parts and each part was used to immunize sheep prior to a challenge infection with *T.hydatigena* eggs.

6.2 MATERIALS AND METHODS.

While the test immunizations to be used were those consisting of antigen contained in polyacrylamide homogenized gel, controls were necessary to show that any resulting immunity was not due to the gel itself or to the presence of SDS, and also to indicate the level of protection resulting from immunization with the unfractionated antigen. For this reason, 2 antigen preparations were made which were not run on SDS-PAGE. Both were FTS oncosphere antigens and one of these was further treated with SDS.

6.2.1 Preparation of Antigens Not Run on SDS-PAGE.

FTS Oncosphere antigen was prepared as described in Section 2.2.3.3.

SDS-treated antigen was prepared in the same manner but, in addition, 10% (w/v) SDS was added and continuously mixed on a Matburn blood mixer for 2 hr at room temperature. Sample buffer (Appendix 2.1) was then added in the proportion of 1 volume of buffer to 4 volumes of antigen preparation, the 2 were thoroughly mixed and boiled for 5 min.

For injection, both antigens were homogenized with the water-in-oil adjuvant STM (Appendix 2.1) at a ratio of 1:1.

6.2.2 Preparation of SDS-PAGE Antigens.

Sufficient eggs to provide at least 10 million hatched embryos were washed in saline, deposited by centrifugation at 700 x g for 5 min and divided into 2 approximately equal portions for hatching. The portions were hatched separately to make it easier to monitor the hatching process.

The products from the two hatchings were combined and resuspended in McCoy's medium to a final volume of 50 ml. Ten 5 µl aliquots were taken with thorough mixing between each sampling and hatched oncospheres counted in a haemocytometer. The mean count multiplied by 10^4 yielded an estimated total of approximately 17.3×10^6 hatched embryos in the 50 ml. The embryos were concentrated by centrifugation and then frozen, thawed, sonicated, centrifuged and boiled in SDS as described in Section 2.2.1.7. The antigen preparation for use contained the equivalent of approximately 4.2 million oncospheres/ml.

Two 1.5 mm thick, 5-25% gradient SDS-PAGE gels were poured (Section 2.2.1.7); the stacking gels formed 2 troughs and 3 marker lanes (Figure 6.1). On one gel, 8 µl of Pharmacia low molecular weight markers were added to each of the 3 lanes and 1.2 ml of the SDS-treated oncosphere antigens (approximately 5×10^6 oncosphere equivalents) were loaded into each trough. The second gel was to be run without antigen to provide a control of SDS-polyacrylamide gel alone. Both gels were run for 1 hr at 100 V followed by 16 hr at 200 V.

The stacking gels were then carefully removed from the resolving gels. The marker lanes were cut from the gel (Figure 6.2), stained with Amido Black and destained

Figure 6.1 **The positioning of the troughs and lanes of the stacking gels.**

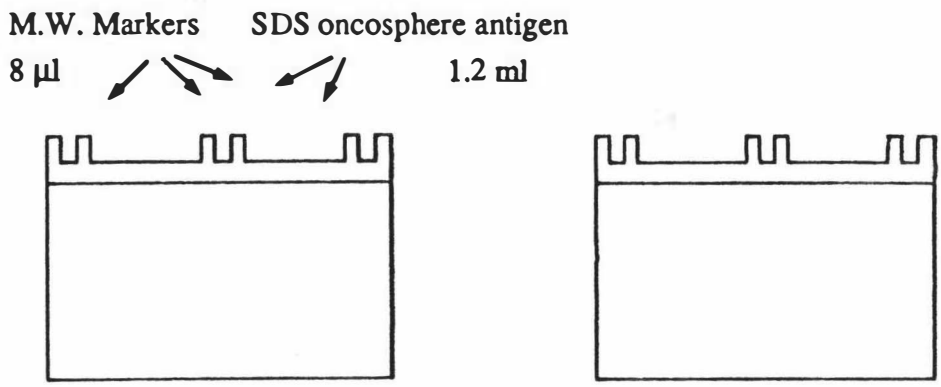


Figure 6.2 **The stacking gel was discarded and the marker lanes were removed to be stained.**

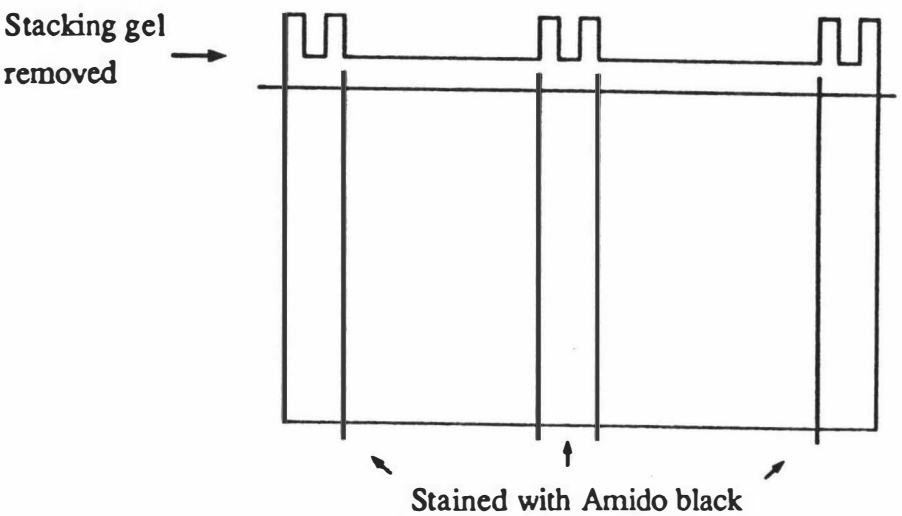
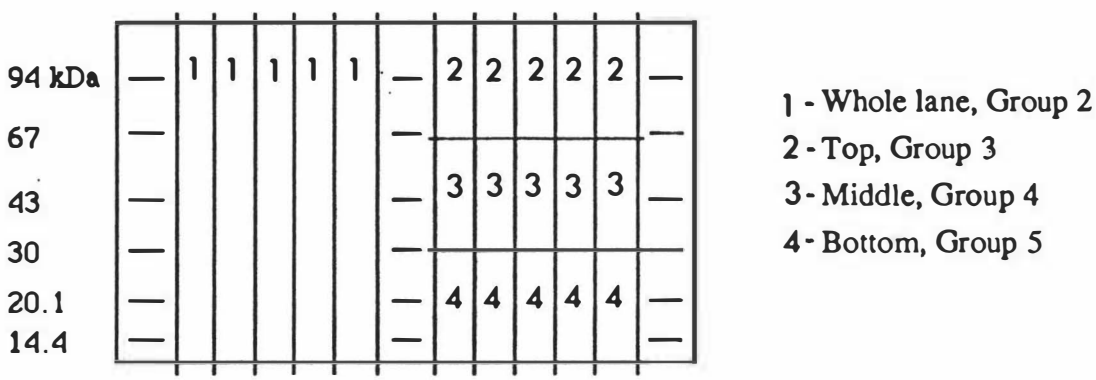


Figure 6.3 **The division of the gel containing *T.hydatigena* oncosphere antigen for use in immunizing 12 sheep.**



while the rest of the gel was kept in running buffer at 4°C. The alcohol in the staining and destaining solutions causes some shrinkage of the strips of gel containing the markers. This was reversed by soaking the strips in 50% methanol/50% distilled H_20 for approximately 10 min until the strips returned to their original sizes. The markers were then placed in their original positions alongside the gel containing the antigen (Figure 6.3). The right half of the gel was cut transversely just below the 67 kDa marker and also in line with the 30 kDa marker, and both the left and right sides were cut into 5 equally-sized vertical strips (Figure 6.3). The outermost strips were transferred onto nitrocellulose membrane using the transblot system (Section 2.2.1.7) and probed with immune sheep serum (Figure 6.4). The proteins remaining on these gel strips were stained with Amido Black (Figure 6.5).

The remaining strips were individually chopped into small pieces, made up to 3 ml with saline and homogenized with 3 ml of the water-in-oil emulsion (STM, Appendix 2.1), before being loaded into a syringe.

6.2.3 Immunization of Experimental Animals.

Twenty-six 1- to 2-year-old ewes and wethers were run on pastures and divided into 5 groups of 4 sheep, which were to receive preparations of homogenized SDS-PAGE gel, and 2 groups of 3, receiving antigens not run on an SDS-PAGE gel.

Table 6.1 The nature of the immunizations given.

Group	n	Immunizing Antigens
1	4	Control - SDS-polyacrylamide gel containing no antigen.
2	4	Whole Lane - The complete antigenic profile of *T.hydatigena* oncosphere antigen.
3	4	Top - Antigens of molecular weights of 67 kDa and greater.
4	4	Middle - The antigens from below 67 kDa down to and including 30 kDa.
5	4	Bottom - The antigens of molecular weights of below 30 kDa.
6	3	FTS oncosphere antigen.
7	3	SDS treated-FTS oncosphere antigen.

Figure 6.4 **Western blot of *T.hydatigena* oncosphere antigen probed with immune sheep serum and showing the division between top, middle and bottom fractions of the gel.**

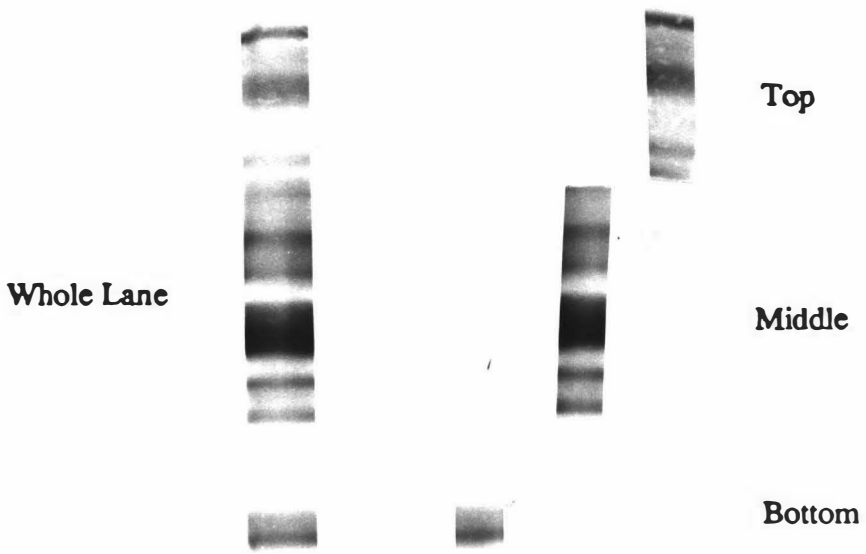
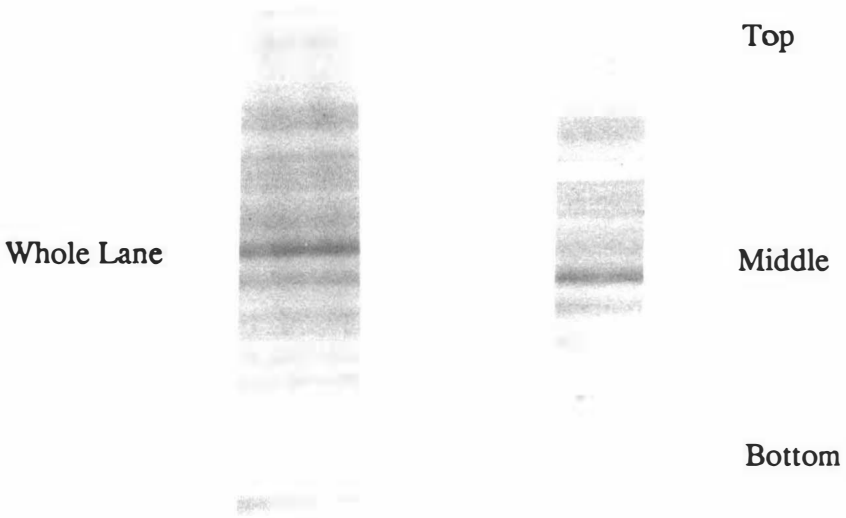


Figure 6.5 **The SDS-PAGE gel stained with Amido black.**



Each sheep in Groups 2 - 7 received antigen fractions from a total of 10^6 oncosphere equivalents. The antigen preparation for each sheep was divided into 2 equal volumes; these were given on day 0 and day 14 and each was divided into intramuscular and subcutaneous injections of approximately equal volume.

6.2.4 Monitoring the Antibody Response.

Western blots of *T.hydatigena* oncosphere antigen run on 5 - 25% gradient SDS-polyacrylamide gels were probed with the serum from pre-immunization blood samples (day 0) and samples taken 14 days after the second injection (day 28). Sera from blood samples taken on day 0, day 14 and day 28 were also tested for anti-oncosphere antibody by ELISA using FTS oncosphere antigen on the plates.

6.2.5 Challenge Infections.

Since the blots and ELISA results indicated that all sheep had produced anti-*T.hydatigena* antibody in response to the immunizations, the sheep were challenged orally on day 30 with 150 activatable oncospheres and 5 weeks later were necropsied as previously described (Section 2.2.1.12).

6.2.6 Statistical Analysis.

The numbers of cysts present in the peritoneal cavities of the sheep were compared by analysis of variance and Tukey's test for pairwise comparisons (Appendices 6.2, 6.3 and 6.4) following square-root transformation of the data as described in Section 2.2.4. The rejection level used for significance was 5%.

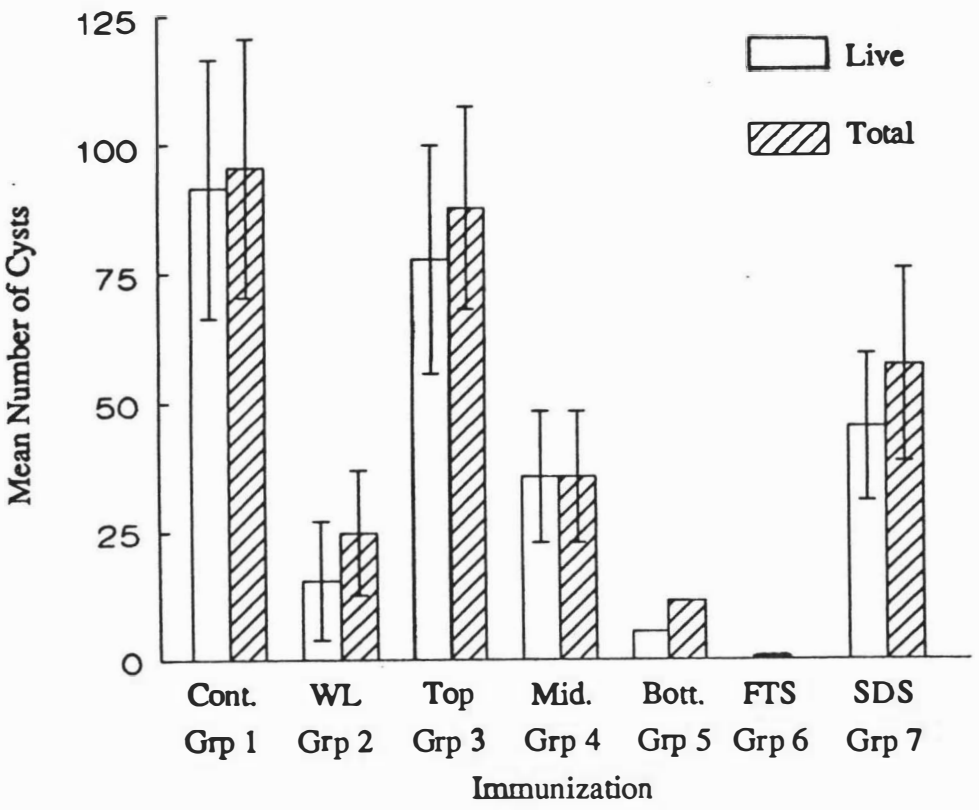
6.3 RESULTS.

6.3.1 Cyst Numbers Found at Necropsy.

The results are shown in Figure 6.6 and the data are presented in Appendix 6.1. Due to the accidental breakage of some containers, the peritoneal washings of one animal in Group 4 and 2 of the animals in Group 5, could not be assessed and therefore the total numbers of cysts could not be determined.

The number of dead cysts found in the peritoneal cavities were few and the number of them was not significantly different in any of the groups ($p > 0.05$). Due to the

Figure 6.6 **Numbers of cysts present in the peritoneal cavities of the sheep immunized with SDS-PAGE gel fractions and controls.** (Mean ± S.E.).



low numbers of replicates in some groups, statistically significant differences in total cyst counts were only shown to exist when comparing Groups 1, 3 or 7 (Controls, Top and SDS, respectively) with Group 6 (FTS) (Appendix 6.4). However, when comparing the numbers of live cysts recorded, other significant differences were also present (Appendix 6.3). Immunization with Groups 2, 5 and 6 (Whole Lane, Bottom and FTS, respectively) resulted in significantly lower numbers of cysts surviving compared with Group 1 (Control), and the numbers surviving after immunization with Groups 5 and 6 (Bottom and FTS, respectively) were significantly fewer than those surviving after immunization with both Groups 1 and 3 (Control and Top, respectively). The sheep immunized with FTS oncosphere antigen (Group 6) were completely protected against challenge infection, with no cysts being found, and no lesions or scars were seen on the livers of any of the sheep.

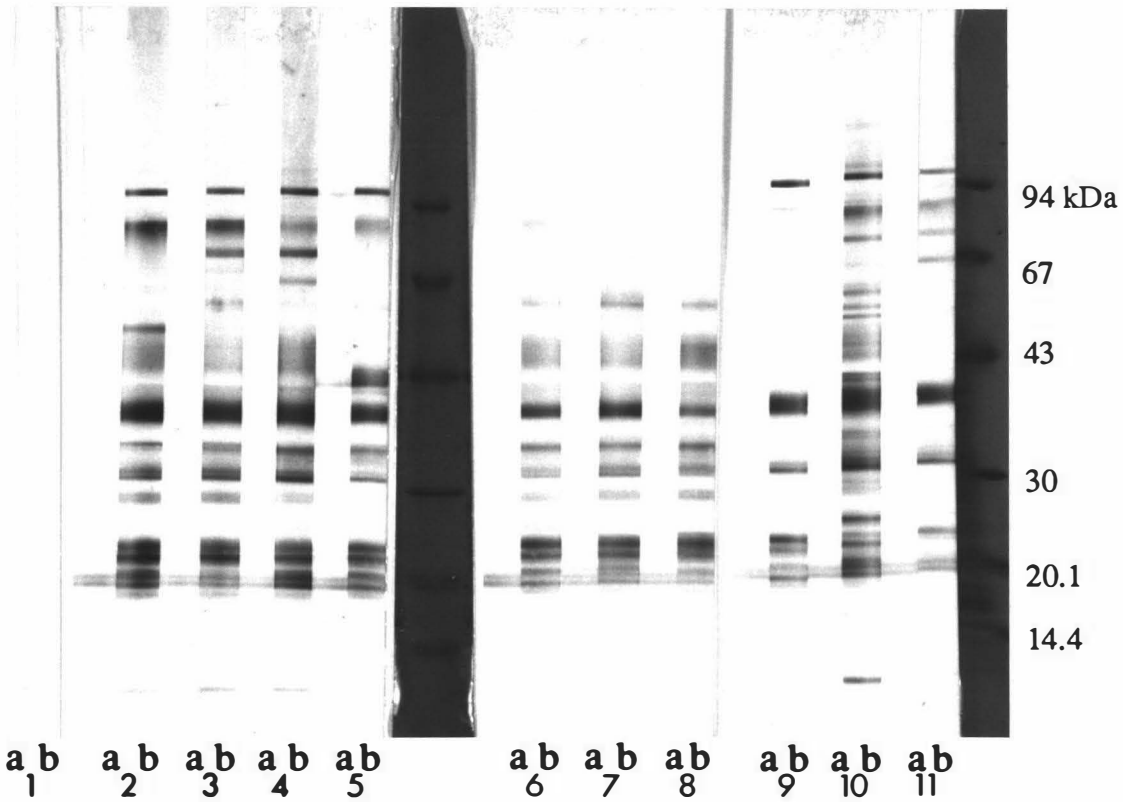
The cyst numbers in the recipients of the middle fraction (Group 3) appear to be substantially reduced compared with the numbers of cysts in the controls, however, this reduction was not found to be significant. The numbers of cysts found in the recipients of the top fraction (Group 4) were not significantly different from the controls either.

6.3.2 **Antibody Responses, Before and After Immunization.**

Figure 6.7 shows the antibody responses of all the sheep tested on Western blots. The sera from all groups, apart from Group 1 and one sheep in Group 3 (1502), reacted with the *T.hydatigena* oncospheral antigen by 28 days after the first immunization. Sheep 1502 also showed very little antibody response on ELISA (Figure 6.8) as did another from this Group (1518). These sheep also had the largest numbers of cysts in the Group (105 and 94 cysts, respectively).
The sheep in Group 3 which was found to have been infected previously (1672), gave no indication of previous infection in the pre-infection serum sample, but after immunization, several bands were present at lower molecular weights compared with the other animals in this group which had none in this region (Figure 6.7). None of these bands present at the lower molecular weights could be identified as protective when compared with the bands of antigen recognized by the serum of sheep which were protected.

The 28 day sera of the sheep which received FTS antigen (Group 6) reacted with very few antigens above 67 kDa. All of these sheep were protected. The sheep of Group 3, which received gel from this high molecular weight area, and had

Figure 6.7 **Western blot of *T.hydatigena* oncosphere antigen probed with the serum collected from each sheep before immunization and 28 days after immunization.**



Lanes	Recipients of immunizations.
a	Serum collected before immunization began
b	Serum collected 28 days after the first immunization
1	Pooled controls (Group 1)
2	Whole Lane (Group 2) sheep no. 1619
3	Whole Lane (Group 2) sheep no. 1559
4	Whole Lane (Group 2) sheep no. 1633
5	Whole Lane (Group 2) sheep no. 1720
6	FTS (Group 6) sheep no. 1565
7	FTS (Group 6) sheep no. 1693
8	FTS (Group 6) sheep no. 1741
9	SDS (Group 7) sheep no. 1582
10	SDS (Group 7) sheep no. 1641
11	SDS (Group.7) sheep no. 1699

Figure 6.7 (ctd)

Lanes	Recipients of immunizations.
a	Serum collected before immunization began
b	Serum collected 28 days after the first immunization
12	Pooled controls (Group 1)
13	Top (Group 3) sheep no. 1502
14	Top (Group 3) sheep no. 1646
15	Top (Group 3) sheep no. 1518
16	Top (Group 3) sheep no. 1672
17	Middle (Group 4) sheep no. 1621
18	Middle (Group 4) sheep no. 1533
19	Middle (Group 4) sheep no. 1660
20	Middle (Group 4) sheep no. 1697
21	Bottom (Group 5) sheep no. 1539
22	Bottom (Group 5) sheep no. 1649
23	Bottom (Group 5) sheep no. 1538
24	Bottom (Group 5) sheep no. 1508

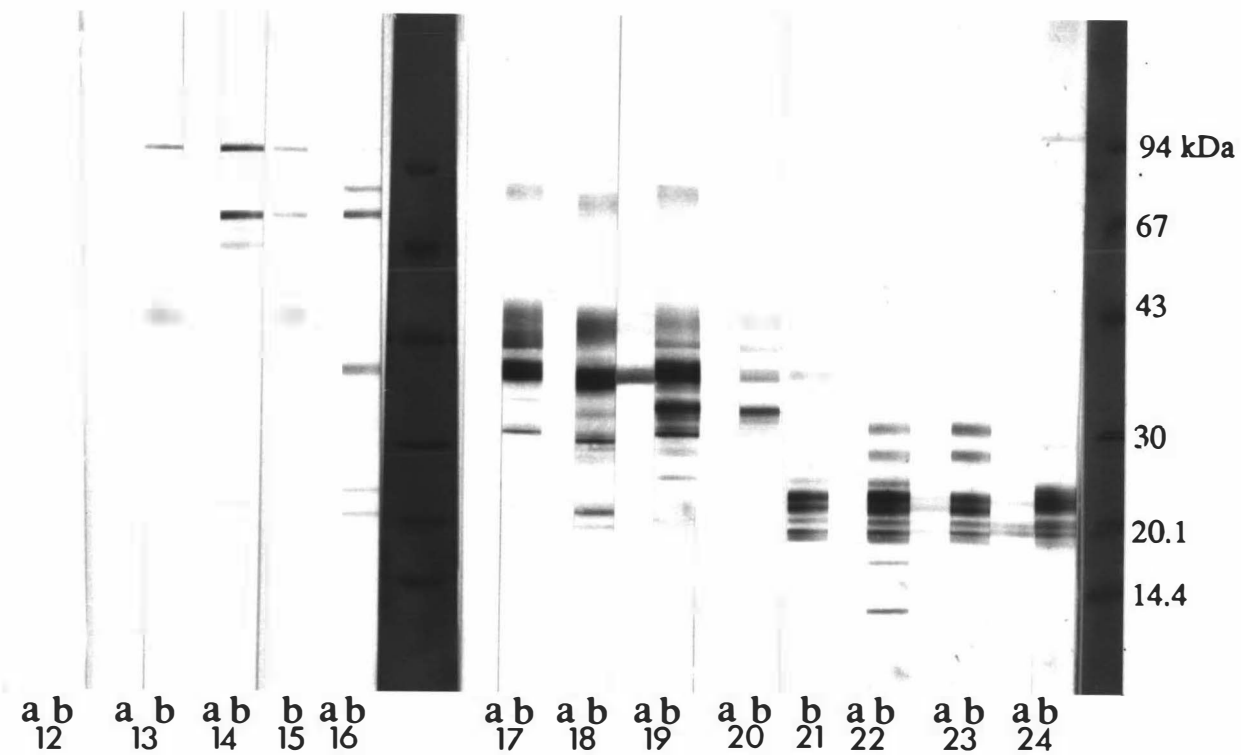
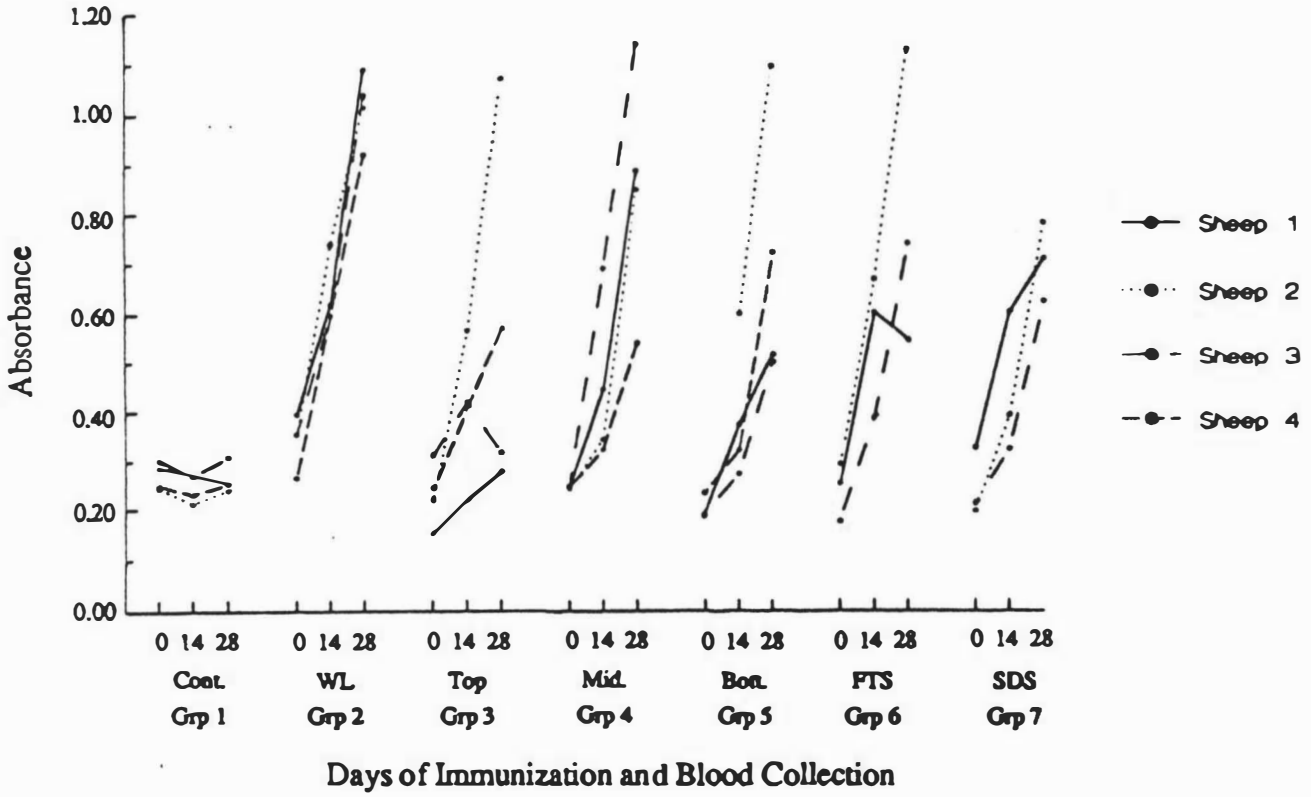


Figure 6.8 The level of anti-*T.hydatigena* antibody in the sera of the immunized sheep.



Sheep Number:		Sheep Number:	
Controls		**Top**	
1	1521	1	1502
2	1558	2	1646
3	1773	3	1518
4	1542	4	1672
Whole Lane		**Middle**	
1	1619	1	1621
2	1559	2	1533
3	1633	3	1660
4	1720	4	1697
FTS		**Bottom**	
1	1741	1	1539
2	1693	2	1649
3	1565	3	1538
		4	1508
SDS			
1	1699		
2	1641		
3	1582		

produced antibody against these antigens, were not significantly protected against the challenge infection. While the numbers of cysts in the recipients of the middle fraction (Group 4) were fewer than the in controls, this reduction was not significant.

6.4 DISCUSSION

The size of oral challenge infection given to the sheep in this experiment was 150 activatable oncospheres; this number was given in an attempt to produce approximately 100 cysts in each of the control sheep. It was thought that the effects of the different immunizing antigens might be distinguished with a challenge of this size, keeping in mind the extreme variation in the number of cysts established in the different sizes of challenge given in the experiments described in Chapter 2. Four of the 7 sheep in the 2 groups which shows the least protection, Groups 1 and 3 (Control and Top, respectively) harboured between 101 and 163 cysts, while the range of cyst numbers in the remaining 3 sheep was 49 to 66.

One of the sheep which received immunizations of the fraction of gel above 67 kDa (Group 3) was found to have been previously infected with *T.hydatigena*. The Western blot probed with the serum collected from this sheep 28 days after the first immunization showed that several bands of antigens below the 67 kDa molecular weight marker were recognized (Figure 6.7). Two of the sheep which received the middle fraction of gel also recognized some lower molecular weight antigens and the serum of one of the sheep receiving the bottom fraction of gel recognized a higher molecular weight antigen. There was no evidence that these latter sheep had been previously infected. An explanation for the recognition of these antigens which were not included in the fraction of gel used to immunize the animals, may be that epitopes contained in the gel which induced the production of antibodies, were shared by antigens of other molecular weights and therefore recognized by the serum.

The *T.hydatigena* oncosphere antigens used to immunize the sheep in Groups 2 (Whole Lane) and 7 (SDS Treated) were prepared in very similar ways yet the Whole Lane preparation induced significant protection while the SDS preparation did not. The Whole Lane Preparation had been centrifuged at 100 000 x g to remove insoluble material and while it also had been boiled in SDS sample buffer (approx. 10% SDS), the process of separating the antigens on SDS-PAGE would have reduced this SDS content to 0.1%. The reduction of the SDS content may have

allowed proteins which had been denatured by the SDS to become sufficiently reconstituted to enable the induction of protection. Another difference between these preparations was that the Whole Lane antigen was incorporated into the polyacrylamide gel which may have acted as a second adjuvant and therefore induced a stronger immune response.

The successful immunization of sheep with oncosphere antigens separated on SDS gel was only achieved with the fractions containing antigens below 30 kDa. There may also be antigens of between 67 and 30 kDa which may contribute to the induction of protective immunity but in these experiments were not able to induce significant protection. The bottom fraction contained only about 3 bands of antigen which were recognized by the immune serum (Figure 6.7). More experiments are needed to investigate this further since the results strongly suggest the presence of protective antigens in this fraction.

CHAPTER 7

GENERAL DISCUSSION.

T.hydatigena is prevalent in most areas of the world and results in economic losses in many countries involved in livestock production, primarily through the damage to infected lambs' livers. Theoretically, control of this parasite could be achieved by simply preventing the access of canids to the larval stages in the abdominal cavities of intermediate hosts. In practice, this is more easily said than done, even in a country such as New Zealand where there are no feral canids and wild intermediate hosts are comparatively scarce, and where there is a relatively high level of control of dog feeding. As a result of the hydatids eradication campaign, *T.hydatigena* is now very much less common in New Zealand than it was. However, with recent changes in the dog treatment system, introduced by the National Hydatids Council, and further changes likely to follow the introduction of *T.ovis* vaccination in the near future, it is possible that there will be a resurgence in the prevalence of *T.hydatigena*. In most other countries, no effective control measures are employed against this parasite.

A high degree of protection results in sheep when they are immunized with antigens derived from the oncospheres of *T.hydatigena* (Gemmell, 1962a, 1964a, 1964b, 1965b, 1966, 1969a; Heath, 1978; Onawunmi and Coles, 1980) indicating that vaccination could be a practical control procedure. However, it would not be practicable to produce a vaccine from these antigens because of the large numbers of oncospheres which would be required. On the other hand, it should now be possible to genetically engineer protective antigens by means of recombinant DNA technology. Gemmell *et al.* (1969) suggested that antibody may be important in the expression of resistance to *T.hydatigena* in sheep. Obviously, the involvement of antibody would greatly facilitate the identification of potentially protective antigens. One of the aims of this study was, therefore, to establish, clearly, the importance of antibody in the immune response.

The production of an effective vaccine would not only require the identification of the appropriate antigens, but also knowledge of the mechanisms involved in the immune response of the host to the infection since this may affect the way in which the antigens are presented to the recipient animal to achieve maximum efficiency. For this reason, the involvement and interactions of complement and sheep leukocytes with antibody were also examined.

Antibody was, indeed, found to be important in the immunological destruction of oncospheres both *in vivo* and *in vitro*. The findings described in Chapter 2 are consistent with those of Blundell *et al.* (1968) who showed that serum from sheep immunized with activated oncospheres was able to protect the recipients of this serum. It was also found in the experiments reported here that the serum of sheep immunized by oral infection is able to protect the recipients; this has not been shown previously. The short-acting protection in lambs suckling ewes which had been orally infected with *T.hydatigena* and the lack of this by hyperinfection of the ewes, appears to be very similar to the results of Heath (1978) in which the lambs from infected ewes were protected for a short time and the protection of the lambs from ewes which had been hyperimmunized was not increased. This contrasts with the results of Gemmell *et al.* (1969) colostral transfer experiments, which showed that naturally infected ewes did not confer any immunity to their lambs via colostrum but that ewes immunized with activated oncospheres did provide short-acting immunity. This may be explained by the finding in the present study that the level of antibody at the time of challenge is important, a possibility suggested by Gemmell *et al.* 1969). This was clearly evident from comparisons of the levels of antibody present in the serum donors and recipients and the protection achieved in Experiments 1, 2 and 3 of Chapter 2. It is probable, therefore, that the naturally infected ewes in Gemmell's experiments had lower levels of antibody in their serum than did those of Heath's experiments. The stimulation of Gemmell's naturally infected ewes by immunization evidently boosted their antibody levels sufficiently to enable the lambs to obtain enough antibody from the colostrum to protect them from infection. In contrast, the hyperimmunization of Heath's ewes could not enhance the immunity passed to the lambs from the orally infected ewes.

It also appears that there is a critical level of antibody required before significant protection is achieved, and below which little protection operates. This is indicated by the relationship between the antibody levels and the number of *T.hydatigena* larvae resulting from challenge infection as described in Chapter 4 and illustrated in Figure 4.7. Interestingly, a similar situation was found in the culture experiments described in Chapter 5 in which a critical level of IgG_2 was apparently needed before significant reductions in the survival of the oncospheres resulted.

In vitro, antibody was shown to be ineffective in the absence of complement or leukocytes. The most significant decreases in oncosphere survival occurred when complement was present together with antibody, a situation which has also been shown to be the case with other taeniids, such as *T.pisiformis*, *E.granulosus*, and *T.taeniaeformis* (see Section 1.3.2.6 for references). The dependence of antibody

activity on complement would be difficult to demonstrate *in vivo* in sheep although, theoretically, an experiment similar to that of Musoke and Williams (1975) involving injections of cobra venom factor into rats, could be repeated on a larger scale.

While the manner in which leukocytes have an effect *in vitro* is unclear, their effectiveness in reducing larval survival in the presence of antibody was evident. The particular types of leukocytes involved were not identified in the present experiments but neutrophils have been reported to cause the death of *T.hydatigena* oncospheres in the presence of immune serum *in vitro* (Beardsell and Howell, 1984). *In vivo*, eosinophils have been implicated in the immune response against other taeniid oncospheres (Freeman, 1964; Heath and Pavloff, 1975; Ansari and Williams, 1976; Siebert *et al.*, 1979), and helper and cytotoxic T-lymphocytes are present in the lesions surrounding migrating *T.hydatigena* larvae in the liver (Meeusen *et al.*, 1989).

To increase what is known of the immunology of the sheep's response to infection with *T.hydatigena*, the involvement of leukocytes requires more investigation. The types of leukocytes involved could be examined by culturing activated oncospheres and assessing their survival in the presence of purified populations of leukocytes, or by identifying by electron microscopy or specific antibodies or stains, the leukocyte types which adhere to the oncospheres in cultures containing mixed leukocytes. Electron microscopy could also be used to study the nature of the damage inflicted by antibody, complement and leukocytes *in vitro*. It is unfortunate that a method of assessing oncosphere death within 24 - 48 hr in the presence of leukocytes, could not be established. It is generally accepted that the destructive action of antibody and complement is very rapid. Assessing oncosphere death within 24 hr and at intervals throughout the culture period, with the appropriate controls, would provide a great deal of information concerning the kinetics of the effects of these components of the immune system *in vitro*. If leukocytes prove to be an important part of the sheep's immunity to infection, the timing of the leukocyte attack on oncospheres is of interest, since inducing early destruction of the oncospheres or larvae is preferable in order to minimise liver damage.

The apparently greater ability of IgG_2, rather than IgG_1, to kill *T.hydatigena* oncospheres is of particular interest in relation to the duration of colostral immunity. With *T.ovis* infections, the duration has been recorded as ranging from 6-16 weeks (Rickard and Arundel, 1974; Rickard *et al.*, 1977b; Heath *et al.*, 1979c; Sutton, 1979). This strongly suggests that IgG_1, which is the major immunoglobulin

present in colostrum, plays a much greater role in protecting against *T.ovis* infections than in protecting against *T.hydatigena*. It would be interesting to compare the activities of these two immunoglobulin classes against *T.ovis* oncospheres *in vitro*.

The involvement of IgG_2 in protection against *T.hydatigena* infection as described, is entirely consistent with what is known of its biological characteristics. It is able to fix sheep complement (as does IgG_1) (Jonas, personal communication) and it binds to the Fc receptors of neutrophils (Watson, 1975).

The nature of an antigen may be what determines the class of antibody which is produced. Unlike the protective antigens of *T.ovis* (Johnson *et al.*, 1989), a large proportion of the antigens of *T.hydatigena* which are recognized by immune sheep serum on a Western blot (particularly below 30 kDa in molecular weight), are probably not proteins. This conclusion is based on the fact that these major bands recognized by serum did not correspond to any of the bands stained by protein stains. Unfortunately, nitrocellulose containing separated *T.hydatigena* antigens was not available at the time for staining with a carbohydrate stain to confirm the nature of these major bands. It has been reported that of the IgG subclasses in mice, predominantly IgG_3 is produced in response to carbohydrate antigens (Slack *et al.*, 1980). It is possible that, in sheep, IgG_2 may be the predominant subclass stimulated in response to carbohydrate antigens.

A preliminary experiment to identify the range of molecular weights of the antigens which induce protection, provided information upon which further experiments may be based. The antigens of lower molecular weights (below 30 kDa) in these experiments induced significant protection and it was found that very few protein antigens exist in this region. While the protection achieved in this experiment was significant, inconsistencies in the recognition of bands of antigens revealed by the Western blots probed with the immune serum of different sheep, show that difficulties may arise in the identification of antigens which will induce protection in the majority of sheep. For this reason the gel-cut-out experiments described in Chapter 6 need to be repeated and extended to ensure that this result was not just peculiar to the few sheep involved.

Apart from the mechanics of the immune response to an infection with *T.hydatigena*, also of interest, is where this immunity might be operating. Campbell (1936) was the first to suggest that two phases of immunity may be operating against the larvae of *T.taeniaeformis*: one which acts on the oncospheres and larvae

before they become established in their site of predeliction, i.e. pre-encystment or early; and the other which acts on the larvae after they have become established in their site of predeliction, i.e. post-encystment or late. In a range of experiments involving the immunization of sheep with a variety of antigen prepartations, and the colostral transfer of immunity, Gemmell and his collegues (see Section 1.3.2.1 for references) have produced evidence suggesting that both pre- and post-encystment immunity may operate although this appeared to vary with the type of antigen preparation used. It appears that the immunity induced in each of the experiments described in this thesis operated before the larvae became established in the peritoneal cavity since there were no significant differences between the numbers of dead larvae found at necropsy in the groups within each experiment, and these were generally few. The early effect of the immunity was also shown by the absence of larvae, in the peritoneal cavities of the immune lambs of Experiment 3 in the serum transfer experiments of Chapter 2, and in some of the lambs born to immune ewes in Chapter 4. The fact that the livers of these animals also showed little evidence of an infection indicates that the larvae were destroyed before they had caused enough damage for gross lesions to result. The number of dead larvae in the peritoneal cavities of the sheep in these experiments may not, however, be comparable to those in Gemmell's experiments since necropsy was performed only 4 weeks after challenge, whereas in Gemmell's experiments necropsy was after 8 or 12 weeks.

Given that this immunity against *T.hydatigena* infections occurs before the larvae become established in the peritoneal cavity, the death of the invading parasites must occur in the intestinal wall, the blood stream or the liver parenchyma. The indications that taeniid oncospheres are destroyed in the intestinal wall of an immune animal before entering a venous capillary, are far from conclusive (see Section 1.3.2.7). The likely sequence of events leading to the rapid death of oncospheres would involve immunoglobulin (possibly IgG_2 being the most effective) binding to the oncospheral surface leading to complement fixation. If *T.hydatigena* oncospheres secrete the complement inhibiting factors which are contained in the larvae (Hammerberg *et al.*, 1976), this may enable some oncospheres to survive at this stage. Leukocytes can adhere to the surface of antibody coated oncospheres and may destroy oncospheres which have avoided the initial effect of the complement. They may also aid in the destruction of oncospheres previously damaged by the complement. In animals with lower levels of anti-*T.hydatigena* oncosphere antibody, destruction of oncospheres may take longer and the prevention of the oncospheres reaching the stage where they become resistant to the antibody-mediated damage may be less effective. This resistance to antibody-mediated damage has been shown to occur within a few days of activation

in *T.taeniaeformis* and *Hymenolepis nana* (see Section 1.3.2.6 for references). It is possible, therefore, that the addition of complement and leukocytes to the cultures described in Chapter 3, on the third and sixth days of culture may have had no effect on the survival of the larvae.

Once in the liver, the developing and burrowing larvae damage the liver tissue and evoke a cellular response. Not all larvae, having reached the liver, are destined to survive. In the present study, degenerating larvae were found in some of the liver lesions. Whether death at this stage is attributable to the damage caused by the earlier complement and cellular attack, or to subsequent immunological attack is unknown but may involve a combination of the two. The cause of the death of larvae which have reached the peritoneal cavity and become established there is also, as yet, unknown.

The information presented here provides a considerable advance in the understanding of the basis of the immune response of sheep to *T.hydatigena* and a preliminary step in the identification of protective antigens. This represents just the beginning of the development of a vaccine and further research is now needed. Given the results of the work presented here, and the recent successful production of a genetically engineered vaccine for *T.ovis*, there is every reason to expect that an effective vaccine for *T.hydatigena* can also be produced.

Appendix 1.1 Estimated potential cost of uncontrolled *T.hydatigena* to New Zealand per annum. (Lawson *et al.*, 1986)

Assuming that 60% of lamb and sheep livers are damaged and downgraded to pet food:

Average price of lamb's liver	= 92c
Average price of sheep's liver	= 45c
Average price received for a downgraded liver	= 42c

therefore:

Average loss for a downgraded lamb liver	= 50c
Average loss for a downgraded sheep liver	= 03c

Total Kill:

34 million lambs.	60% of 34 mill. = 20.4 mill.		
	20.4 mill. x 50c loss	=	$10,200,000
9 million sheep.	60% of 9 mill. = 5.4 mill.		
	5.4 mill x 03c loss	=	162,000
	Total Loss	=	$10,362,000

Appendix 2.1 Reagents.

1. **Acetate buffer, 0.06 M, pH 4.**

Solution A.	0.2 M acetic acid (11.55 ml in 1 L distilled H_2O)
Solution B.	0.2 M sodium acetate (16.4 g of $C_2H_2O_2Na$ or 27.2 g of $C_2H_2O_2Na.3H_2O$ in 1 L distilled H_2O)

Add 41 ml of A to 9 ml of B, adjust to pH 4, dilute to 600 ml.

2. **Amido Black stain**

Amido Black	0.1 g	
Ethanol	20 ml	(20%)
Acetic acid	7 ml	(7%)
Distilled H_2O up to	100 ml.	

Amido Black destain

Ethanol	20 ml
Acetic acid	7 ml
Distilled H_2O up to	100 ml

3. **Amino-ethyl carbazole (AEC)** (High sensitivity peroxidase substrate)

a. Stock AEC solution:

AEC	100 mg
dimethylformamide	10 ml

Dissolve AEC and store at 4°C in the dark.

b. 50 mM acetate buffer, pH 5.0

Solution A.	0.2 M acetic acid
Solution B.	0.2 M sodium acetate

Add 14.8 ml of A to 35.2 ml of B, adjust pH to 5.0 with either solution. Dilute the mixture 1:4 to make 50 mM.

c. For use

AEC stock	2 ml
50 mM acetate buffer, pH 5.0	50 ml

Add acetate buffer to the AEC, and add

H_2O_2 (30%)	10 µl

4. **Artificial gastric fluid (AGF)**

Pepsin activity = 1 Anson unit/g (BDH Chemicals Ltd., Poole, England)	1 g
HCl (Concentrated)	1 ml
0.15 M NaCl	99 ml

5. **Artificial intestinal fluid (AIF).**

$NaHCO_3$	0.2 g
Naive Lamb bile (centrifuged)	1 ml
Pancreatin porcine pancreas Grade IV activity = 4 x NF (SIGMA Chemical Co., St Louis)	0.2 g
Distilled H_2O	10 ml

Pancreatin is dissolved in distilled H_2O (2% w/v) and centrifuged at 1,500 x g for 20 min. The $NaHCO_3$, bile and pancreatin are sterilized by being filtered through progressively finer filters up to 0.2 μm. Store frozen at -20°C.

6. **Brilliant Crocein Scarlet**

Brilliant Crocein MOO (Aldrich Chemical Co. Inc., Milwaukee)	250 mg
Brilliant Blue R (SIGMA Chemical Co., St Louis)	15 mg

Dissolve at 60°C in a solution of 5% acetic acid and 3% trichloroacetic acid. Make up to 100 ml.

7. **Buffered water (0.0067 M, pH 6.8) for Giemsa stain.**

KH_2PO4 (0.67 M)	4.5 ml
$Na2HPO_4$ (0.67M)	5.5 ml
Distilled H_2O up to	1000 ml

8. **Carbonate buffer, pH 9.9.**

	<u>10 x Stock</u>
Na_2CO_3	15.895 g
$NaHCO_3$	42.005 g
Distilled H_2O up to	5000 ml

For use, dilute as follows:

Carbonate 10 x Stock	300 ml
Methanol	600 ml
Distilled H_2O	2100 ml

9. **Coating buffer, pH 9.6.**

NaCl	29.22 g
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

Dissolve in distilled H_2O, adjust pH with HCl or NaOH, and make up to 1000 ml. Keep stoppered.

10. **Coomassie Blue stain**

Coomassie Blue R-250 (BDH Chemicals Ltd., Poole, England)	1 g
Methanol	500 ml
Acetic acid	100 ml
Distilled H_2O	400 ml

Filter through Whatman No. 1 filter paper.

Coomassie Blue Destain

Methanol	500 ml
Acetic acid	100 ml
Distilled H_2O	400 ml

11. **Enzyme Inhibitors** (100 x concentrate)

Iodoacetamide	370 mg
Aprotinin	1 ml
Pepstatin	2 mg
TpCK	50 mg
TLCK	50 mg
EDTA	744 mg
PMSF	174 mg
distilled H_2O up to	10 ml

For use in antigen dilute 1:100.

12. **McCoy's 5A medium (modified)** (GIBCO)

Powdered sachet made up as directed with the following added:

Penicillin	100 U/ml
Streptomycin	100 ug/ml
Gentamycin	50 U/ml
Glucose	2 g/L

13. **Phosphate buffered saline, 0.15 M, (PBS).**

NaCl	40 g
KCl	1 g
Na_2HPO_4	5.75 g
KH_2PO_4	1 g

Dissolve in distilled H_2O, adjust to desired pH with HCl or NaOH, and make up to 5000 ml.

14. **Ponceau S stain.**

Ponceau S (BDH Chemicals Ltd., Poole, England)	0.5 g
Acetic acid	1 ml
Distilled H_2O up to	100 ml

Destain with distilled H_2O.

15. **Saline trypsin-versene.**

Trypsin, activity = 1:250 USP (GIBCO, NY. Cat. No. 840-7072)	0.1%
Versene (Tetrasodium EDTA) (SIGMA Chemical Co., St Louis)	0.02%

Dissolve in 1 L PBS, pH7.2 - 7.4, for 1 - 2 hr and filter (0.2 μm). Store frozen.

16. **SDS Polyacrylamide gel electrophoressis (PAGE) stock solutions.**

a. Acrylamide stock.

Acrylamide (2x) (PAGE Purity) (Serva Feinbiochemica GMBH & Co., Heidelberg)	195 g	(48.75%)
BIS (N,N'-Methylene-bis-acrylamide)	5 g	(1.25%)

Electrophoresis Purity Grade
(Bio-Rad Laboratories, Richmond, CA)
Distilled H_2O up to 400 ml

Dissolve in warm water bath with stirring. Filter through Whatman No.1 filter paper. Store at 4°C for up to 2 months in a dark bottle.
If crystallized when stored, warm before use.

b. Tris/glycine pH 8.3 (10 x stock).

Tris (hydroxymethyl)-methylamine Analar Biochemical grade (BDH Chemicals Ltd., Poole, England)	30.3 g	(3.03%)
Glycine	144.1 g	(14.41%)
Distilled H_2O up to	1000 ml	

Dissolve and adjust pH to 8.3 with HCl.
For use in SDS PAGE systems, dilute 1:10 and add

SDS	1 g/L	(0.1%).

c. Tris gel buffers (1 M).

Tris	30.3 g	(12.12%)
Distilled H_2O up to	250 ml	

Adjust the pH with HCl.
Resolving gel buffer = 1 M Tris/HCl, pH 8.8
Stacking gel buffer = 1 M Tris/HCl, pH = 6.8.

d. Tris gel buffer (1.5 M)

Tris	54.45 g
Distilled H_2O up to	300 ml

Adjust to pH 8.8 with HCl. Store at 4°C.

17. **Silver stain** (Blum *et al.*, 1987)

a. Fix

Methanol	100 ml
Acetic acid	24 ml
Formalin	100 µl
Double distilled H_2O	76 ml

b. Pretreatment

Sodium thiosulphate (0.1 M)	2 ml
Double distilled H_2O	248 ml

c. Impregnation solution

Silver nitrate	0.4 g
Formalin	150 µl
Double distilled H_2O	200 ml

d. Developing solution

Sodium carbonate	12 g
Formalin	100 µl
Sodium thiosulphate (0.1 M)	32 µl
Double distilled H_2O	200 ml

e. Stopping solution

Methanol	100 ml
Acetic acid	24 ml
Double distilled H_2O	76 ml

18. **Sodium barbitone buffer, pH 8.6.**

Na barbitone	33.5 g
EDTA	9.5 g
Sodium azide	2.5 g

Dissolve in deionized distilled H_2O, adjust pH to 8.6 and make up to 5000 ml.

19. **Substrate solution for ELISA**

a. Substrate buffer

A 0.1 M citric acid

B 0.1 M Na_2HPO_4

Add approximately 1 volume of A to 2 volumes of B.

Adjust to pH 5 using these two reagents.

b. O-phenylenediamine (OPD) stock.

OPD	100 mg
Methanol	2 ml

Store in the dark at -20°C.

c. Substrate

Substrate buffer (at 25°C)	25 ml
H_2O_2 (concentrated)	10 ul
OPD solution (stock)	200 ul

20. **Tris-buffered saline - high salt (TBS) (500 mM)**

Tris	2.24 g
NaCl	29.22 g
Distilled H_2O up to	1000 ml

Dissolve and adjust pH to 7.5 with HCl.

21. **Water-in-oil emulsion (Span, Tween, Marcol (STM)).**

A = 0.15 M NaCl

B = Mineral Oil Marcol 52^R (ESSO Netherlands) consisting of a mixture of paraffins and cycloparaffins in a ratio of 63:37.

C = Span 85^R (Sorbitan triolate) (ICI) and Tween 85^R (Polyoxyethylene 20 sorbitan triolate) (ICI). Ratio of 54:46.

Mix A, B and C in a ratio of 8:9:1 (v/v/v).

Appendix 2.2a **Analysis of variance table for the square-root transformed total numbers of cysts in the serum donors in Experiment 1.**

Variance Source	DF	F	P
Treatment	1	1.73	0.2592
Replicates	4	2.71	0.1790

Appendix 2.2b **Tukey pairwise comparisons of the square-root transformed numbers of cysts in the serum donors of Experiment 1.**

Treatment	Mean	Treatments not signif. different ($p > 0.05$)
Challenged donors	6.801	I
Unchallenged donors	5.433	I

Appendix 2.3a **Analysis of variance table for the square-root transformed numbers of live cysts in the recipients of Experiment 1.**

Variance Source	DF	F	P
Treatment	2	1.22	0.3436
Replicates	4	0.02	0.9994

Appendix 2.3b **Tukey pairwise comparisons of the square-root transformed numbers of live cysts in the recipients of Experiment 1.**

Treatment	Mean	Treatments not signif. different ($p > 0.05$)
Control Serum	4.580	I
Saline	3.369	I
Immune Serum	2.678	I

Appendix 2.4a **Analysis of variance table for the square-root transformed numbers of dead cysts in the recipients of Experiment 1.**

Variance Source	DF	F	P
Treatment	2	1.13	0.3687
Replicates	4	0.15	0.9579

Appendix 2.4b **Tukey pairwise comparisons of the square-root transformed numbers of dead cysts in the recipients of Experiment 1.**

Treatment	Mean	Treatments not signif. different ($p > 0.05$)
Immune Serum	4.377	I
Control Serum	3.645	I
Saline	2.870	I

Appendix 2.5a **Analysis of variance of the square-root transformed total numbers of cysts in the recipients of Experiment 1.**

Variance Source	DF	F	P
Treatment	2	1.14	0.3657
Replicates	4	0.17	0.9469

Appendix 2.5b **Tukey pairwise comparisons of the square-root transformed total numbers of cysts in the recipients of Experiment 1.**

Treatment	Mean	Treatments not signif. different ($p > 0.05$).
Control Serum	5.948	I
Immune Serum	5.517	I
Saline	4.610	I

Appendix 2.6a **Analysis of variance table for the square-root transformed numbers of dead cysts in the recipients of Experiments 2 and 3.**

Variance Source	DF	F	P
Treatment	3	0.33	0.8032

Appendix 2.6b **Tukey pairwise comparisons of the square-root transformed number of dead cysts in the recipients of Experiments 2 and 3.**

Treatment	Mean	Treatments not signif. different ($p > 0.05$).
Immune Serum, Expt 3	3.625	I
Saline	3.132	I
Control Serum	2.720	I
Immune Serum, Expt 2	2.218	I

Appendix 2.7a **Analysis of variance table for the square-root transformed numbers of live cysts in the recipients of Experiments 2 and 3.**

Variance Source	DF	F	P
Treatment	3	11.14	0.0003

Appendix 2.7b **Tukey pairwise comparisons of the square-root transformed numbers of live cysts in the recipients of Experiments 2 and 3.**

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.05$).	
Control Serum	13.90	I	
Saline	12.98	I	
Immune Serum, Expt 2	5.704		I
Immune Serum, Expt 3	5.436		I

Appendix 2.8a **Analysis of variance table for the square-root transformed total numbers of cysts in the recipients of Experiments 2 and 3.**

Variance Source	DF	F	P
Treatment	3	11.90	0.0002

Appendix 2.8b **Tukey pairwise comparisons of the square-root transformed total numbers of cysts in the recipients of Experiments 2 and 3.**

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.02$).
Control Serum	14.24	I
Saline	13.81	I
Immune Serum, Expt 3	6.806	I
Immune Serum, Expt 2	6.493	I

Appendix 3.1 **The numbers of larvae surviving after 10 days *in vitro* in each well with the mean ± S.E. given for each group.**

Serum and Complement		Leukocytes	Leukocytes from:	
		Absent	Control sheep	Immunized sheep
Control serum	-C'	43, 44, 59	45, 53, 56	31, 35, 39
		48.7 ± 5.2	51.3 ± 3.3	35.0 ± 2.3
	+C'	39, 46, 51	43, 47, 56	29, 33, 37
		45.3 ± 3.5	48.7 ± 3.8	33.0 ± 2.3
Immune serum	-C'	58, 67, 68	19, 22, 27	36, 37, 39
		64.3 ± 3.2	22.7 ± 2.3	37.3 ± 0.9
	+C'	11, 17, 20	1, 1, 4	1, 3, 3
		16.0 ± 2.7	2.0 ± 1.0	2.3 ± 0.7

Appendix 3.2 **Analysis of variance table for the square-root transformation of numbers of larvae surviving.**

Variance Source	DF	F	P
Immune serum (Ab)	1	187.63	0.0002
Complement (C')	1	158.76	0.0002
Leukocytes (L)	2	35.55	0.0028
Replicates (R)	2	0.49	0.6427
Interactions:			
Ab * C'	1	130.21	0.0003
Ab * L	2	30.49	0.0038
C' * L	2	1.05	0.4304
Ab * C' * L	2	1.11	0.4127

Appendix 3.3 **Tukey pairwise comparisons of the square-root transformed numbers of larvae surviving.**

Treatment.			Mean	Treatments in line not sig. diff. ($p > 0.05$).
Ab	C'	Leuk.		
Ab	-	-	8.016	I
-	-	Cont.	7.157	I I
-	C'	Cont.	6.965	I I I
-	-	-	6.957	I I I
-	C'	-	6.723	I I I
Ab	-	Imm.	6.109	I I
-	-	Imm.	5.910	I I I
-	C'	Imm.	5.737	I I
Ab	-	Cont.	4.748	I I
Ab	C'	-	3.971	I
A				

Appendix 4.1. The data resulting from the experiments described in Chapter 4 are presented in this table. The antigen used in the ELISAs was *T.hydatigena* oncosphere antigen.

	LAMB					EWE				
				ELISA Absorbance			ELISA (day 0) Absorbance		No. of cysts	
Group	Lamb Number	No. of cysts	SSTT grade	day 7	chall	Ewe Number	whey	sera	old	new
1	3007	22	3	0.117	0.117	650	0.181	0.006		9
	3013	17	3	0.112	0.112	420	0.218	0.015		11
*	3023	14	2	0.079	0.079	542	0.246	0.048		0
	3076	15	3	0.112	0.112	415	0.296	0.044		113
	3009	16	2	0.015	0.015	1654	0.305	0.007		23
	3010	14	2	0.017	0.017	1654				
2	3006	2	2	0.356	0.356	344	0.461	0.261	38	
	3095	3	2	0.208	0.208	447	0.106	0.142	215	
	3097	0	3	0.378	0.378	409	0.974	0.197	12	
	3021	0	3	0.358	0.358	408	0.60	0.136	1	
	3022	0	3	0.427	0.427	408				
3a	3008	25	1	0.005	0.005	309	0.563	0.105	170	
	3014	0	3	0.285	0.285	656	0.435	0.135	107	
	3017	0	3	0.574	0.574	760	0.765	0.355	102	
	3020	0	2	0.400	0.400	463	0.833	0.225	74	
	3077	14	2	0.212	0.212	326	0.683	0.145	144	
3b	3011	9	3	0.185	0.167	363	0.656	0.234	109	
	3015	6	3	0.272	0.234	656	0.435	0.135	22	
#	3018	8	2	0.188	0.226	368	0.653	0.177	0	
	3025	0	2	0.597	0.446	586	0.564	0.205	151	
3b controls										
	926	7				630				119
	928	13				255				20
	930	15				226				70
	932	13				582				110
	934	13				200				32

	LAMB					EWE				
				ELISA Absorbance			ELISA (day 0) Absorbance		No. of cysts	
Group	Lamb Number	No. of cysts	SSTT grade	day 7	chall	Ewe Number	whey	sera	old	new
3c	3012	23	2	0.138	0.118	260	0.521	0.144	45	
	3016	4	3	0.250	0.089	445	0.475	0.146	30	
	3019	6	2	0.324	0.136	468	0.856	0.164	88	
	3024	13	2	0.607	0.197	511	0.974	0.238	18	
3c controls										
	927	15				239				116
	929	1 track				255				20
	931	18				231				94
	933	39				31				33

* = Although this sheep contained no cysts in its peritoneal cavity, its liver contained large numbers of lesions, indicating that the larvae of the challenge infection had developed to a considerable extent before succumbing to the host reponse.

= The liver of this sheep also showed considerable scarring and lesions indicating that larvae from the first infection induced immunity, perhaps before many had emerged into the peritoneal cavity.

Appendix 4.2a **Analysis of variance table for the square-root transformed numbers of dead cysts.**

Variance Source	DF	F	P
Treatment	6	4.49	0.0049
Replicates	5	1.39	0.2707

Appendix 4.2b **Tukey pairwise comparisons of the square-root transformed numbers of dead cysts.**

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.05$)
Control, week 5	2.735	I
Control, week 1	2.569	I
Control, week 3	2.170	I I
Group 3b	1.647	I I
Group 3c	1.381	I I
Group 3a	0.921	I I
Group 2	0.441	I

Appendix 4.3a Analysis of variance table for the square-root transformed numbers of live cysts.

Variance Source	DF	F	P
Treatments	6	8.44	0.0001
Replicates	5	1.23	0.3320

Appendix 4.3b Tukey pairwise comparisons of the square-root transformed numbers of live cysts.

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.05$).
Control, week 1	3.065	I
Control, week 5	2.971	I
Control, week 3	2.585	I I
Group 3c	2.583	I I
Group 3b	0.735	I I
Group 3a	0.259	I
Group 2	0.168	I

Appendix 4.4a Analysis of variance table for the square-root transformed total numbers of cysts.

Variance Source	DF	F	P
Treatment	6	6.22	0.0008
Replicates	5	1.16	0.3637

Appendix 4.4b Tukey pairwise comparisons of the square-root transformed total numbers of cysts.

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.05$)
Control, week 1	4.028	I
Control, week 5	3.495	I
Control, week 3	3.436	I
Group 3c	3.115	I I
Group 3b	1.974	I I
Group 3a	0.845	I
Group 2	0.604	I

Appendix 4.4c Tukey pairwise comparisons of the square-root transformed total numbers of cysts.

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.06$)
Control, week 1	4.028	I
Control, week 5	3.495	I
Control, week 3	3.436	I
Group 3c	3.115	I I
Group 3b	1.974	I I I
Group 3a	0.845	I I
Group 2	0.604	I

Appendix 5.1 **Percentage of freshly activated oncospheres surviving 24 hours culture in the presence of serum or colostrum immunoglobulin preparations, or the pooled IEF-fractions of the immune serum immunoglobulin preparation or the Low Infection colostrum preparation.**

	Immunoglobulin Preparations from	
	Immune Serum (Imm.S)	Low Infection Colostral Whey (Imm.C)
% Survival	10, 29, 17, 12	52, 39, 33, 30
Mean	17	36.6

	Control Serum (Ctrl.S)	Control Colostral Whey (Ctrl.C)
% Survival	74, 52, 45, 50	65, 78, 61, 63
Mean	55.3	66.8

Pooled Groups of IEF-Fractions from Immunoglobulin Preparations

Serum Groups	S1	S2	S3	S4	S5
% Survival	69, 80	52, 47	14, 37	0, 5	0, 4
Mean	74.5	49.5	25.5	2.5	2

Colos um Groups	C1	C2	C3	C4	C5
% Survival	85,70,54,50	50, 52	39,29,52,37	73,49,57	66,61,57
Mean	64.8	51	39.3	59.7	61.3

Appendix 5.2 **Analysis of variance table for the proportions of oncospheres surviving: Data transformed to arcsines.**

Variance Source	DF	F	P
Treatment	13	27.29	0.0000
Replicates	3	2.91	0.0541

Appendix 5.3 **Tukey pairwise comparisons of the arcsine transformed proportions of oncospheres surviving 24 hr in cultures containing the immunoglobulin preparations.**

Treatment	Mean	Treatments in line not sig. diff. (p >0.05)
S1	57.53	I
Ctrl.C	54.72	I I
C1	54.02	I I
C5	50.25	I I
C4	49.31	I I
Ctrl.S	48.14	I I
C2	43.43	I I I
S2	42.54	I I I
C3	38.66	I I I
Imm.C	38.29	I I I
S3	27.43	I I
Imm.S	23.90	I
S4	4.127	I
S5	3.493	I

Appendix 5.4 **ELISA absorbances of anti-*T.hydatigena* IgG classes with the percentage of oncospheres surviving and the arcsine transformation of this data.**

Treatment	Mean IgG_1 Absorbance	Mean IgG_2 Absorbance	Proportion Living	Arcsine of Proportion
Imm.S	1.7890	0.2860	0.1000	18.43
			0.2917	32.71
			0.1739	24.65
			0.1154	19.82
			mean = 0.1700	
Ctrl.S	0.7250	0.0610	0.7391	59.28
			0.5200	46.15
			0.4500	42.13
			0.5000	45.00
			mean =0.5525	
S1	0.7760	0.0410	0.6875	56.04
			0.8000	63.43
			mean = 0.7450	
S2	1.6270	0.0720	0.5238	46.38
			0.4667	43.11
			mean = 0.4950	
S3	1.7140	0.1400	0.1379	21.81
			0.3704	37.46
			mean = 0.2550	
S4	1.7340	0.256	0.0000	0.00
			0.0476	12.66
			mean = 0.0250	
S5	1.4530	0.6020	0.0000	0.00
			0.0385	11.39
			mean = 0.0200	

Appendix 5.4 (ctd):

Treatment	Mean IgG_1 Absorbance	Mean IgG_2 Absorbance	Proportion Living	Arcsine of Proportion
Imm.C	1.4230	0.0370	0.3889	38.59
			0.3333	35.24
			0.5238	46.38
			0.2963	32.96
			mean = 0.3856	
Ctrl.C	0.8050	0.0300	0.6471	53.55
			0.7778	61.89
			0.6071	51.18
			0.6250	52.24
			mean = 0.0.6675	
C1	0.2750	0.0390	0.8500	67.21
			0.7000	56.79
			0.5357	47.06
			0.5000	45.00
			mean = 0.6475	
C2	0.7750	0.0170	0.5000	45.00
			0.5217	46.26
			mean = 0.5100	
C3	0.9820	0.0330	0.3913	38.70
			0.2857	32.33
			0.5200	46.15
			0.3704	37.46
			mean = 0.3925	
C4	0.6050	0.0220	0.7308	58.76
			0.4878	44.31
			0.5714	49.08
			mean = 0.5967	
C5	0.4130	0.0140	0.6667	54.76
			0.6061	51.12
			0.5714	49.08
			mean = 0.6133	

Appendix 5.5 **The equation used to analyse the significance of the difference between the slopes of the 2 linear regression lines.**

$$t = \frac{b_1 - b_2}{\sqrt{\frac{(S^2_{y.x})_p}{(\Sigma X^2)_1} + \frac{(S^2_{y.x})_p}{(\Sigma X^2)_2}}}$$

Where:

residual DF = n - 2

b = regression coefficient

$$(S^2_{y.x})_p = \frac{(\text{residual SS})_1 + (\text{residual SS})_2}{(\text{residual DF})_1 + (\text{residual DF})_2}$$

$$= \frac{2558.5 + 2498.5}{12 + 12}$$

$$= 210.7$$

$$t = \frac{-36.13 - 116.45}{\sqrt{\frac{210.7}{23.33} + \frac{210.7}{0.0697}}}$$

t = 8.1 with $\nu = 24$

$p < 0.001$

Appendix 6.1 Number of cysts found in the peritoneal cavity.

Group & Sheep no.		Immunizing Antigen	Cysts in Peritoneal Cavity Living	Total	Mean of Total ± SE
1	1521	Control gel	157	163	95.5 ± 25.1
	1558		103	103	
	1773		63	66	
	1542		43	50	
2	1619	Whole lane gel	0	0	24.8 ± 12.1
	1559		7	39	
	1633		5	9	
	1720		50	51	
3	1502	Top gel fraction	105	113	87.7 ± 19.6
	1646		34	49	
	1518		94	101	
	1672 *		4	8	
4	1621	Middle gel fraction	14	14	35.7 ± 12.7
	1533		35	35	
	1660		58	58	
	1697		ND		
5	1649	Bottom gel fraction	1	4	11.5
	1508		10	19	
	1539		ND		
	1538		ND		
6	1565	FTS antigen	0	0	0 ± 0
	1693		0	0	
	1741		0	0	
7	1582	SDS treated FTS antigen	49	78	57.3 ± 18.7
	1641		19	20	
	1699		68	74	

* This sheep was found to be carrying a mature *T.hydatigena* cyst indicating a previous infection, therefore its data were excluded from the experiment.

ND Due to the accidental breakage of some containers, the peritoneal washings of these animals could not be assessed and the total numbers of cysts determined.

Appendix 6.2a Analysis of variance table for the square-root transformed numbers of dead cysts.

Variance Source	DF	F	P
Treatment	6	2.33	0.1001
Replicates	3	0.07	0.9727

Appendix 6.2b Tukey pairwise comparisons of the square-root transformed numbers of dead cysts.

Treatment	Mean	Treatments not Signif. Different ($p > 0.05$).
Group 3	3.077	I
Group 7	2.907	I
Group 5	2.229	I
Group 2	2.167	I
Group 1	1.709	I
Group 4	-.028	I
Group 6	-.028	I

Appendix 6.3a Analysis of variance table for the square-root transformed numbers of live cysts.

Variance Source	DF	F	P
Treatment	6	7.69	0.0015
Replicates	3	0.40	0.7527

Appendix 6.3b Tukey pairwise comparisons of the square-root transformed numbers of live cysts.

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.05$).
Group 1	9.293	I
Group 3	8.815	I I
Group 7	6.759	I I I
Group 4	5.982	I I I I
Group 2	2.991	I I I
Group 5	2.556	I I
Group 6	0.234	I

Appendix 6.4a Analysis of variance table for the square-root transformed total number of cysts.

Variance Source	DF	F	P
Treatment	6	7.00	0.0022
Replicates	3	0.08	0.9690

Appendix 6.4b Tukey pairwise comparisons of the square-root transformed total numbers of cysts.

Treatment	Mean	Treatments in line not sig. diff. ($p > 0.05$).
Group 1	9.528	I
Group 3	9.324	I
Group 7	7.400	I
Group 4	5.855	I I
Group 2	4.099	I I
Group 5	3.417	I I
Group 6	0.108	I

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