STUDIES ON *CRYPTOSPORIDIUM* INFECTION AND IMMUNITY

By

Ruhangiz Taghi Kilani Damavandi

A Thesis submitted to the Faculty of Graduate studies of the University of Manitoba in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

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TO

MY WONDERFUL SONS

ALI, AMIR, AHMAD

AND

TO MY DEAR AND SUPPORTIVE HUSBAND

AZIZ

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Abbreviations

ABA	azobenzenearsonate
ADH4	anti- ly 2.2 (CD8) mAb producing subclone of HO 2.2 ATCC (TIB 150)
AIDS	acquired immune deficiency syndrome
anti- u	rabbit anti- mouse IgM antibody
ATCC HB170	anti- mouse IFN- gamma mAb
BSA	bovine serum albumin
C	centigrade
CFA	complete Freund's adjuvant
CMI	cell mediated immunity
Con A	concanavalin A
cpm	counts per minute
CRP.	Cryptosporidium
CsCl	cesium chloride
DFR	delayed footpad reaction
dH_2O	distilled water
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTH	delayed type hypersensitivity
EDTA	disodium ethylene diamine tetra- acetate
EITB	enzyme linked immunoelectrotransfer blot
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Fig.	figure

FITC	fluorescein isothiocyanate
g	gram
x g	gravitational force
GK1.5	rat anti- mouse- L3T4 (CD4) mAb (ATCC TIB 207)
HBSS	Hank's balanced salt solution
HMW	high molecular weight
HO13.4	anti- Thy 1.2 mAb (ATCC TIB 99)
hr	hour
H-S	high molecular weight standard
IEL	intra- epithelial leukocyte
IFA	indirect immunofluorescent antibody test
IFN-gamma	gamma- interferon
Ig	immunoglobulin
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
I.M.	intra- muscular
I.P.	intra- peritoneal
I.V.	intra- vascular
kDNA	kinetoplast DNA
Kd	kilodalton
Kds	kilodaltons
LK	lymphokine
LMW	low molecular weight

log	logarithm
LPS	lipopolysaccharide
L-S	low molecular weight standard
M	molar
mA	milliampere
mAb	monoclonal antibody
M cells	membrane- like epithelial cells
uCi	microcurie
ug	microgram
min	minute
ml	milliliter
ul	microliter
MLR	mixed lymphocyte response
mm	millimeter
mM	milli- molar
um	micrometer
MW	molecular weight
NK	natural killer
nm	nanometer
NRIg	normal rabbit immunoglobulin
NZW	New Zealand white rabbit
O.D.	optical density
OPD	orthophenylamine diamine
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid- schiff stain
PBS	phosphate buffer saline

PCA	passive cutaneous anaphylaxis
PFGE	pulse field gradient gel electrophoresis
p.i.	post infection
p.ir.	post irradiation
rad	radiation
r IL	recombinant interleukin
S.C.	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
sec.	second
SI	stimulation index
sIg^+	surface immunoglobulin positive
sp.	species
spp.	species (plural)
TBS	Tris- buffer saline
TBST	Tris- buffer saline Tween 20
TE	Tris- EDTA
TH	T helper
U	unit
V	volume
WB	western blot
wk	week

SUMMARY

Cryptosporidium is an important and sometimes fatal pathogen in patients with AIDS as well as in newborn calves. The sources of human infections are not fully recognized, though a zoonotic transmission is suspected. More research is needed especially on the species/strains infective to man. In the absence of a cure, AIDS patients must avoid all potential sources of infections.

Cryptosporidium is a self-resolving infection in immunocompetent persons but the mechanisms involved are unknown. Understanding the protective response could be of value to immunodeficient hosts who are unable to clear the infection. Since Cryptosporidium is an intracellular pathogen, immunotherapeutic agents may have a vital role.

The objectives were to study the infection with Cryptosporidium and the immune response to it.

Experimental infections of calves were carried out to obtain adequate amounts of fecal oocysts. Ten calves were orally infected. Fecal oocysts were collected, concentrated and purified. An ELISA for the detection of specific antibodies was established. SDS-PAGE and WB were used to compare human, calf and chicken isolates.

For the study of humoral and cellular immune responses to Cryptosporidium, normal, NRIg-treated, anti-u treated, anti-CD4 treated and irradiated BALB/c mice and NK cell deficient (C3H/HeJ-Bg/Bg) mice were used.

The infecting dose was unrelated to the pattern of oocyst shedding and to the level of antibodies produced in calves. Antibodies appear to be not protective. The CsCl gradient procedure was found to be useful for the purification of oocysts and sporozoites. Mammalian isolates appear to belong to the same species while the chicken isolate may be a separate one. A 25 Kd antigen was identified which can be used for serodiagnosis and serosurveys.

Using B cell deficient mice, it appears that humoral immunity plays no role in the resolution of Cryptosporidium infection.

CD4 deficient mice cleared the infection at the same time as control BALB/c mice, though their Cryptosporidium infection was significantly more severe. In response to in vitro restimulation with Cryptosporidium oocysts, normal infected BALB/c mice were shown to produce IFN-gamma. We suspect that CD4 deficient mice do not produce IFN-gamma. The absence of IFN-gamma needed to activate radioresistant macrophages, may explain the severity of cryptosporidial infection in CD4 deficient mice.

Irradiation of neonatal BALB/c mice did not significantly affect the intensity and duration of Cryptosporidium infection. The non - specific activation of macrophages following lethal irradiation, reported by Roesler et al., may explain the above finding.

Natural Killer cells appear to have no involvement in the

recovery from cryptosporidiosis.

All B and T cell deficient adult BALB/c mice remained resistant to Cryptosporidium infection. Again, this age related resistance may be explained by the deficiency in macrophages reported in neonates.

Further studies are needed to document the role of macrophages and IFN-gamma in the control of life threatening Cryptosporidium infection in immunosuppressed hosts, particularly AIDS patients.

INTRODUCTION

Organisms of the genus Cryptosporidium are small coccidian parasites that invade and replicate within the epithelial cells lining the digestive and respiratory organs of vertebrates. Cryptosporidium is an important cause of diarrhea in animals, and is a major concern to the cattle industry. It has been recognized as a cause of enterocolitis in humans only late in the seventies (64, 151, 227, 231, 299, 300).

Cryptosporidium can produce a short-term, flu - like, self - limited gastro-intestinal illness in immunocompetent individuals which contrasts sharply with the prolonged course of life- threatening, cholera- like, diarrhea in most immunodeficient subjects, especially those with acquired immune deficiency syndrome (AIDS) (10, 41, 98, 106, 142, 145, 167, 187, 226, 239, 285).

Organisms of the genus Cryptosporidium are unique among the Coccidia of warm-blooded vertebrates because:

1- Their developmental stages are confined to the microvillus border of epithelial cells.

2- They exhibit little or no host-specificity.

3- Occasionally, they may infect a variety of tissues (63).

HISTORY

In 1895, Clark may have been the first to observe a species of Cryptosporidium, which he described as "Swarm spores lying upon the gastric epithelium of mice". These minute organisms were probably the motile merozoites of Cryptosporidium muris (64), described by Tyzzer in 1907 in the gastric glands of

asymptomatic laboratory mice. Tyzzer identified it as a new genus called Cryptosporidium, or " hidden sporocyts " in 1910, and in 1912 he described much of the morphology and life cycle of a second species, Cryptosporidium parvum, found in the small intestine of laboratory mice (298).

Clinical illness was first associated with cryptosporidiosis in 1955 when Slavin reported severe diarrhea in turkeys infected with Cryptosporidium (279).

Of 15 reports published prior to 1975 describing cryptosporidiosis in eight different species of animals, only 5 were associated with some illness, 3 of these 5 were in calves (299).

The first case of human cryptosporidiosis was reported in 1976 by Nime et al, in a 3 year old child who developed a severe, acute, self - limited enterocolitis (230). Later in 1976, Meisel et al. reported another case of human cryptosporidiosis (205). Only 7 human cases had been published until cryptosporidiosis was reported to be a life-threatening infection in AIDS patients in 1982 and the number of reported cases increased dramatically since then (227).

Between 1968 and 1981, species of Cryptosporidium in fish, reptiles, birds , and mammals were named on the assumption that each host species harbored a separate species of Cryptosporidium (88).

Taxonomy

1- Classification

Cryptosporidium spp. are protozoa, belonging to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, suborder Eimeriorina, family Cryptosporidiidae, genus Cryptosporidium (169). Eimeria, Isospora and Toxoplasma are important genera of Eimeriorina (299).

2. Number of Species

Twenty species of Cryptosporidium were named according to the host in which the parasite was found (88).

Recent cross-transmission studies have invalidated the criterion of host specificity (76, 88, 299). Tzipori et al. suggested that Cryptosporidium is a single species genus (302). Levine concluded that there are four valid species, one for each vetebrate class (168). Based on oocysts size and morphology, Upton and Current (313) have suggested that two different species can infect mammals, C. muris, the less common form with large oocysts, and C. parvum with smaller oocysts, responsible for most mammalian cryptosporidiosis. Upton et al. (314) believe that two species of Cryptosporidium can infect birds, C. baileyi and C. meleagridis, while the species found in the small intestine of quails (Colinus virginianus) may be distinct. Hoover et al. gave the name C. nasorum to the species first found in fish (129); whether the two other reported Cryptosporidium in fish (160, 237) belong to the same species is not known. According to Upton

et al., statistical evaluation of oocyst structure suggests that multiple species of Cryptosporidium may exist among the reptiles examined (314).

Further studies are needed to determine the number of species infecting fish, reptiles, birds and mammals.

Hosts

Cryptosporidium spp. have been found in four classes of animals; mammals (69, 135, 149, 266, 315), birds (77,103,108, 232), reptiles (38, 314), and fish (160, 237) with no reports in amphibia to date (314).

Site of Infection

Cryptosporidium differs from other Coccidia in that its developmental stages do not occur deep within host cells; instead they are confined to an intracellular, extra-cytoplasmic location in epithelial cells (63). Each stage is within a parasitophorous vacuole of host cell origin (i.e. intracellular), but this vacuole remains at the microvillus surface of the host cell (i.e. extracytoplasmic) (195). While cryptosporidia are restricted to the apex of absorptive cells, they may be found deep within the cytoplasm of M cells overlying Peyer's patches (195).

In mammals, Cryptosporidium has been found in the gastrointestinal tract; mainly in the villous enterocytes and crypt enterocytes of the small and large intestine, where extensive mucosal changes can occur (120, 299). It can be found sometimes in the stomach, and occasionally in the bile

and even in the pancreatic ducts. In some cases it was found in the respiratory tract (4, 72, 156, 161).

In immunodeficient persons, Cryptosporidium sp. has been found in all parts of the gastrointestinal and respiratory systems (30, 37, 92, 96, 105, 106, 146, 240, 243, 244, 280, 322). Cryptoporidium has also been observed in the tonsillar region (11). In immunocompetent persons an association with laryngotracheitis and reactive arthritis was suspected (110, 113).

Pavlasek claimed that various developmenal stages of this parasite could be observed in the liver, lung and heart of four experimentally infected mice and three spontaneously infected calves and in blood smears from another two spontaneously infected calves (236).

In birds there are some reports of its occurrence in the conjunctiva, nasal cavity, nasal sinus, salivary gland, esophageal glands, kidney, and bursa of Fabricius (91, 95, 101, 197, 254). Additionally, intestinal and/or respiratory infections of Cryptosporidium are common in poultry, specially broiler chickens (70).

Life Cycle

The life cycle of Cryptosporidium is characteristic of Coccidia, i.e. an asexual followed by a sexual stage. It is monoxenous (one host for each life cycle); the life cycle begins with the intake of oocysts orally, and possibly by inhalation (64, 152, 299, 302).

The Cryptosporidium oocyst releases its sporozoites in response to stimuli that are presently unknown, although, excystation occurs when the wall of the oocyst is digested in the gastrointestinal tract of a new host (227). It is known that in vitro excystation of oocysts requires the combined action of trypsin and bile salts (65). Some investigators suggest that temperature- activated oocysts of Cryptosporidium initiate their own excystation with enzymes present in the sporozoite or oocyst (86). The organism completes its life cycle on the mucosal lining by adhering to the brush border of enterocytes and begin development into trophozoites below the surface membrane. All other developmental stages appear to take place in the resultant parasitophorous vacuole (138, 299).

Recent light and electron microscopic studies of mice, chicken embryos, and cell cultures infected experimentally with human and calf isolates of Cryptosporidium have clarified its life cycle and developmental biology (64, 102). Cryptosporidium is similar to most Coccidia (Eimeriorina) in that its life cycle can be divided into 6 major developmental events:

1- Excystation (release of infective sporozoites).

2- Merogony (asexual multiplication).

3- Gametogony (formation of micro-and macrogametes).

4- Fertilization.

5- Oocyst wall formation.

6- Sporogony (sporozoite formation).

Oocysts of Cryptosporidium sporulate while they are within the host cells and are immediately infective when released in the feces (64, 138, 301). Most genera of Coccidia, including Eimeria, Hammondia, Isospora, and Toxoplama undergo sporogony outside the body. Others such as Frenklia, Sarcocystis, and Caryospora sporulate endogenously but the latter is the only genus other than Cryptosporidium known to initiate autoinfection (88).

The life cycle of Cryptosporidium has been studied by Current et al. (66). The infective stage is the viable oocyst containing sporozoites. The diagnostic stage in feces is the oocyst. Thick- walled oocysts are believed to transmit the infection from one host to another, while thin- walled oocysts are responsible for autoinfection (66, 70). Auto-infection, which is important in immunosuppressed patients, not only can result from the release of sporozoites from thin-walled oocysts, but also from the release of merozoites from type 1 meronts (66).

It has been shown that corticosteroid treatment of chickens prolongs the life cycle of Eimeria mivati by 24 days (179). Extended oocyst production has also been noted in dexamethasone - treated calves infected with Eimeria spp. (229). Prolonged cryptosporidiosis in immunologically compromised humans may be due to a continuation of schizogony since schizonts were present in biopsies taken over a period of 4 months in one case and 1 year in another case;

however, the possibility of repeated reinfections of these individuals cannot be ruled out (256).

The host's species, immune status, and the age at which infection is acquired can affect the duration of the incubation period (i.e. interval between infection and oocyst shedding) (11, 88, 227). In general, the incubation period ranges from 2 to 14 days. In human, it ranges from 2 to 21 days (7, 140, 256, 273).

The patent period (i.e. duration of shedding of fecal oocysts) ranges from 1 - 33 days, in different animal species (88). In immunocompetent humans a range of 2 to 25 days was reported (139) but shedding may last for more than 30 days (88).

Morphology

As the parasite enters the host cell, the microvillus of the host cell extends along its surface and finally covers it completely, resulting in the formation of a parasitophorous vacuole (102, 134, 301). At the ultrastructural level, the membrane surrounding the parasite appears to be fused with the host cell membrane at one point forming a specialized attachment zone (151). The parasite derives its nutrients directly from the host cytoplasm via a "feeder organelle" in the attachment zone (102).

Trophozoite

Trophozoites are ovoid, about 2 by 1 um with a conspicuous nucleus (134). They are transitional stages from

sporozoites and merozoites to meronts (88).

Schizonts

Each trophozoite undergoes three nuclear divisions producing a mature schizont with eight merozoites (32).

Two types of schizonts have been described:

1- Mature primary schizonts (meronts type I), each is round, 4-5 um in diameter, and contains 6-8 falciform (banana shaped) merozoites, attached to a small residual body. Merozoites are released without destroying the host cell and start meront type I or II.

2- A second generation of schizonts (meronts type 2), each containing four merozoites (134, 151). Through a split in the wall, the detached merozoites escape into the gut lumen to either become attached to another mucosal cell, start a sexual stage, or be voided in the feces (32).

Merozoite

Each merozoite is ovoid, about 5 by 1 um, and possesses an oval nucleus (134). Type II merozoites are shorter and broader than type I merozoites (67).

Micro- and Macrogamonts

Microgametocyte and macrogametocyte are similar in size to the early schizont (i.e. 2-5 um in diameter) (151). Microgametocytes are an infrequently found male stage, which produce 16 microgametes during microgametogenesis, each is 0.95 by 0.4um in size, wedge- shaped and unflagellated (32,

102).

Macrogametocytes result in only one macrogamete which is round, 5 um in diameter and has a large eccentric nucleus (102, 134).

Fertilization is achieved by protrusion of parts of the macrogamete membrane towards the microgamete, and may involve the formation of enzymes (32, 301).

Oocyst Formation and Sporogony

Oocyst formation takes place in the parasitophorous vacuole. Oocysts are formed from the fertilized macrogametes which undergo successive changes (301). Approximately 80% of the oocysts have a thick, two-layered wall, while 20% are thin- walled, having only a single unit wall (67).

Oocysts are 4-6 um in size and each contains four sporozoites, which contrary to most other enteric Coccidia, are naked and have no sporocysts (231).

Sporozoites are generally crescent- shaped, 1 by 5 um in size, with the anterior end slightly pointed and the posterior end rounded (88, 134).

Mode of Transmission

Studies of experimental infections in farm and laboratory animals have clearly established that cryptosporidiosis is transmitted by the ingestion of oocysts which are fully sporulated and infective at the time they are passed in the feces, (i.e. a fecal- oral transmission) (63).

Aerosols may be implicated in the transmission of

Cryptosporidium (299), though the frequency and significance of respiratory tract infections in mammals, as well as the infective stage, are not known (35, 37, 92, 126, 212).

In birds, respiratory cryptosporidiosis is well documented and may be explained by air - borne transmission (227).

A hematogenous transmission of sporozoites or merozoites is another possibility (92). The presence of Cryptosporidium in blood, liver, heart, lungs of experimentally infected mice and naturally infected calves has been reported (236).

The presence of Cryptosporidium in the pharyngeal mucosa of one patient indicates that infection may be transmitted through contact with oral secretions, however, the presence of Cryptosporidium has not been reported in saliva (227).

Sources of Infection

1- Zoonosis

Recent studies have shown that calves and perhaps other companion animals serve as potential sources of human infection (11, 22, 154, 246).

The possibility of transmission of Cryptosporidium from animals to humans is supported by:

1- The experimental transmission of Cryptosporidium from humans to several animal species; 2- The accidental infection of man exposed to calves infected with Cryptosporidium (68, 69, 249, 252, 257, 276).

These reports indicate that calves with diarrhea due to cryptosporidiosis should be considered as potential sources of

human infection and that proper precautions should be taken by individuals who have contact with such animals (68).

2- Human to Human Transmission

This may occur through direct (more commonly between children) or indirect contact with contaminated feces (33, 49, 56, 63). There are several reports of cryptosporidiosis among children attending day care centres (2, 34, 57, 61, 118, 293).

Transmission of Cryptosporidium from patients with cryptosporidiosis to attending hospital staff and to other patients have been reported (22, 63, 153). This type of transmission has been reported in household contacts of infected individuals (118, 127). Asymptomatic carriers of Cryptosporidium can possibly act as important reservoirs (227).

3- Water, Food and Vector Transmission

Ingestion of oocysts through contaminated food, untreated water, ice cubes, and milk or milk products has been implicated as the mode of transmission (8, 208, 218).

D'Antonio et al. reported the first waterborne outbreak of cryptosporidiosis occurring in Texas (71).

Examination by immunofluorescence of both raw and filtered drinking water samples from British Columbia revealed Cryptosporidium oocysts (133). From 109 surface water samples collected in six western states in the USA, 77 were positive for Cryptosporidium oocysts. In that report, two of

three outbreaks of waterborne cryptosporidiosis were linked to drinking water and the third, to surface water (261). Cryptosporidium oocysts were also identified in river waters from California and Washington states (234).

Musial et al. developed a method for the detection of Cryptosporidium oocysts in water by using polypropylene cartridge filters (224).

Recent reports suggest that Cryptosporidium is an important cause of traveller's diarrhea (141, 290). Cryptosporidiosis was reported in tourists returning from African countries, the Caribbean, the USSR - particularly Leningrad, and Mexico (49, 172, 184, 218) .

The possible role of insects in the transmission of Cryptosporidium requires investigation.

The presence of Cryptosporidium in blood (236), suggests the possibility of transmission of Cryptosporidium via blood products.

In summary, the sources of human infection are numerous and not fully understood.

Cryopreservation and Infectivity of Cryptosporidium Oocysts

Cryptosporidium oocyst is highly resistant to adverse conditions (224).

Oocysts may remain viable for long periods in the environment (246). The elimination of Cryptosporidium from potable waters is complicated by the fact that, because of their small size, filtration is difficult; as well, they are

resistant to chlorination and to a number of other chemical disinfectants (13, 40, 64, 65).

The relationship between infectivity of oocysts and climate is not clear. An Australian study of hospitalized patients with gastroenteritis showed that 7% were excreting Cryptosporidium oocysts in feces during the summer months, while only 2% were excreting oocysts during the remainder of the year (299). Mann et al. reported that, in Manitoba, infections appear to be seasonal, winter/spring for calves, and summer/fall for humans (190), while Rahman et al. found that most cases of cryptosporidiosis in calves were detected during the warm, wet season (252).

Freezing destroyed the infectivity of oocysts, irrespective of the cryopreservation method used. There was also a progressive loss of infectivity at 4° C after 2 months of storage in distilled water. Complete loss of infectivity occurred within 2 weeks at 15 to 20° C and within 5 days at 37° C (276).

Infectivity of oocysts was reported to be destroyed by ammonia, formalin saline (238), freeze drying, exposure to temperatures below freezing for 30 min, as well as by warming to 45° C for 20 min (5). Drying of Cryptosporidium - laden calf feces reduced its infectivity for infant mice (6). Storage of oocysts at 4° C could maintain the parasite viable for a longer period than storage at a higher temperature (276).

The most stable preparation was a 2.5% potassium

dichromate solution, which retained oocysts infectivity for 4-6 months (276). Recently, a method for the cryopreservation of oocysts was reported by Rossi et el. They claimed that oocysts cryopreserved in 10% dimethyl sulphoxide and frozen in liquid nitrogen can be used to infect laboratory animals (263).

Laboratory Diagnosis

From 1976-1981, the diagnosis of cryptosporidiosis was based primarily on the electron microscopic examination of intestinal or rectal biopsies (321). For histological demonstration of the endogenous stages by light microscopy, intestinal tissues were fixed in either 10% formalin or Bouin's solution. Hematoxylin and eosin, toluidine blue, phosphotungstic acid-hematoxylin, Giemsa, and trichrome stains have all been shown to stain Cryptosporidium oocysts adequately (151).

Staining Techniques

A non - invasive procedure for the diagnosis of cryptosporidiosis was first reported in 1978 for calves (250). In 1980, Tzipori et al. were the first to diagnose human cryptosporidiosis by stool examination using Giemsa- stained fecal smears to detect oocysts (303).

There is little consensus on which is the best staining method. According to Navin et al. acid- fast stains are the most useful ones because they can differentiate between yeasts

(blue-green) and Cryptosporidium oocysts (red) (227). Five staining procedures were compared by Garza et al. (97) and a modified acid-fast stain found to be the most effective one. Henricksen et al. introduced the Ziehl - Neelsen acid-fast technique (121). Comparing 15 different methods for the recovery and identification of Cryptosporidium oocysts, Garcia et al. (94) recommended a modified Ziehl-Neelsen carbolfuchsin staining of stools preserved in 10% formalin. Auramine-phenol can be used as an alternative to the Ziehl-Neelsen stain (239, 294). Fluorescence staining with acridine orange causes both oocysts and yeasts to fluoresce (186), while using fluorochrome Truant auramine- rhodamine stain, only oocysts fluoresce (185). All positive auramine stains must be confirmed using acid- fast stain (60). During routine screening of sputum for acid-fast bacilli, Cryptosporidium oocysts were detected by auramine fluorescent stain (212). A modified periodic acid - schiff stain (PAS) method was suggested as a negative stain to facilitate the differentiation of fungal spores and pollen grains from Cryptosporidium oocysts (130). A study comparing negative staining method using nigrosin solution with the modified Ziehl-Neelsen method showed almost identical results (245).

Concentration and Purification Techniques

In some fecal specimens, especially the semi-solid or formed ones, the number of oocysts may be low, hence the need for concentration procedures.

The most widely used technique for concentrating Cryptosporidium oocysts is Sheather's sugar flotation, which employs a dense sugar solution to levitate the oocysts (3). Iseki used zinc sulphate flotation technique (134). Ma and Soave found Sheather's sugar coverslip flotation more effective than formalin - ether sedimentation (186). In contrast, the formalin - ethyl acetate concentration procedure followed by a modified cold Kinyoun acid-fast stain was reported to be equivalent to Sheather's flotation (201).

Zierdt used a new disposable plastic tube device for parasite concentration. He has shown that this technique is less messy, less time consuming and much safer than gauze filtration (330).

Ma and Soave recommended a three step process for the detection of Cryptosporidium oocysts; 1) iodine wet mount of fecal material, which will leave oocysts unstained while yeasts stain brown, 2) a modified Kinyoun acid-fast to be used as the permanent record, and 3) concentration with Sheather's sugar coverslip flotation (186).

The sporozoites within the oocyst become easier to recognize after incubation for one week in potassium dichromate solution, simply because most of the oocyst residuum disappears during this time (227). When preserved at 4^{o} C in this solution, oocysts could remain alive for up to 12 months (65). Calf samples preserved in dichromate solution for over 6 months can be concentrated using flotation method (326).

Purification of oocysts and sporozoites is a vital preliminary step for biochemical and immunological studies of Cryptosporidium. Separation of oocysts from fecal material by formalin-ether sedimentation, followed by discontinuous density gradient centrifugation, has been used to obtain purified oocysts for use in immunological studies, as well as for propagation in cell culture and chick embryo preparations (318). However, formalin- ether may alter the antigenicity of the oocysts (60). Heyman et al. developed a method in which oocysts were initially collected by centrifugation through a sucrose density gradient and further purified by passage through glass bead columns. They reported that these purified oocysts were antigenically active and sufficiently pure to be used in immunological studies (123). Isolation of oocysts and sporozoites by discontinous sucrose and isopycnic percoll gradients was suggested by Arrowood and Sterling (16). They reported that the recovered oocysts were essentially free of debris and bacteria; however they represented 34% of the original oocyst and 63% of the original sporozoite suspensions, with only 2.2% contamination by intact oocysts and none by oocysts walls.

To obtain purified oocysts suitable for IFA, and for ELISA, different protocols were used by Casemore (46), and by Ungar et al. (311), respectively. Casemore used a modified formalin- ether method, omitting formalin in the preliminary cleaning step and concentrating pure oocysts by percoll density gradient (46). Ungar et al. used distilled water

saturated with NaCl and several washes with water, followed by treatment of the pellet containing oocysts with 1% sodium hypochlorite solution (311). Both authors did not clarify whether the above mentioned purification steps altered the oocyst antigenicity.

Serological Techniques

Techniques for the serodiagnosis of Cryptosporidium infection were developed, using indirect fluorescent antibody (IFA) test and enzyme linked immunosorbent assay (ELISA) techniques (33, 48, 153, 191, 304, 311).

Cryostat sections of infected lamb intestine (304) and paraffin-embedded intestinal sections from infected mice (41) were used as antigen for IFA tests. Mann et al. (191) suggested that using fecal oocysts rather than intestinal sections as antigen may improve the specificity of the IFA test, by eliminating the presence of host tissues, and by providing oocysts with a uniform morphology as opposed to the variety of morphologies exhibited by Cryptosporidium tissue stages. However, Koch et al. (153) suggested that the sensitivity of the test may be lowered when fecal oocysts are used as antigen. Using an IFA, Casemore et al. (50) showed rising titers of IgA and IgM antibodies. In primary infections of humans, when serially collected serum specimens were examined, the expected rise in IgG antibodies did not occur and the levels remained low. Low titers of IgA and IgM antibodies were also present in some control subjects. In

another study, Casemore (46) examined paired sera obtained from eight immunocompetent individuals with Cryptosporidium positive stools, who had their acute sera drawn during the first 2 weeks of illness. He reported that the rise in IgG antibodies was inconsistent (detected in four of the eight), while the rise in IgE, IgA, and IgM antibodies was noticed more often in the acute stage (in six out of the eight persons tested). The same study, showed that the sensitivity of the IFA (using as criterion for positivity, any level of IgE, IgA, IgM, or IgG antibodies) was 100% in 12 stool-positive persons, while the specificity of the IFA was only 40%, with antibodies detected in 15 persons who were stool-negative.

Specific Cryptosporidium IgM and IgG antibodies were detected by ELISA in 95% of patients with cryptosporidiosis at the time of onset of disease and in 100% within 2 weeks of onset (311). However, the 90% specificity for ELISA reported by these investigators was not accepted by others (60), since a high percentage of the seropositive individuals had no Cryptosporidium oocysts in the feces, while other enteric parasites were detected. Serosurveys show that more than 50% of the persons not known to be currently infected with Cryptosporidium may have specific antibodies, suggesting that past infections may be common (304, 309, 311). According to Ungar et al. the simultaneous presence of IgM and IgG antibodies probably correlates with recent Cryptosporidium infections (309).

Using an ELISA system, Cryptosporidium antibody levels were measured in the serum and feces of experimentally infected calves (325).

The serodiagnosis of cryptosporidiosis is of limited value in patients with hypogammaglobulinemia (41, 227).

Oocysts in fecal or in environmental samples can be detected by a direct fluorescent assay. Procedure involves the use of a rabbit antiserum against Cryptosporidium oocysts of calf origin (291), or of human sera containing anti-Cryptosporidium IgA antibodies (48). Only a very weak cross - reactivity with coccidian oocysts of other genera was reported (291).

Garcia et al. (93) used monoclonal antibodies (mAb) and a direct fluorescent antibody assay for the detection of Cryptosporidium oocysts in human fecal specimens. They reported a sensitivity of 100% in heavy as well as in light infections. All positive and negative specimens were previously tested by the acid-fast technique. Similarly, McLauchlin et al. (200) using mAb in an IFA test reported that their procedure is highly specific and more sensitive than conventional techniques. However, Baron et al. believe that the routine use of such an expensive and time consuming procedure should not be recommended (21).

Pohjola et al. (248) reported that latex agglutination can be adapted as a rapid immunoassay for the diagnosis of oocysts in the feces of infected persons, but further studies concerning the specificity and sensitivity of the method are

required.

An ELISA has been reported for the detection of Giardia lamblia antigen in fecal specimens (312). To date such a system has not been reported for the detection of Cryptosporidium oocysts in fecal samples.

Antigenic and Biochemical Properties

1- Antigenic characteristics

The immunological and biochemical characteristics of sporozoa can be used to study their taxonomy (147).

SDS-PAGE and western blot techniques were widely used for characterization of protozoan antigens. Walzer and Linke (319) showed that rat Pneumocystis carinii antigens were recognized by human sera and human P. carinii antigens were recognized by rat sera, thus suggesting the existence of shared or cross- reacting determinants; however, the markedly different patterns of immunoreactivity of rat and human P. carinii suggested species or strain-specific determinants. In another study (155) human and rat Pneumocystis were shown to be antigenically not identical.

Toxoplasma gondii was also shown to exhibit antigenic strain variations (320). On the other hand, Araujo et al. (14) detected antigenic similarities between the coccidian parasites T. gondii and Hammondia hammondi. These organisms shared cell surface and cytoplasmic antigens which may explain the fact that animals immunized with H. hammondi resist challenge with virulent T. gondii.

SDS-PAGE and WB analysis of Giardia lamblia isolates from widely differing geographic locations showed gross similarities; however, subtle differences were detected by crossed electrophoresis and ELISA, suggesting the existence of potentially important antigenic differences (282).

It is only in very recent years that some information has been published regarding the antigenic characteristics of Cryptosporidium.

The antigenic structure of Cryptosporidium is complex (46). Lazo et al. (165) analysed extracts of bovine- derived Cryptosporidium oocysts in silver-stained 5-25% gradient gels in SDS-PAGE. They showed 41 polypeptide bands, with MW ranged from <14 to >330 Kd. They found that 40 days post infection, the serum from a Cryptosporidium infected calf reacted with nine oocyst antigens ranging from 60 to >300 Kd. They believe that the EITB technique was not very effective in transferring proteins located near the origin of the electrophoretic run which may explain the absence of any reactive band below 60 Kd.

Luft et al. (183) used immune sera from orally and intra-peritoneally infected mice to characterize the Cryptosporidium antigens. A variable number of antigens was recognized by immune sera, ranging in MW from 43 to greater than 100 Kd. However, similar to Lazo et al.'s findings, none of the sera from immunized mice recognized the low molecular weight antigen (i.e. 23 Kd) reported by Ungar and Nash (310).

Mead et al. (204) detected a total of 46 bands ranging

from 3 to 300 Kd, using silver - stained SDS-PAGE gels. In western blots, convalescent sera from Cryptosporidium infected calves, horses and humans recognized a 20 Kd sporozoite antigen.

Luft et al. (183) reported that plant lectins, which have been used as specific probes for carbohydrates, revealed the presence of carbohydrates in as many as 10 components present in C. parvum antigen preparations. Treatment of the antigen preparation with mixed glycosidases reduced the reactivity of these antisera to antigens with a MW >60 Kd. They interpreted these data as suggesting that the antigenic composition of C. parvum is complex and that carbohydrates alone, or in association with lipids or proteins, may be important in the immune response to C. parvum.

There are few reports on the stage and species specificity of coccidian antigens. Plasmodium berghei sporozoites have specific antigens on their surface; as well, sporozoites possess antigens shared with the blood stages (125). Monoclonal antibodies to circumsporozoite proteins were capable of identifying the species of malaria parasite in infected mosquitoes (328).

Stage- specific sporozoite antigens of T.gondii were recognized using monoclonal antibodies (144). However, the tachyzoite and the sporozoite stages share minor antigens.

Antibody cross- reactivity between sporozoites and merozoites of Cryptosporidium may exist, since one monoclonal antibody to a 20 Kd sporozoite surface antigen recognized

merozoites recovered from the ileum of infected mice (204).

2- Biochemical characteristics

The biochemical methods for parasite characterization have been divided into two main categories (228). A) those that are concerned with the cell phenotype (e.g. isoenzyme analysis) and B) those that are related to cell genotype (e.g. DNA buoyant density studies). However, the applicability of methods for genotype characterization of parasites has, in general, been limited by the costly equipment and specialized techniques needed (217). Often a combination of various methods are needed for the characterization of protozoal species and/or strains.

For trypanosomatids, including Trypanosoma and Leishmania, the following have been used: A) determination of isoenzyme patterns (18, 148, 180, 324); B) determination of the buoyant density of nuclear and kinetoplast DNA (kDNA) (19, 228); C) restriction endonuclease fingerprinting of kDNA and non-kDNA (20, 36, 136); D) agglutination reactions by various lectins (107, 269); E) protein typing by disc electrophoresis (80); F) comparative study of ribosomal genes (253); G) study of chromosomal DNA composition (DNA karyotype) using pulse field gradient gel electrophoresis (PFGE) (270).

Electrophoretic isoenzyme patterns were used in the differentiation of Giardia and Entamoeba isolates, particularly invasive and non- invasive strains of E. histolytica (31, 52, 84, 198, 251).

Restriction endonuclease analysis of DNA was used in the

differentiation of Giardia isolates (225).

It was demonstrated that, within the Eucoccidia, the haploid amount of DNA/species may be a useful criteria for quantitative taxonomy (59).

Using PFGE, Mead et al. (203) analyzed Cryptosporidium DNA prepared from purified sporozoites and intact oocysts. In this study, the chromosomal DNA migration patterns of five C. parvum isolates were indistinguishable, whereas, differences were evident between C. baileyi and C. parvum isolates.

Treatment

More than 90 agents have been tried, but so far no drug has been shown to consistently improve the condition of Cryptosporidium patients (43, 60, 88). In the absence of a cure, it is vital that AIDS patients be made aware of the potential sources of infection and avoid them as much as possible (43).

Recently, attention has turned to immunological rather than pharmacological approaches. Bovine transfer factor, which is believed to confer cell - mediated immunity to humans, may have a role in the treatment of cryptosporidiosis in AIDS patients (181). A therapeutic role for Cryptosporidium antibodies present in hyperimmune colostrum (87, 305, 306) or purified monoclonal antibodies (258) has been suggested. However, the oral administration of bovine colostrum containing Cryptosporidium IgG antibodies was of no value to an AIDS patient with intestinal cryptosporidiosis

(64).

Immunity

The specific immune responses and the network of non-specific inflammatory events generated in infections are complex (25). Little is known about the immune mechanisms in cryptosporidiosis . Immunologically incompetent persons (e.g. the very young, the malnourished, AIDS patients, persons receiving immunosuppressive therapy) tend to exhibit more severe and extended symptoms, sometimes leading to severe dehydration and death (60, 63, 64, 128, 202, 205, 210, 301). In contrast, immunologically healthy persons have a shorter duration of symptoms and complete recovery occurs spontaneously (301). However, the possibility of reinfection has been suggested (309). Asymptomatic carriers have also been documented (71, 127, 293).

Patients with reversible immune deficiencies usually recover when the cause of immunosuppression is removed (171). However, exceptions have been reported (233).

Persistent infections have been reported in patients with high titers of Cryptosporidium antibodies and low T cell counts (41), as well as in hypogammaglobulinemic patients with normal T cell functions (69).

1- Humoral Immunity

A role for antibodies in the natural resolution of cryptosporidial infections is suggested by the observation that many of the cases of cryptosporidiosis were patients with

Ig deficiencies (154, 162, 280, 322). Antibody production is commonly noted in self- resolving human and animal infections (41, 46, 304, 311). A recent study showed that immuno-compromised patients also develop an antibody response to Cryptosporidium (311).

Immune bovine sera at an IFA titer of 1:10,000 were reported to be able to neutralize the infectivity of sporozoites (259).

To study the role of humoral immunity in a murine model of Cryptosporidium infection, B cell deficient BALB/c mice can be used. To abolish B cells and their functions in the circulation as well as in mucosal surfaces, these mice are continuously treated with rabbit anti- mouse IgM antibodies (anti-u), beginning within 24 hours of birth. This widely used strategy yields mice which are virtually B cell deficient (58, 115, 163, 193, 283). These mice display very few (< 5%) surface immunoglobulin (sIg) expressing B cells in spleen or lymph nodes, cannot generate responses to the B cell mitogen LPS, and have been reported to have undetectable or severely reduced levels of serum and gut immunoglobulins of all isotypes (58, 150, 194, 283). The majority of T cell and macrophage functions are intact in these anti- u treated mice (104, 114, 115, 116, 117, 137, 196).

2- Local Immune and Inflammatory Responses

The main components of mucosal immune response are IgA antibody, mucosal mast cells, and intra- epithelial

leukocytes (IEL) including natural killer (NK) cells and macrophages (i.e. non- specific factors of immune system).

A) IgA Antibody

Some of the functions of mucosal IgA antibodies that have been identified to date include neutralization, inhibition of mucosal colonization, stimulation of cellular cytotoxicity, and antigen clearance (25). In Taenia taeniaformis infections, IgA antibody will confer resistance to challenge with eggs (178). Recent studies have suggested that IgA antibodies may facilitate a cytotoxic response to Giardia muris (143). A protective role of IgA antibodies was also suggested in coccidial infections (74). Specific IgM and IgA antibodies were found in the feces of Cryptosporidium infected calves (325). However, the role of secretory IgA and IgM in Cryptosporidium infection is not known (46).

B) Mucosal Mast Cells

Intestinal mucosal mast cells and basophils were shown to be mostly involved in killing the parasites in gastro-intestinal helminthic infections (17, 166).

C) Intraepithelial Leukocytes (IEL)

Significant increases in the numbers of IEL occur in human and murine giardiasis (89). In coccidial infections, sporozoites appear to invade IEL and reside within these cells for some time before developing in epithelial cells (209). However, the role of IEL in Cryptosporidium immunity is not

known.

3- T Cell Immunity

Cell- mediated immune mechanisms (CMI) are important in the immune response to intracellular parasites. Protective immunity against most coccidian parasites depends primarily on CMI; for example, it was shown that immunity to Eimeria falciformis was thymus dependent, and T- lymphocytes seemed to be required for protection. It is believed that the immune response to Eimeria falciformis does not interfere with penetration of the host cells, but acts against the parasitic stages developing within the intestinal epithelial cells (206, 207).

Persistence of cryptosporidiosis in nude mice and AIDS patients suggests that immunity is T lymphocyte dependent (probably the T helper- inducer subset). Whether the role of T cell dependent immunity is regulatory or effectory or both is not known (216, 300). The T helper cell depletion described in patient with AIDS appears to involve the small bowel mucosa where the immunologic reaction to Cryptosporidium occurs (138).

In experiments with a murine model using anti-CD4 mAb, it was shown that TH cells mediate immunity to G. muris, Leishmania, Eimeria vermiformis and Toxoplasma gondii (124, 262, 265, 316).

4- Non - Specific Factors of Immune System

Non - specific factors such as natural killer (NK) cells,

phagocytic leukocytes including polymorphonuclear (neutrophils and eosinophils) and mononuclear phagocytes (monocytes and macrophages), and serum factors other than antibodies are critical in the battle against parasites (53, 277).

A) Natural Killer (NK) Cells

NK cells are large granular lymphocytes that lyse certain tumor cells, virus infected cells, and some normal cells (271). Enhanced NK cell activity has been noted in mice infected with trypanosomes of different species (1, 112). Administration of recombinant interleukin 2 (rIL 2), which resulted in increased NK cell activity, significantly reduced the mortality of mice infected with a lethal dose of Toxoplasma gondii (272).

Athymic (nude) mice, which cannot clear Cryptosporidium infections, are known to have high levels of NK cell activity (100, 119); therefore NK cells may not play an important role in the clearance of Cryptosporidium infections.

B) Macrophages

Interaction of parasite antigen and T cells may induce macrophage activation or lymphokine (LK) release, resulting in an important damage of the parasite target (44). Products of LK- activated macrophages are able to inhibit the growth of malaria parasites in erythrocytes in vitro. As well, LK-activated mature tissue macrophages appear to be particularly potent at killing schistosome larvae in vitro. The principal LK responsible for both intracellular and extracellular

parasite killing appears to be gamma- interferon (274). Macrophages may be important in host defense against hepatic amoebiasis and giardiasis (99, 143).

Involvement of macrophages is important for pathogens sequestered within them (42). The growth of Trypanosoma, Leishmania, Eimeria and Toxoplasma may be inhibited by activated macrophages probably via the production of IFN-gamma (131). Eimeria and Toxoplasma are coccidian parasites, closely related to Cryptosporidium.

Macrophages are present in significant numbers in connective tissues, skin and gastrointestinal tract (277).

The ability of alveolar macrophages to kill infective larvae of Nippostrongylus brasiliensis was demonstrated in vitro. Significant increases in their number and activity were detected within 2 days of infection and were present by day 8 p.i. However, the relevance of these observations to events in vivo requires further studies (25, 82).

Antigens present within the small intestine are sampled by a specialized type of cells, termed the M cells (membrane-like epithelial cells or follicle associated epithelial cells), located over each Peyer's patch and bordering on the intestinal lumen. Cryptosporidium have been found deep in the cytoplasm of M cells (60, 307). Partially digested and intact parasites have been seen among macrophages beneath the M cells (195).

Objectives

The actual number of species or strains of Cryptosporidium infecting mammals, birds, reptiles and fish is still controversial. To determine which of the species or strains are capable of infecting humans will help define the potential sources of human infections. As well, the immunological mechanisms involved in Cryptosporidium infections are not fully understood. A study of the mechanisms responsible for the control of cryptosporidiosis in healthy individuals (or in an animal model) is essential for planning of strategies to fight this disease in immunosuppressed hosts.

This thesis is divided into two different but related sections. Part A deals with the experimental infection of calves. It is essential first, to collect and purify enough fecal oocysts to compare the protein banding patterns of calf, human and chicken isolates and second, to use these oocysts for all the other experiments designed. As well, the experimental infection of calves provide an opportunity to study the calf humoral response to Cryptosporidium infection, as well as the relationship of antibody response and course of infection. Part B is a study of the humoral, as well as the specific and non-specific cellular responses to Cryptosporidium infection, in experimentally infected, immuno-competent and immunocompromised mice.

MATERIALS AND METHODS

MATERIALS

Sources of Oocysts: Feces containing Cryptosporidium oocysts were obtained from the following sources. A) Naturally infected calves : feces submitted to Manitoba's Provincial Veterinary Laboratory, Winnipeg, Manitoba and kindly provided by Dr. G.P.S. Nayar. B) Experimentally infected calves : feces collected by Mr. R. Fargey, National Biological Laboratory, Winnipeg, Manitoba. C) Infected humans, one immunocompromized patient from New York, USA, and one immunocompetent child from Winnipeg, Manitoba, kindly provided by Dr. A. Ebrahimzadeh and Dr. P.A. Major, respectively. D) One infected chicken from Alabama, USA, courtesy of Dr. B.L. Blagburn.

Sources of Sera and Colostrum: Sera were obtained from the following sources. A) Experimentally infected calves collected by Mr. R. Fargey, National Biological Laboratory, Winnipeg, Manitoba, and uninfected calves, courtesy of Dr. E.D. Mann. B) Hyperimmune and preimmune NZW rabbits. C) Experimentally infected, hyperimmune, and uninfected BALB/ c mice. Colostrum samples were collected from cows by Mr. R. Fargey.

Animals: Canadian Holstein male calves were obtained from Van Ryssel Dairy,Oak Bank, Manitoba,and maintained at the National Biological Laboratory, Dugald, Manitoba. New Zealand white rabbits were purchased from Blue Farm Rabbitry , Lockport, Manitoba. BALB/c, Swiss, C3H/HeJ-Bg/Bg mice and S-D rats were obtained from breeding colonies maintained at the University of Manitoba Medical School. These animals were

kept and used in strict accordance with the guidelines of the Canadian Council on Animal Care.

Chemicals : Phenol, methylene blue, potassium dichromate, sucrose, sodium chloride, disodium ethylenediamine tetra-acetate (EDTA), sodium carbonate, sodium bicarbonate, sodium hydroxyde , and paraformaldehyde were obtained from Fisher Scientific Co., FairLawn, NJ. Percoll, trizma base, incomplete Freund's adjuvant, bovine serum albumin (BSA), 3,3' - diamino-benzidine, p - nitrophenyl phosphate disodium , concanavalin A (Con. A) and sodium azide were purchased from Sigma Chemical Co., St.Louis, MO. Hanks' balanced salt solution (HBSS), RPMI, and trypsin were products of Flow Laboratories, McLean, VA. Sodium taurocholate, complete Freund's adjuvant (CFA) and lipopolysaccharide (LPS) were purchased from Difco Laboratories, Detroit , MI. Horse serum , fetal calf serum (FCS), L-glutamine, penicillin, streptomycin, and fungizone were products of Gibco Laboratories, Grand Island, NY. Basic fuschin was obtained from Anachemia, Montreal, Canada; Giemsa from Harleco, Gibbstown, NJ. ; Sodium hypochlorite from RW Packaging LTD. Winnipeg, Canada; Cesium chloride from Cabot, Revere, PA.; Tween 20 from BDH Chemicals,Toronto, Canada; Evan's blue from Kallestad, Austin, TX.; orthophenylanine diamine (OPD) from Abbott Laboratories, Chicago, IL.; sulfuric acid from J.T. Baker Chemical Co., Phillipsburg, NJ.; hydrogen peroxide from Ingram & Bell Medical, Don Mills, Ontario; trypan blue from Aldrich Chemical Co., Inc. Milwaukee, Wis.; [^{3}H] - thymidine from ICN radiochemical, Irvine, CA. and

p-arsanilic acid (ABA) from Eastern Kodak Co., Rochester, NY. FITC conjugated goat anti- bovine Ig and IgG , goat anti- rabbit Ig , goat anti- mouse Ig, and peroxidase labelled goat anti- rabbit Ig and IgG were purchased from Cappel, West Chester, PA. FITC conjugated and peroxidase labelled goat anti - bovine IgMs were products of Kirkegaard & Perry Laboratories, InC., Gaitherburg, MD. Peroxidase labelled goat anti - bovine IgG was obtained from Bio/Can Scientific InC., Mississauga, Ontario; FITC conjugated goat anti-rat Ig, rabbit anti - mouse Ig and rabbit anti - goat Ig from Jackson Immuno- Research Laboratories, West Grove, PA.; and alkaline phosphatase conjugated rabbit anti-mouse Ig, IgG, IgM, and IgA from Southern Biotechnology Associates, Birmingham, AL. Bis-acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), tetramethyl ethylene diamine, amido black, and bromphenol blue were products of Bio-Rad Laboratories, Richmond, CA. Methanol, acetic acid, glycerol, formaldehyde (37 %), acetone, and gluteraldehyde were obtained from Fisher Scientific Co., Winnipeg, Manitoba. Low molecular weight (LMW) protein markers were obtained from Pharmacia , Piscaraway, NY., and high molecular weight (HMW) standards, obtained from Bio - Rad, as well as Pharmacia. Anti-u antibody, anti-CD4 and anti- CD8 monoclonal antibodies, anti-thy 1.2 monoclonal antibody, affinity purified goat anti-mouse Ig, normal rabbit Ig, anti- ovalbumin IgE, sheep anti - mouse Ig, rat Con.A supernatant, WEHI - 279, CTLL cells, and anti- IFN- gamma monoclonal antibody (ATCC HB170) were

kindly provided by Dr. K. T. HayGlass, University of Manitoba. CT.4S cells were a gift of W.E. Paul, NIH, Bethesda, MD. and rIL4 was generously provided by Dr. R. Tepper, Harvard Medical School, Boston, MA. Recombinant IL2 was obtained from Genzyme, Boston, MA.

All other standard laboratory reagents were purchased from either Fisher Scientific Co. Winnipeg, Canada, or Sigma Chemical Co. St. Louis, MO.

Equipment: A Hemocytometer was obtained from American Optical, Buffalo, NY.; 16 x 102 mm Ultra-Clear ^{TM} tubes from Beckman Instrument, InC., Palo Alto, CA.; sonicator cell disruptor, model W 185 F, from Ultra Sonics InC., Plainview, NY.; 96 well ELISA plates and polystyrene flat-bottom microtiter plates, Corning 25805, Corning Science Products, Corning, NY.; slab gel electrophoresis apparatus, model 550, power supply, model 550/200, Trans Blot cell, and nitrocellulose paper from Bio-Rad. Kodak technical pan films were used. ELISA microplate reader was a Dynatech Product, Torrance, CA. PHD^{TM} cell harvester, Cambridge Technology, Inc., Cambridge, MA. was used. EPICS V FACS was from Coulter Electronics, Inc., Hialeah, FL. Irradiation was done at the Cancer Institute, University of Manitoba, using a Cobalt 60 source.

METHODS

Feces of naturally infected calves, humans, and chicken were examined microscopically for the presence of oocysts. Oocysts were identified by a Kinyoun acid fast staining (186).

Oocyst Preparation

The oocyst - containing feces were stored at 4° C in 2.5% potassium dichromate solution, and oocysts were concentrated by a modification of the dichromate sucrose flotation procedure of Willson and Acres (326). A solution consisting of 9 ml of 2.5% $K_2Cr_2O_7$-preserved feces, 24 ml of sucrose (825 g in 500 ml dH_2O) and 6 ml of dH_2O was placed in a 50 ml screw-cap centrifuge tube, inverted 10 times and centrifuged at 800 x g for 10 min; the surface layer was decanted and washed with 0.85% saline and centrifuged at 1200 x g for 5 min; washing was repeated 3 times, and oocysts were counted using a hemocytometer.

Experimental Infection of Calves

Newborn, one day old, calves were checked for the presence of fecal oocysts and only uninfected ones were used for the experiment. Each calf was colostrum - fed before oocyst inoculation; then tube-fed a total of 2-4 x 10^6 oocysts (kept in potassium dichromate solution for less than one month) in 1 ml of saline added to 500 ml of milk. Feces were collected daily, concentrated and preserved as described above and used for all other experiments within 6 months period. Oocysts from experimentally infected calves' feces were used to infect different strains of mice. Purified oocysts were used to analyse their protein patterns and compare them with those of human and chicken oocysts. Calves were bled weekly for periods of 3 - 16 weeks and serum samples were collected and kept at - 20 °C until used. As well, colostrum samples were

obtained from 4 randomly selected Holstein cows and kept at -20° C until tested.

Purification of Oocysts

To purify oocysts, the procedures recommended for coccidian parasites purification were first tried: e.g. the petri dish method of Hammond et al. (109), the 5.25% sodium hypochlorite solution recommended by Wagenbach et al. (317), and the saturated sodium chloride procedure of Smith and Herrick (281), but none was found suitable. Different procedures had to be developed.

A- Percoll Gradient Procedure

To obtain pure oocysts, two previously reported procedures for Coccidia were modified and used in tandem. The first step was a modification of Wagenbach et al.(317) procedure and the second step was an adaptation of the Percoll procedure used by Fernando et al. (90) for the purification of Eimeria merozoites. Preserved oocysts were washed three times with saline and treated with 3% sodium hypochlorite by keeping the solution in an ice bath for 20 min, followed by washing three times in 10 mM Phosphate Buffer Saline (PBS), pH 7.2. The gradient of Percoll consisted of 3 ml of stock (9 volumes Percoll and 1 volume of 8.5% NaCl), 4 ml of 70% , and 4 ml of 50% of stock Percoll diluted with saline. Four ml of PBS containing oocysts (pH 7.2) were layered over the gradient in 16x102 mm Ultra-Clear™ tubes and centrifuged at 16000 x g for 10 min at 4° C. Three distinct bands were formed and

recovered separately and washed 3 times with PBS pH 7.2. At each stage of the sodium hypochlorite treatment and Percoll separation, aliquots (200 ul) were examined for bacterial contamination by inoculation of blood agar plates. In addition, 3 smears were prepared and stained by Kinyoun and Giemsa, as well as Gram staining. Proof of viability of oocysts was obtained by excystation and by observing microscopically the motility in the majority of excysted sporozoites. Some of these treated oocysts were used as a source of antigen for an IFA test (191), as well as for protein pattern analysis in a SDS-PAGE.

B- Cesium Chloride Gradient Procedure

This method was established to purify oocysts not pretreated with sodium hypochlorite. Concentrated fecal oocysts, using the modified procedure of Willson and Acres(326), were washed twice with saline and once with TE buffer (50 mM Tris, 10 mM EDTA) and counted. In 16x102 mm tubes a CsCl gradient was prepared, consisting of three ml layers of CsCl in TE buffer with a density of 1.40, 1.10, and 1.05 g/ml in the bottom, middle and top layers, respectively. One ml of TE buffer containing oocysts was layered on top of the CsCl gradient and centrifuged at 16000 x g for 1 hr at 4^{o}C. Three bands were recovered separately and dialyzed against 10 mM Tris buffer containing 1mM EDTA. Bacterial contamination was checked by inoculation of 200 ul from each band onto blood agar medium. At the same time, three smears were prepared and stained as described before.

After 24 hr, bacterial growth was checked by counting the number of colonies. Purified oocysts were aliquoted and stored in 2.5% $K_2Cr_2O_7$ at 4°C until used. These oocysts were used as antigen for IFA and ELISA, as well as for protein separation by SDS-PAGE.

Excystation

Oocysts were excysted by the method of Current et al. (65) with slight modifications. CsCl purified oocysts were pretreated with 1.75% sodium hypochlorite for 12 min in ice-bath and washed with Hanks' balanced salt solution (HBSS),free of Ca^{++} and Mg^{++} as recommended by Reduker and Speer (255). Treated and washed oocysts were incubated at 37°C for 60 min in 15 ml parafilm sealed tubes containing HBSS with 0.25% trypsin and 0.75% sodium taurocholate (each weight to volume). Tubes were agitated for 2-3 sec every 15 min.

Purification of Sporozoites

For the purification of sporozoites using cesium chloride gradient, various concentrations and centrifugation times were tried. The most successful combination was obtained by layering a suspension of 1 ml excysted oocysts in HBSS pH 7.2, over a gradient consisting of four 2 ml layers of CsCl with densities of 1.3, 1.2, 1.1, and 1.05 g/ml , respectively, from bottom to top. The gradient was centrifuged at 16,000 x g for 3 hr at 4°C.

Preparation of Hyperimmune Rabbit Sera

Hyperimmune sera were raised in NZW rabbits against

oocysts and against sporozoites using CsCl purified oocysts and pure sporozoites. The protocol for raising antibodies was as per a personal communication with Dr. W.L. Current. Briefly, $10x10^6$ of either purified oocysts or pure live sporozoites were used for the first injection and for each booster. The oocyst solution was freeze-thawed 5 times, using liquid nitrogen and a water bath (37^oC). Rabbits were injected via three routes, I.V., I.M., and S.C., each with a dose of $3.3x10^6$ Cryptosporidium oocysts or intact sporozoites. Incomplete Freund's adjuvant was used for the I.M. and S.C. injections. Each rabbit received 4 boosters of oocysts and three boosters of sporozoites at intervals of 14 days. The intravenous injection was omitted in the booster injections.

Determination of Cryptosporidium Specific Antibody by Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed on: a) Serum samples from experimentally infected and uninfected (IFA seronegative) calves. b) Hyperimmune and preimmune rabbit sera. c) Colostrum samples.

1- Preparation of Sonicated Antigen

CsCl purified oocysts recovered from experimentally infected calf feces, were suspended in bicarbonate buffer pH 9.6, sonicated 30 times at set 7, 30 sec each.

2- ELISA Procedure

Development of an ELISA allowed for a more precise and

sensitive assessment of Cryptosporidium - specific serum Ig class levels, as compared to indirect immunofluorescent antibody assays (IFA) (191).

The method described by Turunen et al. (296) was slightly modified. Briefly, 100 ul bicarbonate buffer (0.05 M, pH 9.6) containing antigen from 50,000 sonicated oocysts (approximately 2.5 ug/ml protein) was added into each well of 96-well ELISA plates. The plates were incubated at 4^{o} C overnight and stored for up to 7 days. The plates were emptied and 200 ul of 1% horse serum in PBS pH 7.2 added into each well and incubated at 37^{o} C for 2 hr. After washing the wells 5 times with PBS pH 7.2, containing 0.05% Tween 20, each well received 100 ul of either 1:100 dilution of calf sera, 1:100 dilution of colostrum, or serial dilutions of rabbit sera in PBS. The plates were incubated at 37^{o}C for another 2 hr. The wells were washed 5 times with PBS-Tween 20 and filled with 100 ul of a 1:16,000 dilution of peroxidase labelled goat anti-bovine IgG, or a 1:8,000 dilution of peroxidase labelled goat anti-bovine IgM, or a 1:16,000 dilution of peroxidase labelled goat anti-rabbit Ig or IgG. The above dilutions were selected to reduce the background noise. The plates were incubated at 37^{o}C for 1 hr. After washing the wells 5 times with PBS-Tween 20, each well received 100 ul of orthophenylanine diamine (OPD) substrate as directed by the manufacturer and the plates were incubated at room temperature in the dark for 30 min. The reaction was stopped by adding 100 ul/well of 2 N Sulfuric acid. The

results were recorded using an ELISA microplate reader, at absorbance of 490 nm. The cutoff value was obtained by testing, in triplicate, two IFA negative sera (uninfected calf or rabbit), at dilution of 1:100 and then using as cutoff three times the mean of their O.D. values. To plot the standard curve for experimentally infected calf serum IgM or IgG antibodies, two sera with the highest O.D. value were chosen, and an ELISA was done for each sample, individually, by using serial doubling dilutions starting at 1:50 up to 1:1600.

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot analysis

1- Protein Determination

The protein content of oocysts was determined by the method of Lowry et al. (182). The oocyst pellet was solubilized with 0.1 ml of o.1 N NaOH and bovine serum albumin was used as a protein standard.

2- SDS-PAGE

SDS-PAGE was performed as described by Laemelli (159) with the following specifications. Recently purified oocysts were dissolved in sample buffer containing 2% SDS and 2% 2-mercaptoethanol, then boiled for 5 min and kept at -20^{o} C until used. Electrophoresis was performed by using either a 12% or a 7.5% polyacrylamide vertical slab (140 x 85 x 0.75 mm), both with a 4% stacking gel. The above frozen oocyst samples were thawed, boiled for five min, and loaded at

concentration of $1x10^6$ oocysts (5 ug protein) per lane. Phosphorylase b (94 Kd), Bovine serum albumin (67 Kd), Ovalbumin (40 Kd), Carbonic anhydrase (30 Kd), Soybean trypsin inhibitor (20.1 Kd) and Lactalbumin (14.4 Kd) were used as low molecular weight protein markers (LMW) and Thyroglobulin (330 Kd), Ferritin (220 Kd), Myosin (200 Kd), and E.coli- galactosidase (116.25 Kd) were used as high molecular weight protein markers(HMW). As recommended by the manufacturers, protein markers were also boiled and denatured as described for oocyst samples. A running buffer of 0.025 M Tris, 0.19 M glycine and 1% SDS, pH 8.3 was used. The electrophoresis was carried out on a slab gel apparatus at 20 mA /gel for approximately three hours at room temperature until the tracking dye reached the bottom of the gel. To optimize resolution of the high molecular weight proteins, the tracking dye was allowed to run for another 30 minutes after reaching the bottom of the 7.5% gel. The gels were then stained using the silver stain decribed by Morrissey (220). Gels were kept in deionized water and photographed wet. Calculation of molecular weight markers was done as recommended by Pharmacia (242).

3- Enzyme- Linked Immunoelectrotransfer Blot (EITB) Technique

Protein blotting was performed by transfer technique as per Bio-Rad instructions. Separated proteins were transferred from either 12% or 7.5% gel to nitrocellulose paper in the Trans Blot cell, containing transfer buffer (0.192 M glycine, 0.025 M Tris, 20% V/V methanol, pH 8.3) at

150 mA for 16 hr and then for 1 hr at 200 mA at room temperature.

The electrophoretic blots were either stained with amido black or blocked by 1% skim milk in Tris-buffer saline (TBS) for 1 hr at 37°C. The blots were incubated at 37° C for 1 hr in TBS containing 0.2% Tween 20 (TBST), with either a 1:100 dilution of control sera or 1:20000 dilution of rabbit IgG antibody raised against oocysts, or a 1:1000 dilution of rabbit IgG antibody raised against sporozoites or a 1:25 dilution of experimentally infected calf serum positive by ELISA. The above dilutions were selected to reduce the background staining. The blots were rinsed once in TBST at room temperature and washed twice in the same solution at 37°C for 10 min each time. The blots were incubated for 1 hr at 37°C with a 1:1000 dilution of either highly purified peroxidase - conjugated goat anti- bovine IgG or goat anti-rabbit IgG, in TBST. The blots were rinsed once and washed twice in TBST, at 37°C for 10 min each time. The color was developed with 0.5 mg/ml 0f 3,3' - diaminobenzidine in PBS pH 7.4 containing 0.1% of 3% hydrogen peroxide (H_2O_2). The reaction was stopped by extensive washing of blots in distilled water. These blots were photographed immediately or stored in dark until photographed. All washes and incubations were performed with constant agitation.

<u>Cryptosporidium</u> Infection in B Cell- Deficient Mice

1- Animals

Adult (6-7 wk old) and neonate (7-21 days old) BALB/c

(inbred), adult (6-7 wk old) Swiss (outbred) mice and adult S - D rats were used. Mice were placed in an isolated infection room.

2- Preparation of Anti-u or Normal Rabbit Ig(NRIg)-Treated Mice

Anti-u treatment was carried out as described by HayGlass et al. (116). Newborn BALB/c mice were injected intraperitoneally, starting within 24 hr of birth, with 150 ul of either concentrated anti-u antibody containing 700-1000 ug of mouse red blood cell - absorbed, $(NH_4)_2\ SO_4$ precipitate of rabbit anti-mouse IgM antibodies per injection or with a mouse erythrocyte - absorbed, $(NH_4)_2\ SO_4$ precipitate of normal rabbit serum (NRIg) . The mice were injected three times per week until sacrificed. Untreated mice served as additional normal controls in all experiments.

3- Inoculation of Mice with Cryptosporidium Oocysts

Four different age groups of BALB/c mice (4-9/ group) were studied. Neonatal (7 day old) mice were given orally 1.5 x 10^5 oocysts/ mouse, in 10 ul of saline; mice 11-12, 14-15, and 21-22 days old were fed with 3 x10^5, 6 x 10^5 and 1 x 10^6 oocysts/ mouse, in 20 ul of saline, respectively.

To determine the amount of oocysts required to infect normal adult mice (4-6 / group), groups of adult (42 - 49 days old) BALB/c , and Swiss mice were fed 50 ul saline containing oocysts varying in number from 2 x10^4 to 1.5 x 10^6/mouse. The above mentioned maximum dose was selected

for inoculation of B cell - deficient adult BALB / c mice. In addition, age matched controls received saline alone. Each experimental adult and each group of neonates were caged separately.

4-Stools and Blood Collection

Stools were collected from each individual adult or each group of neonates every day for at least 3 weeks. The presence of oocysts in stools and their numbers were recorded, using Kinyoun acid fast staining. Two slides were prepared for each sample, a minimum of 60 fields (400 x magnification) were counted, and data recorded as the mean number of oocysts observed / 10 fields ($\pm$ SE). In addition, a sensitive concentration procedure, Sheather's flotation method, was used for adult stool samples (201, 256).

All experimental adults and neonates were anesthesized and bled by cardiac puncture from 14- 31 days p.i. Serum samples were collected and stored at - 20 ^{o}C until analysed.

5- B Cell Proliferative Responses

Spleen or lymph node cells from normal, NRIg - treated and anti-u-treated animals were stimulated <u>in vitro</u> with lipopolysaccharide (LPS), to monitor mature B cell activity (292). To prepare cell suspensions, animals were sacrificed and then spleens and lymph nodes were removed aseptically. Washed, single cell suspensions were prepared in serum free medium RPMI 1640 and the number of viable cells determined by counting, using trypan blue and acetic acid. Cells were cultured in duplicate at $6x10^5$ cells/ well in a 96 well flat

bottom plates in the presence of mitogen (LPS) at final concentrations of 0, 2, 10, and 50 ug/ml. Culture medium consisted of RPMI 1640 supplemented with 100 ug/ml penicillin, 100 ug/ml streptomycin, 0.25 ug/ml of fungizone, 2 mM glutamine, $5x10^{-5}$ M 2-mercaptoethanol,and 10% pre-selected, heat-inactivated fetal calf serum. Cultures were incubated for 48hr in 5% CO_2 at 37°C with saturated humidity. After 48 hr, cultures were pulsed with 1 uCi [^{3}H]- thymidine/ well and harvested 8-18 hr later. [^{3}H]-thymidine uptake was determined by liquid scintillation counting.

6- Fluorescence Analysis of Surface Ig^+ Cells

The percentage of spleen or lymph node cells expressing cell surface (murine) Ig^+ cells was determined by the method described by HayGlass et al. (114). Cells were stained with affinity purified FITC-conjugated goat anti-mouse Ig (recognizing heavy and light chains) or FITC - conjugated rabbit anti-goat Ig as a control. After extensive washing, these cells were analysed immediately or fixed and stored in a solution of 2% paraformaldehyde and 1% BSA until analysed. The percentage of sIg^+ cells and their relative fluorescence intensity were determined using an EPICS V fluorescence-activated cell sorter (FACS). Approximately 5,000-10,000 cells were analysed per sample. Duplicate preparations of each sample were independently stained and analysed.

7- Determination of Cryptosporidium Specific Antibody Production

In preliminary experiments serum antibody levels were assessed by indirect immunofluorescent antibody assays (IFA). Sera obtained from Cryptosporidium oocysts- CFA immunized mice were used as positive controls for the experiment.

In most experiments, Cryptosporidium antibody concentrations in the sera of uninfected normal, infected normal, NRIg treated and anti-u treated mice were measured by a sensitive, antigen specific, isotype specific ELISA. This ELISA was similar to that described for calf and rabbit sera except that 1% BSA in PBS was used as blocker and a solution of 0.5% BSA, 0.5% Tween 20 and 0.02% sodium azide in PBS was used as dilution buffer. Alkaline phosphatase conjugated rabbit anti- mouse Ig (panspecific) (1:1000 dilution), anti-IgG, anti- IgG1, anti- IgG2a, anti- IgG2b, anti- IgG3, anti-IgM and anti- IgA (1:500 dilution) were utilized. P-nitrophenyl phosphate substrate was used as directed by the manufacturer. The reaction was allowed to proceed for 60 min and stopped by adding 100 ul of 3N NaOH solution.

Background control values from wells missing one component in turn, did not exceed 0.07 absorbance units. Results were expressed as ELISA titers compared to a common internal standard run in each assay. Each serum sample was assayed at least twice. Geometric means of the end point titer were expressed as the dilutions of serum yielding an absorbance of 0.5 at 630 nm provided an arbitrary point well within the

linear portion of the titration curve.

IgE levels were determined by passive cutaneous anaphylaxis (235) and expressed as geometric mean PCA titers. Briefly, S-D rats were injected intradermally with 0.15 ml of serial dilutions of sera from Cryptosporidium infected mice, and as a positive control, serial dilutions of mouse serum with known titers of anti - ovalbumin IgE, in saline. After 48 hr, they were challenged by the I.V. injection of 5×10^6 sonicated oocysts and 200 ug ovalbumin in 1.0 ml physiological saline and 2% Evan's blue. The highest dilution giving a significant blueing reaction was taken as the end point.

ELISA and PCA titers were log transformed and geometric means were compared.

8- T Cell Responses In Vitro

Unfractionated or T cell enriched spleen and lymph node cells, from normal, NRIg -treated, and anti-u treated animals were prepared. T cell- enriched populations were obtained by "panning" with affinity -purified goat anti-mouse Ig-coated petri dishes (170), typically yielding preparations with < 5% sIg^+ cells.

To examine T cell proliferative capacity, cells were stimulated in vitro with different stimuli as follows:

A) T cell responses to concanavalin A (Con A):

Unfractionated spleen and lymph node cell preparations were cultured in duplicate at $6x10^5$ cells / well, in the

presence of the T cell mitogen Con A, at final concentrations of 0, 1.2, 2.5, 5, ug/ml. Cultures were incubated in 5% CO_2 at 37^{o} C for 24 hr. They were pulsed with 1 uCi of [^{3}H]-thymidine and harvested 8-18 hr later. Thymidine uptake was determined by liquid scintillation counting.

B) Antigen - specific T cell proliferative responses to Cryptosporidium oocyst antigens:

Unseparated or T enriched populations of spleen or lymph node cells from naive and Cryptosporidium-infected normal or anti-u treated BALB / c mice were cultured 12, 14, 15, and 45 days post inoculation at 6 $x10^{5}$ cells/well in the presence of 0, $0.25x10^{5}$, $1x10^{5}$ and $4x10^{5}$ intact oocysts / well or 0, $3x10^{3}$, $1x10^{4}$, $3x10^{4}$, $1x10^{5}$, and $3x10^{5}$ sonicated oocysts/ well. Cultures were maintained for 3, 4, 5, or 6 days at 37^{o}C, 5% CO_2, pulsed with 1 uCi of [^{3}H]-thymidine and harvested 8-18 hr later. Thymidine uptake was then determined by liquid scintillation counting.

C) Cytokine Assay

1) Cytokine production in response to Cryptosporidium sp. oocyst antigens:

T enriched spleen cells from normal infected and age matched non-infected mice were prepared at days 8, 11 and 14 p.i. (i.e. peak infection, decline and recovery). Cells were cultured in 24 well flat bottom plates at 6 x 10^{6} cells/ well in the presence of 4 ug Con A, as a positive control, or of 0, $0.062x10^{6}$, 0.25 x 10^{6}, 1 x 10^{6} and 4 $x10^{6}$ sonicated

oocysts. Supernatants were collected after 24 and 48 hr and kept at -20°C for later evaluation of IFN-gamma, IL- 2, and IL- 4 production.

2) Bioassays of IFN-gamma, IL- 2 and IL- 4 production

To determine the level of IFN-gamma , IL-2, and IL-4 production in the culture supernatants described above, bioassays were performed utilizing WEHI - 279, CTLL, and CT.4S cells respectively.

The level of IFN- gamma was determined by mixing 75 ul of supernatant, 75 ul of medium or medium with anti- IFN- gamma mAb and 50 ul of growth media containing 10,000 WEHI cells / well and incubating at 37° C, 5% CO_2. Mouse Con A supernatant containing IFN-gamma was used as a standard positive control. Cells were pulsed with 1 uCi [^{3}H] - thymidine after 48 hr and harvested 8 - 18 hr later. The growth of WEHI - 279 cells is inhibited in a dose dependent fashion by IFN- gamma with the specificity of the assay assured by the blocking these effects upon addition of anti- IFN- gamma mAb (ATCC HB170) to duplicate wells. The lower limit of detection of IFN- gamma by this assay is 1-2 U/ml.

IL-2 production was assessed by culturing CTLL cells (10^4 cells in 50 ul of growth media) for 24 hr at 37° C and 5% CO_2 in the presence of 150 ul tissue culture supernatant, harvested from Cryptosporidium stimulated lymphocytes, rIL- 2, or rat Con A supernatant containing IL-2. The cultured CTLL cells were pulsed with [^{3}H]-thymidine and harvested after 8-18 hr. The CTLL cells used were absolutely dependent upon

the presence of IL-2, proliferating in a dose dependent fashion in response to this cytokine. Although some CTLL sub-lines proliferate in response to either IL-2 or IL-4, the CTLL cells used in this study did not respond to rIL-4 at doses up to 2000 U/ml (personal communication, Dr. K. T. HayGlass).

IL-4 production was assessed in a similar assay recommended by Hu-Li et al. (132), by culturing CT.4S cells, an IL-4 dependent subclone of CTLL cells. CT.4S cells are highly responsive to murine IL-4 in a dose dependent fashion, but display very limited sensitivity to IL-2 under the conditions used. CT.4S cells were cultured (5-10 x 10^3/well) for 48 hr in 150 ul tissue culture supernatants, harvested from Cryptosporidium stimulated spleen cell cultures or rIL-4 (4 - 266 U / well) as a positive control.

9- T cell Responses In Vivo

To test for delayed type hypersensitivity (DTH), normal uninfected controls and normal, NRIg-treated and anti-u treated mice infected with Cryptosporidium sp., were challenged by S.C. injection in the footpad with $2x10^6$ intact or sonicated oocysts, 7-35 days after the peak of Cryptosporidium infection. Footpad swelling of these mice and uninfected controls was measured 24 hr later using a micrometer to compare the thickness of the injected and contralateral foot. As a positive control, normal adult BALB/c mice were immunized with 0.2 ml of activated azobenzenearsonate solution (ABA) subcutaneously. These mice, and mice not previously immunized with ABA, were injected in

the footpad with 25 ul of ABA 7 days later. ABA specific footpad swelling was recorded 24 hr later.

Studies of Cryptosporidium sp. Infection in Anti-CD4, and Irradiation Treated BALB/c Mice

1- Preparation of Anti- CD4 Treated Mice

Supernatant from the hybridoma GK 1.5, producing rat monoclonal antibody (mAb) against CD4, was concentrated 12 fold and injected every other day into BALB/c neonates (150 ul/ mouse), I.P. from day 3 after birth until the mice were sacrificed . Untreated BALB / c neonates were used as controls in these experiments as treatment with an unrelated rat IgG had previously been shown to be without effect (personal communication, Dr. K. T. HayGlass). At the age of 7 days, mice were inoculated with 1.5×10^5 oocysts/ mouse.

2-FACS Analysis for CD4 Cells Ratio

The percentage of spleen cells expressing cell surface CD4 or CD8 was determined in normal and CD4 depleted mice. T enriched spleen cells (200,000) were exposed to GK 1.5 supernatant (anti-CD4), ADH 4 supernatant (anti-CD8) or HO 13.4 supernatant (anti-thy 1.2) for 45 min at $4^{o}C$ in 96 well plates. Cells were then washed and stained with FITC-conjugated rabbit anti-rat or rabbit anti- mouse Ig for an additional 45 min at $4^{o}C$. Cells were then washed, fixed in 2% paraformaldehyde, and stored in the dark at 4^{o} C until analysed by flow cytometry using EPICS V FACS.

3 - Preparation of Irradiated Mice

Adult BALB/c mice and neonates (7 days old) were irradiated with 550 rad, using a Cobalt 60 source. One third of irradiated adults were reconstituted with B cell enriched spleen cells, and one third with T cell enriched spleen cells, and one third was not reconstituted. Irradiation was followed immediately by inoculation of 1.5×10^6 oocysts/ mouse.

To evaluate the extent of B and T cell reconstitution following irradiation, spleen cells of irradiated and non-irradiated mice were compared on day 4, 8 , 10, and 14 post-irradiation in terms of their capacity to respond to the B and T cell mitogens LPS and Con A, as described above, or to generate mixed lymphocyte responses.

Cryptosporidium sp. Infection in Natural Killer (NK) Cell Deficient Mice

Mice genetically deficient in NK cell activities (i.e. C3H/HeJ-Bg/Bg) were used. To examine the role of NK cells in the resolution of Cryptosporidium infection, neonates (7 days old) were inoculated with oocysts at concentration of 1.5×10^5 oocysts/ suckling mouse. Stool samples were collected daily and checked for the presence of oocysts as described above.

Statistical Analysis

Differences of statistical significance between tests and their controls were determined, using Student's t test.

RESULTS

PART A

I- Experimental Infection of Calves

Post infection (p.i.) all ten colostrum- fed infected calves excreted oocysts in their feces, starting on day 4 and peaking between day 7 and 8. Eight calves survived and stopped excreting oocysts after 13 or 14 days, while the two other calves developed a severe diarrhea and died at day 12 p.i. The cause of death was probably cryptosporidiosis since neither pathogenic bacterial nor viruses were found in stools examined at the Cadham Provincial Laboratory. Feces from infected calves were collected and oocysts recovered.

II- Purification of Oocysts

Five different procedures for the purification of Cryptosporidium oocysts were evaluated ; three of these were found to be impractical. In the glass petri dish method, only 10% of the oocysts were recovered, while the sodium hypochlorite (5.25%) and the saturated sodium chloride procedures resulted in the excystation of more than 95% of the oocysts.

1- Percoll Gradient Purification

Pretreatment of oocysts with 3% sodium hypochlorite followed by a percoll gradient resulted in the recovery from band 2 of 75% of the oocysts (Fig.1). Band 1, 2, and 3 (Fig.1) were examined microscopically. Direct smears and

smears stained by Kinyoun acid fast and Giemsa showed that band 1 contained debris, band 2 had very clean oocysts with no debris, and band 3 contained oocysts with some debris. This procedure resulted in the recovery of 75% of the oocysts. Oocysts recovered from band 2 were incubated at 37°C for 1 hr. Microscopic examination revealed motile, spontaneously-excysted sporozoites indicating that oocysts pretreated with 3% sodium hypochlorite were viable. Tests for bacterial contamination were all negative when pure oocysts from band 2 were inoculated into blood agar plates and incubated at 37°C for 24 hr. When evaluated as a source of antigen in an IFA test using a 1:100 hyperimmune rabbit serum, these pretreated oocysts exhibited a fluorescence of 1^+ compared to the 4^+ shown by untreated oocysts, i.e. a reduction of > 75%. Thus sodium hypochlorite treatment appears to have modified the surface antigenic make up of oocysts. This observation was confirmed by SDS - PAGE analysis, as described later on in this section. Sodium hypochlorite pre - treated oocysts had very few protein bands compared to oocysts purified by the cesium chloride method described below (Fig.13).

Treatment with sodium hypochlorite prior to percoll gradient appears to be necessary, as clean oocysts were not recovered from any of the bands whenever percoll gradient was attempted on untreated oocysts, even when the centrifugation time was increased from 10 to 20, 30, or 60 min.

2- Cesium Chloride (CsCl) Gradient Purification

A- Purification of Oocysts

In the CsCl gradient, 3 bands and a distinct pellet could be recognized. Clean, viable oocysts, free of bacterial contamination (shown by lack of bacterial growth on blood agar), were recovered from band 1 (Fig.2). Lack of debris was demonstrated microscopically. Direct smears, Giemsa, and Kinyoun acid fast stained smears showed a good concentration of oocysts (Fig.3). Viability was demonstrated by the fact that those purified oocysts were successfully used to infect 2 newborn calves. When exposed to an excystation solution containing trypsin (0.25%) and sodium taurocholate (0.75%) at $37^{o}C$ for one hr, these oocysts were shown to excyst.

Band 2 contained oocysts contaminated with debris, band 3 had debris, bacteria, and very few oocysts, while the pellet was full of bacteria mixed with debris. These were shown by either direct examination of solutions recovered from each band or by examination of stained smears or by bacterial growth on blood agar. Approximately 94% of the oocysts were recovered from band 1, when oocysts varying in number from 1×10^{7} up to 1×10^{8} were passed through the CsCl gradient. These oocysts were suitable sources of antigen for IFA and ELISA as will be discussed later, and were used to raise hyperimmune antisera in rabbits. The 94% recovery of oocysts was recorded only when oocysts were stored for less than 60 days. Recovery was reduced to 17.5% for oocysts stored for 10 months and to 5% after one year storage.

B- Purification of Sporozoites

The CsCl gradient used for the purification of sporozoites showed 2 bands. When examined microscopically, pure viable sporozoites were recovered mainly from band 2 while intact oocysts were found in the pellet (Fig.4). Recovery of approximately 70% was possible when 140 x 10^6 sporozoites were layered on the top of the gradient on each of the three occasions.

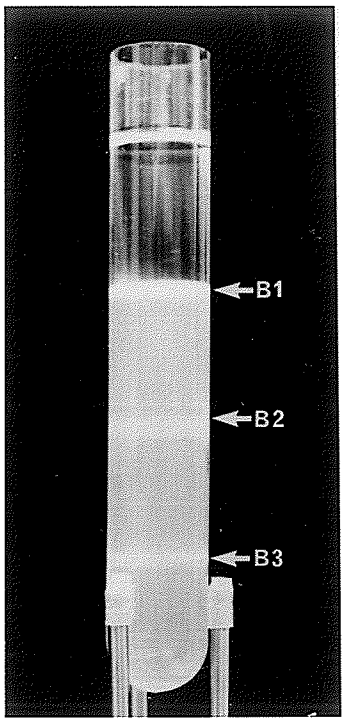


Figure 1. Percoll gradient for the separation of sodium hypochlorite - treated oocysts. Four ml of the oocyst suspension in PBS were layered over the Percoll gradient, and centrifuged at 16,000 x g for 10 min at 4° C. B1 contained mostly debris; B2 and B3 contained clean oocysts with no bacteria nor debris.

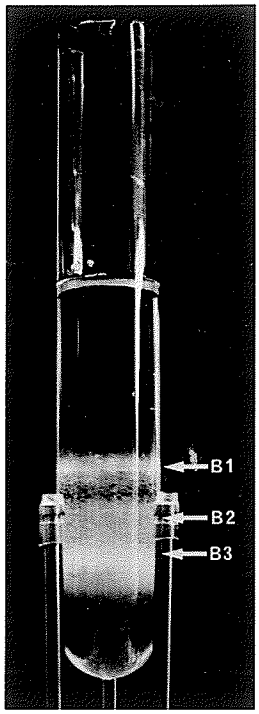


Figure 2. Cesium chloride gradient for the separation of oocysts. One ml of oocyst suspension in 50 mM Tris and 10 mM EDTA were layered on top of a CsCl gradient, and centrifuged at 16,000 x g for 60 min at 4° C. B1 contained clean oocysts free of bacteria and debris; B2 contained oocysts contaminated with debris; B3 had debris, bacteria, and very few oocysts.

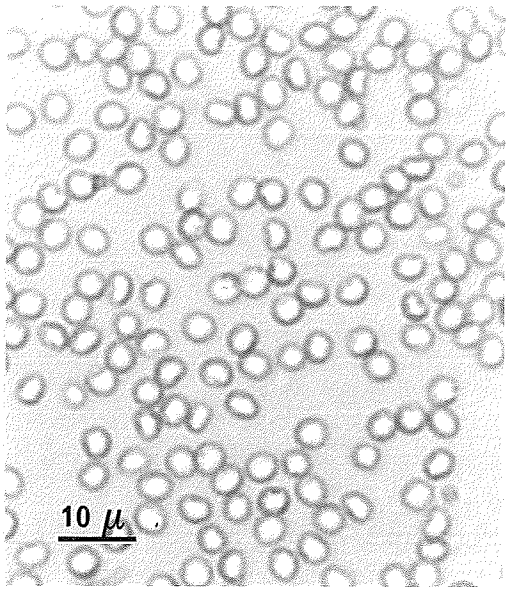


Figure 3. Unstained Cesium chloride - purified oocysts. Following Cesium chloride gradient purification, a direct smear of band 1 (B1) shows a heavy concentration of clean oocysts.

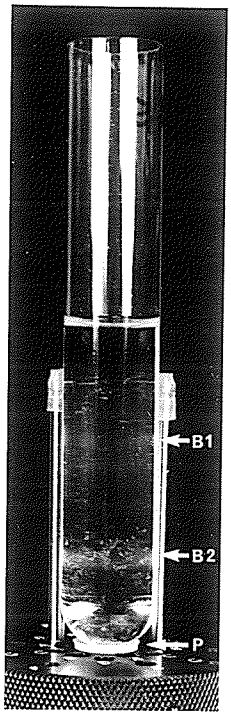


Figure 4. Cesium chloride gradient for the separation of sporozoites. One ml of excysted oocysts in HBSS was layered over a gradient of CsCl, and centrifuged at 16,000 x g for 3 hr at 4° C. B1 contained oocysts wall and few sporozoites; B2 contained clean sporozoites; and pellet (P) contained intact oocysts.

III-Enzyme-Linked Immunosorbent Assay (ELISA)

1- ELISA on Calf Serum Samples

An ELISA was performed on a 1:100 dilution of sera collected over a period of 3-18 weeks, from ten experimentally infected calves.

The mean of O.D. of two negative sera was 0.05. Sera having an O.D. of $\geq$ 0.15 were considered as ELISA IgM or IgG positive. Very high, high, and low positive sera had O.D. of $\geq$ 1.0, 0.5 - 0.99, and 0.15 - 0.49, respectively.

A- IgM Antibodies

a) Fig. 5 shows the IgM antibody levels in samples collected from 4 colostrum - fed infected calves followed up for 3 - 4 months. On the day of inoculation, 3 calves had low levels of IgM antibodies with O.D. = 0.25 - 0.34 (Fig. 5A, B, C). One week post infection, the level of IgM antibodies had dropped in all these 3 calves. This drop was followed by a rise in IgM antibody levels. The peak level was reached within 2 - 3 weeks post infection for calves A and C and was maintained for the period of serum collection in calf C but dropped after 13 weeks in calf A. In calf B, IgM antibodies reached an O.D. level of 0.27 ± 0.01 only 8 weeks post infection. Calf D had no IgM antibodies on the day of inoculation nor one week post infection; IgM antibodies appeared at 3 weeks and then peaked 12 - 13 weeks p.i.

b) Fig.6 shows the IgM antibody levels in serum samples collected weekly from 6 calves followed up for a short period

of time. The serum collection on calves E, F, G, H was continued for 3 weeks, while calves I and J died on day 12 p.i. At the time of oocyst inoculation, the 4 calves which survived had detectable IgM antibodies while the two calves which died had no such antibodies; one of these 2 calves developed a low level of antibodies one week p.i. Post infection, the level of IgM antibodies decreased in each of the weekly samples in calves E and F, while calves G and H exhibited an opposite pattern, with IgM antibodies peaking at 3 weeks.

c) To assess the titer of IgM antibodies, two calf sera with high O.D. values for IgM antibodies (calf E, preinoculation and calf A, 12 weeks p.i.) were serially diluted from 1:50 to 1:1600 and IgM antibodies measured by ELISA. Fig.7 shows that IgM antibodies were still detected at a dilution of 1:1600 in calf E and at a dilution of 1:400 in calf A. The end point was found only in calf A (ie. 1:800).

B- IgG Antibody

a) Fig. 8 shows the levels of IgG antibody in samples obtained from the 4 colostrum- fed calves followed up for 3 - 4 months. At zero time, all calves had detectable IgG antibodies with the strongest IgG response in calf C (O.D.= 0.69 ± 0.01); this calf also had the strongest IgM response, compared to calves A, B, and D (Fig. 5). In all 4 calves , the levels of IgG antibodies declined one week p.i., then started to rise again. IgG antibodies reached their peak

after 5 weeks in calves A and B, after 8 weeks in calf C, and after 9 weeks in calf D. The level of IgG antibodies stayed at this maximum level for the period of serum collection.

b) Fig. 9 illustrates the IgG antibody responses in the six colostrum- fed calves followed up for a shorter period of time. A very strong positive IgG antibody response (O.D.= 1.09 $\pm$ 0.03), was detected initially in calf E, then declined gradually during the period of 3 weeks. A similar pattern was detected in calf F. The remaining four calves had detectable IgG antibodies at the time of oocyst inoculation; the level of IgG antibodies declined one week p.i. in calves G, H, I,and J. In calves G and H, IgG antibodies increased and peaked within three weeks; calves I and J died at day 12 p.i.

c) To assess the titer of IgG antibodies, two serum samples with strong O.D. values for IgG antibodies from calf E, preinoculation and calf A, 12 weeks p.i. were serially diluted from 1:50 to 1: 1600 and the IgG antibodies measured by ELISA. Fig. 10 shows that IgG antibody was still detected at a dilution of 1:1600 in sample of calf E and at a dilution of 1:800 in serum of calf A. The end point was found only in calf A (ie. 1:1600).

2- ELISA on Colostrum

The IgG and IgM antibodies present in 4 randomly selected colostrum samples, tested at a dilution of 1:100, are shown in Table 1. All had high O.D. values for both IgG and IgM antibodies. No Cryptosporidium IgG and IgM negative colostrum

could be obtained.

3- ELISA on Rabbit Serum Samples

An ELISA was performed to obtain the end point titer of Cryptosporidium antibodies in hyperimmune rabbit sera. The mean of O.D. for two preimmune rabbit sera was 0.07. Any serum having an O.D. of $\geq$ 0.2 was considered ELISA positive.

Total Ig and IgG antibody levels in serum dilutions of rabbits injected with either oocysts or sporozoites were measured. For oocysts, the end point dilution for IgG antibodies was 1:160,000 (log = - 5.2), and for total Ig was 1:80,000 (log = -4.9), as shown in Fig. 11. For sporozoites, the end point dilution was 1:10,000 (log= -4) for both IgG antibody and total Ig, as shown In Fig. 12.

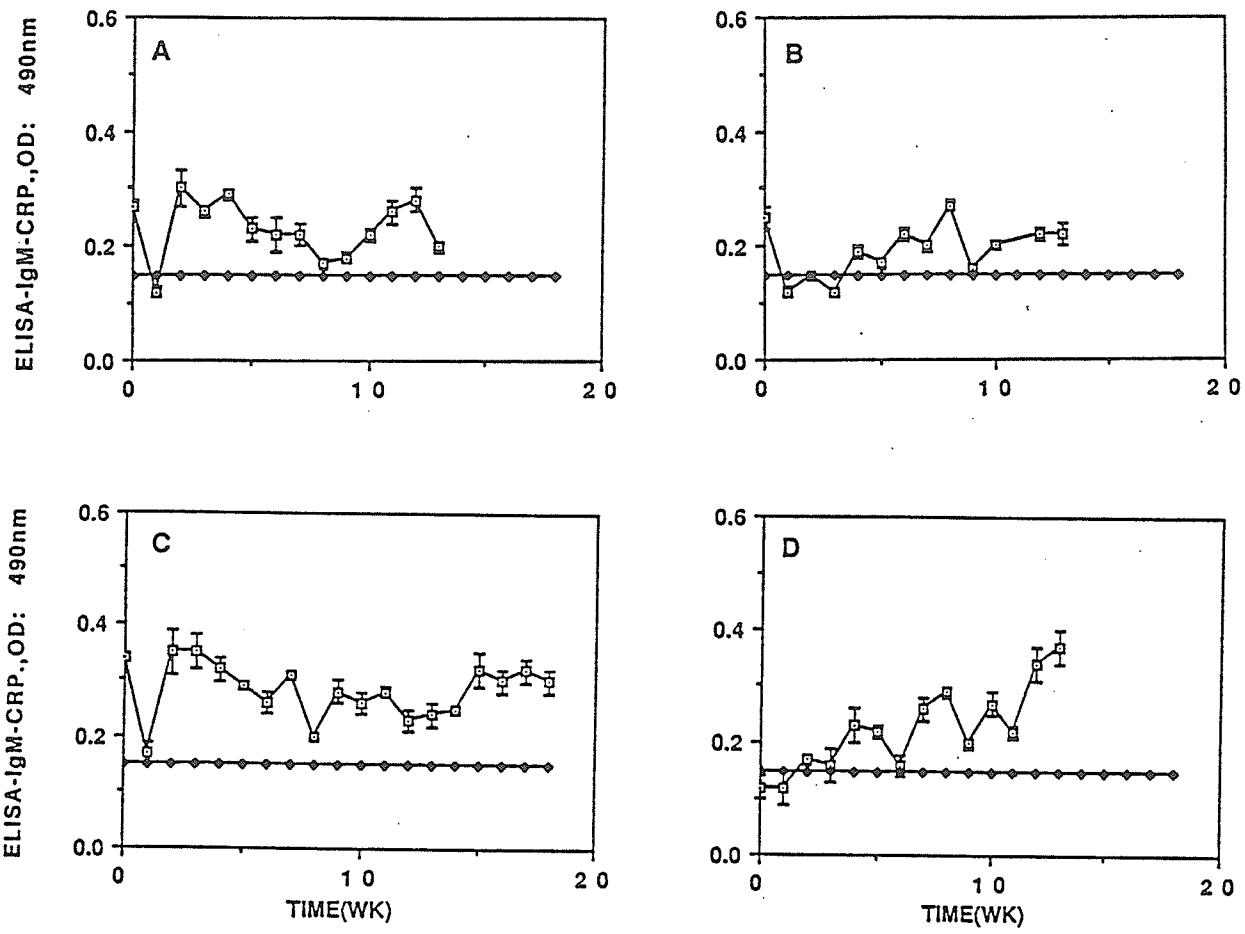
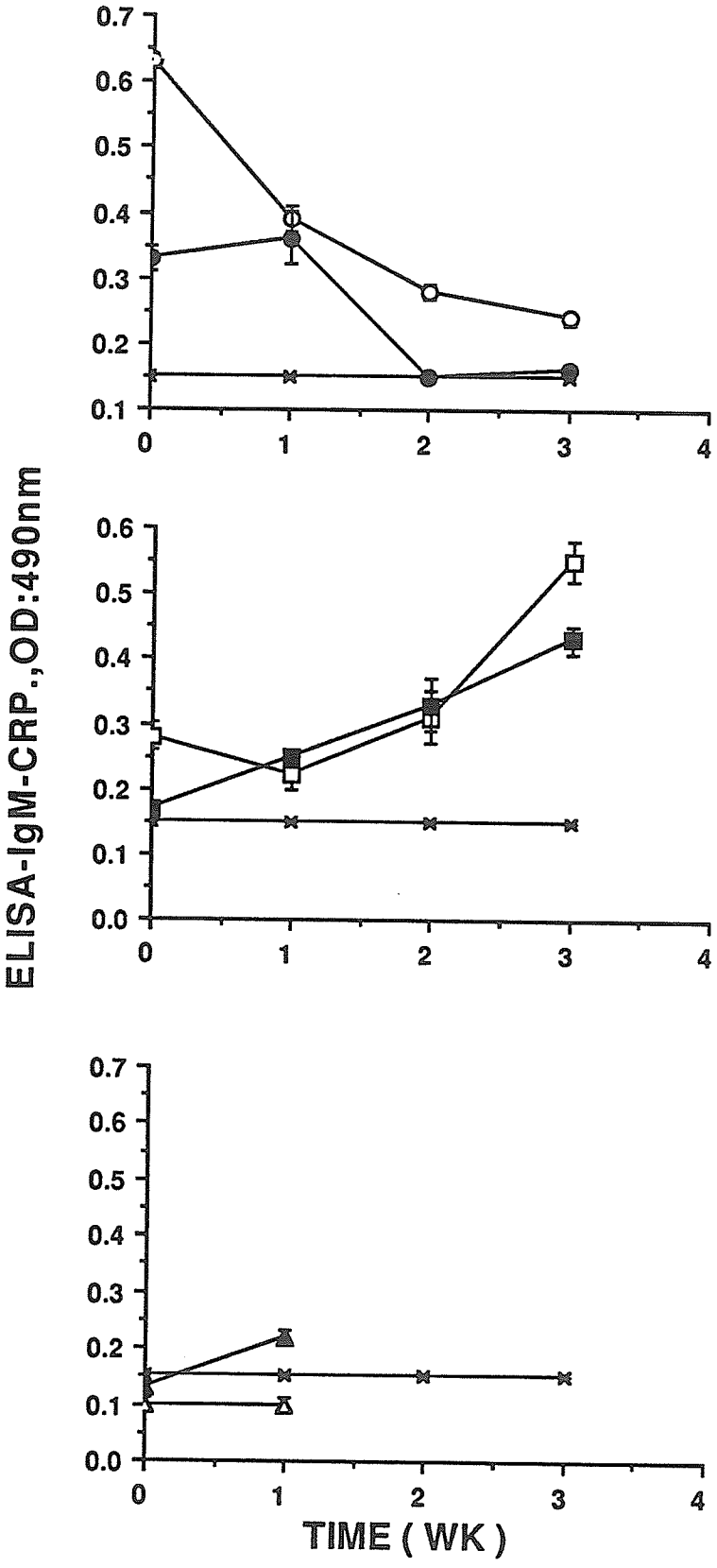


Figure 5. IgM antibodies detected by ELISA in the sera of experimentally infected calves. IgM antibodies to Cryptosporidium were measured by ELISA on serum specimens from four experimentally infected calves. Sera were collected weekly for a period of 13 weeks in calves A, B, D, and 18 weeks in calf C. O.D.$\geq$ 0.15 are considered positive. The cut off point (-◆-◆-) obtained, is three times the mean of O.D. of two IgM negative sera from uninfected calves. Each serum is tested in triplicate and the mean ($\pm$ SD) of their O.D. in a typical experiment is represented by a point on the graph.

Figure 6. IgM antibodies detected by ELISA in the sera of experimentally infected calves. IgM antibodies to Cryptosporidium were measured by ELISA on serum specimens collected weekly from four calves for a period of 3 weeks; calf E (—○—); calf F (—●—); calf G (—□—); calf H (—■—); and for a period of one week from calf I (—△—); and calf J (—▲—). Calves I and J died at day 12 p.i. O.D.$\geq$ 0.15 are considered positive. The cut off point (—✱—) obtained, is three times the mean of O.D. of two IgM negative sera from uninfected calves. Each serum is tested in triplicate and the mean ($\pm$ SD) of their O.D. in a typical experiment is presented by a point on the graph.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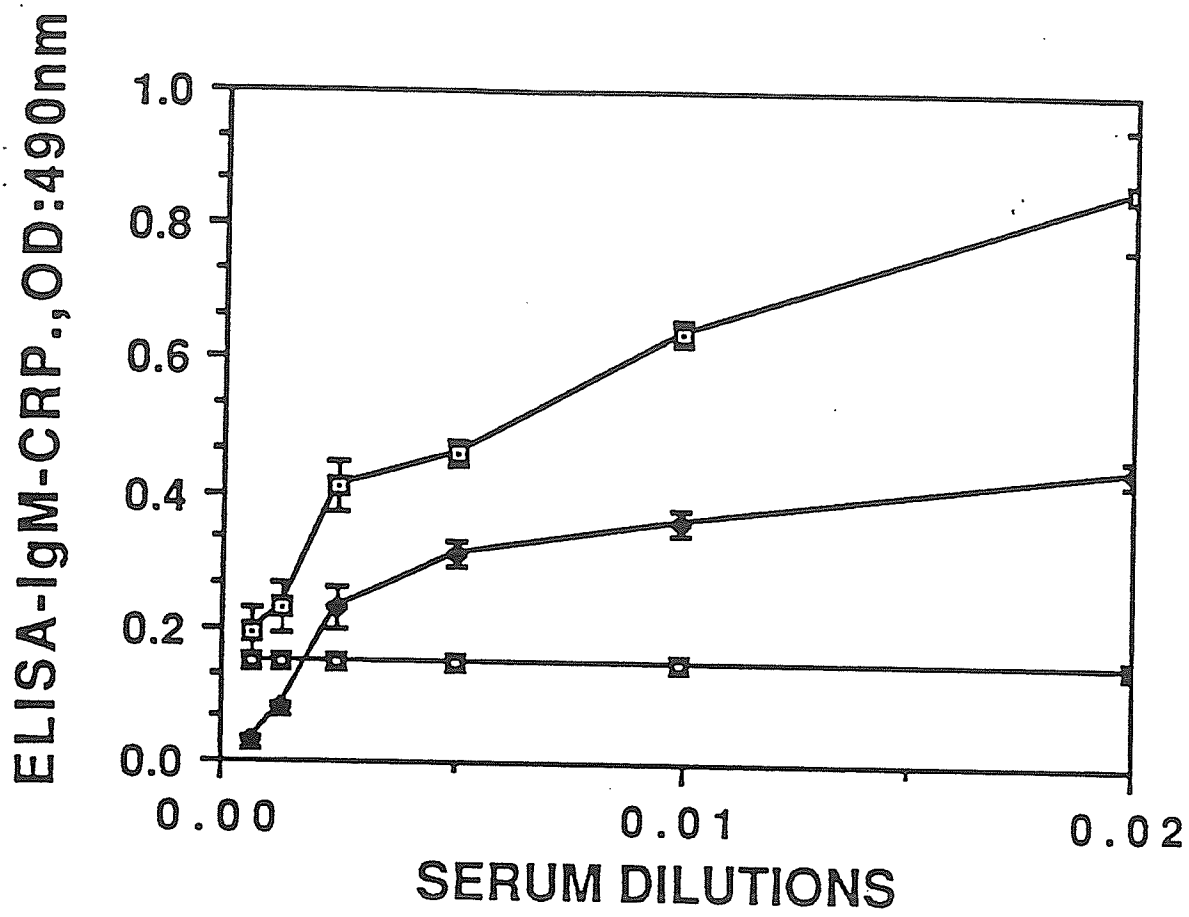
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
TIME (WK)



to Cryptosporidium were mesured by ELISA on serially diluted sera (1:50 - 1:1600) of the two calves with highest IgM O.D. values. Calf A (—◆—) is IgM positive at 1:400 dilution and calf E (—▣—) is positive at 1:1600 dilution. O.D.$\geq$ 0.15 are considered positive. The cut off point (—▣—) obtained, is three times the mean of O.D. of two IgM negative sera from uninfected calves. Each dilution of serum is tested in triplicate and the mean ($\pm$ SD) of their O.D. in a typical experiment is presented by a point on the graph. The 0.00 represents an infinite dilution.

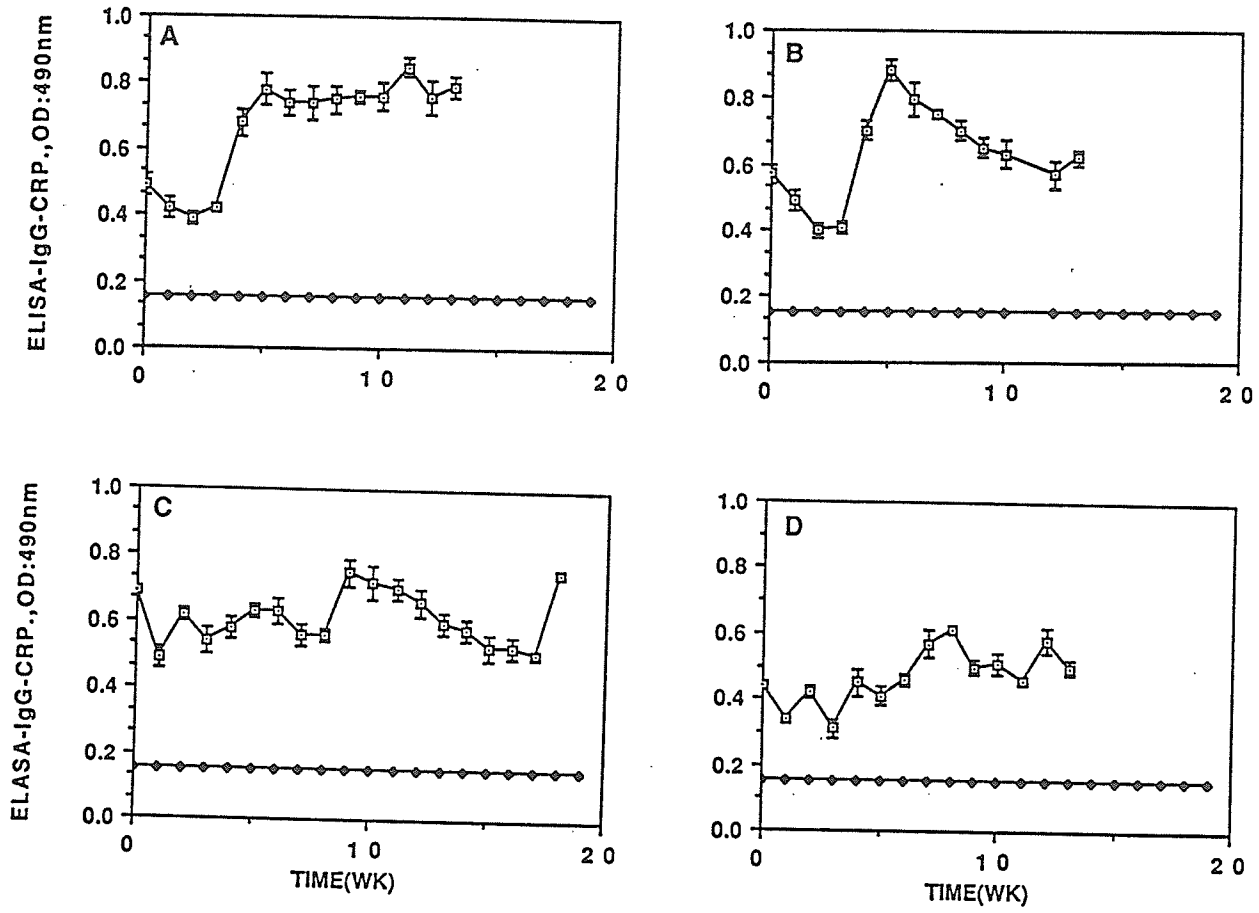
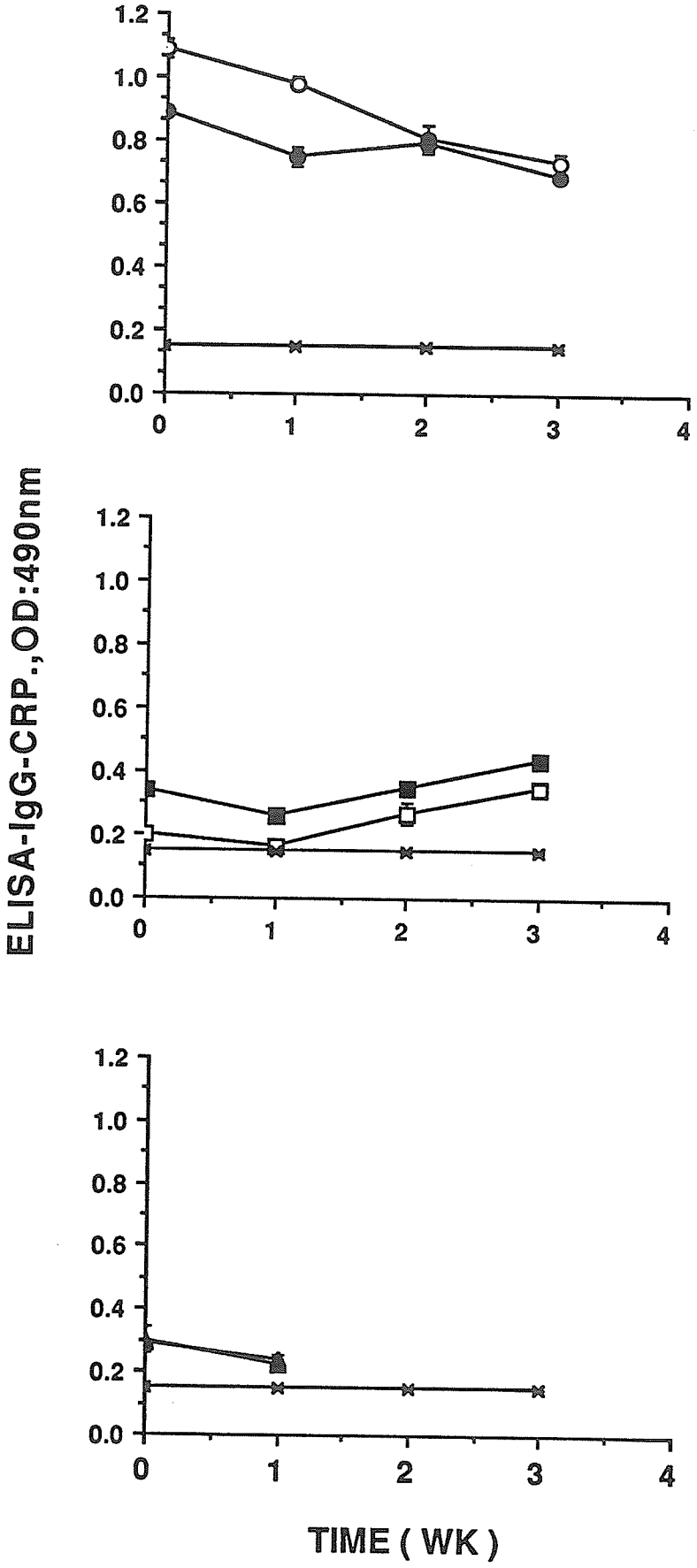


Figure 8. IgG antibodies detected by ELISA in the sera of experimentally infected calves. IgG antibodies to Cryptosporidium were measured by ELISA on serum specimens collected weekly from four calves for a period of 13 weeks in calves A, B, D, and 18 weeks in calf C. O.D. $\geq$ 0.15 are considered positive. The cut off point (-◆-◆-) obtained, is three times of O.D. of two IgG negative sera from uninfected calves. Each serum is tested in triplicate and the mean $\pm$ SD of their O.D. in a typical experiment is represented by a point on the graph.

Figure 9. IgG antibodies detected by ELISA in the sera of experimentally infected calves. IgG antibodies to Cryptosporidium were measured by ELISA on serum specimens collected weekly from four calves for a period of 3 weeks; calf E (—○—); calf F (—●—); calf G (—□—); calf H (—■—); and a period of one week from calf I (—△—); and calf J (—▲—). Calves I and J died at day 12 p.i. O.D. $\geq$ 0.15 are considered positive. The cut off point (—×—) obtained, is three times the mean of O.D. of two IgG negative sera from uninfected calves. Each serum is tested in triplicate and the mean ($\pm$) SD of their O.D. is represented by a point on the graph.


ELISA-IgG-CRP.,OD:490nm
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0.4
0.2
0.0
0
1
2
3
4
TIME (WK)

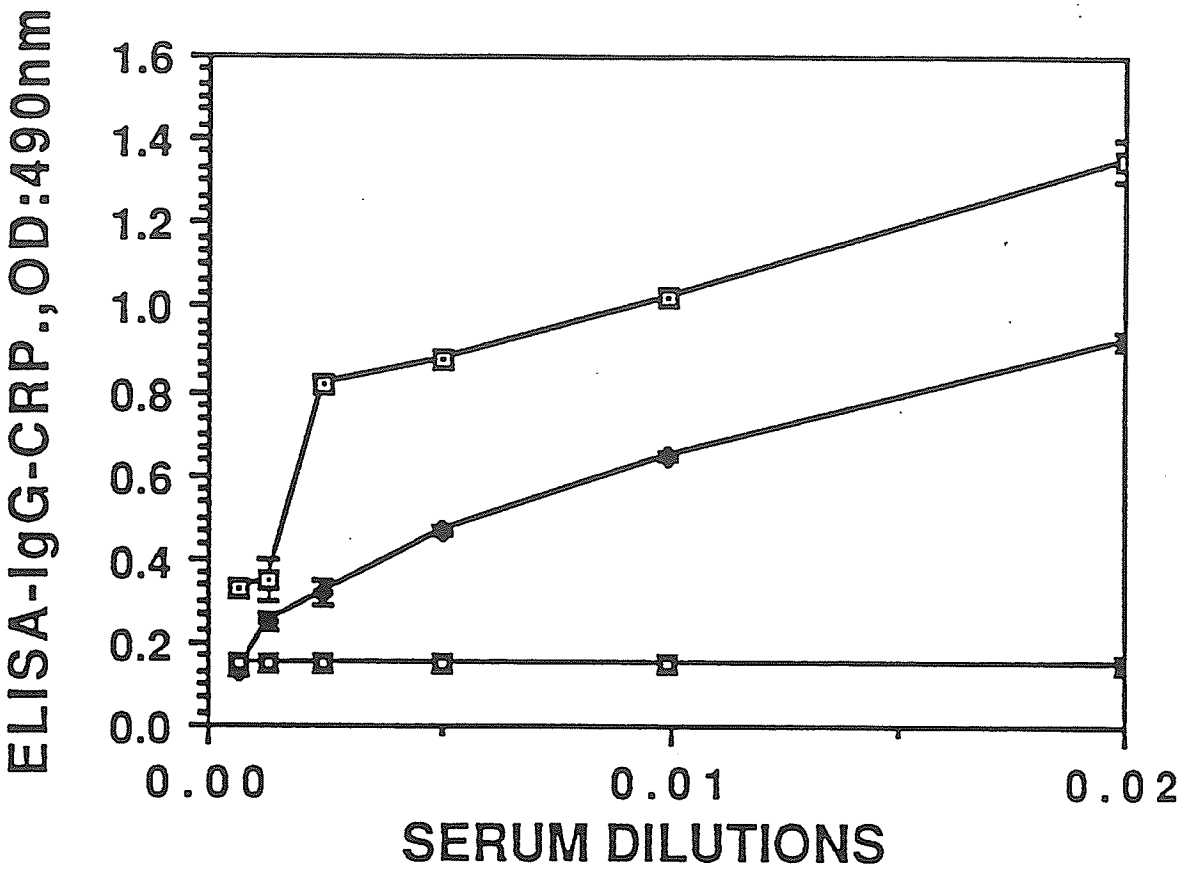


Figure 10. Standard curve of IgG antibodies detected by ELISA in the sera of experimentally infected calves. IgG antibodies to Cryptosporidium were measured by ELISA on serially diluted sera (1:50 - 1:1600) of the two calves with highest IgG O.D. values. Calf A (—◆—) is IgG positive at 1:800 dilution and calf E (—▣—) is positive at 1:1600 dilution. O.D.$\geq$ 0.15 are considered positive. The cut off point (—▣—) obtained, is three times the mean of O.D. of two IgG negative sera from uninfected calves. Each serum dilution is tested in triplicate and the mean ($\pm$ SD) of their O.D. in a typical experiment is presented by a point on the graph. The 0.00 represents an infinite dilution.

Table 1. IgM and IgG antibodies detected by ELISA in samples of colostrum.

Colostrum	IgM (O.D. $\pm$ SD)	IgG (O.D. $\pm$ SD)
1	0.39 $\pm$ 0.03	0.52 $\pm$ 0.01
2	0.37 $\pm$ 0.006	0.47 $\pm$ 0.02
3	0.60 $\pm$ 0.04	0.71 $\pm$ 0.02
4	0.47 $\pm$ 0.006	0.78 $\pm$ 0.06

Four samples of cow's colostrum were diluted 1:100 and tested in triplicate by an ELISA for the detection of IgM and IgG antibodies. Mean optical density (O.D.) $\pm$ standard deviation (SD) are shown for each sample.

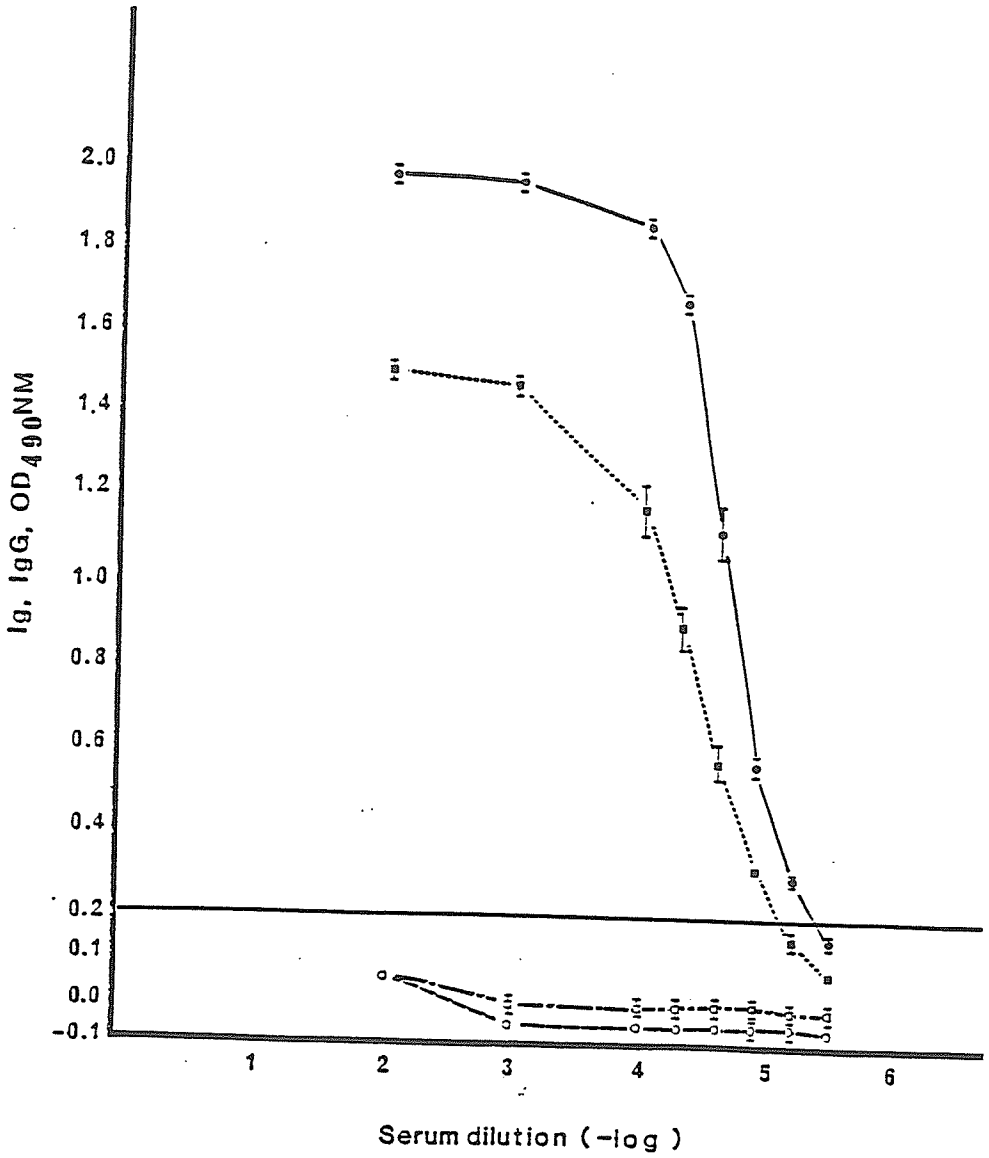


Figure 11. Total Ig and IgG antibodies detected by ELISA in the sera of a rabbit hyperimmunized with freeze - thawed oocysts and in pre- immune rabbits. Each serum was diluted serially from 1:100 to 1:320,000 and tested in triplicate for each serum dilution. The mean O.D. ($\pm$ SD) is represented by a point on the graph. Three times the mean O.D. of negative pre- immune rabbit sera is taken as the cut off point (——). Positive sera are those with O.D. $\geq$ 0.2. The end point titer for IgG antibodies (—●—) was 1:160,000 and for total Ig (—■—) antibodies was 1:80,000. Total Ig (—□—) and IgG (—○—) antibodies in pre- immune rabbit serum are shown.

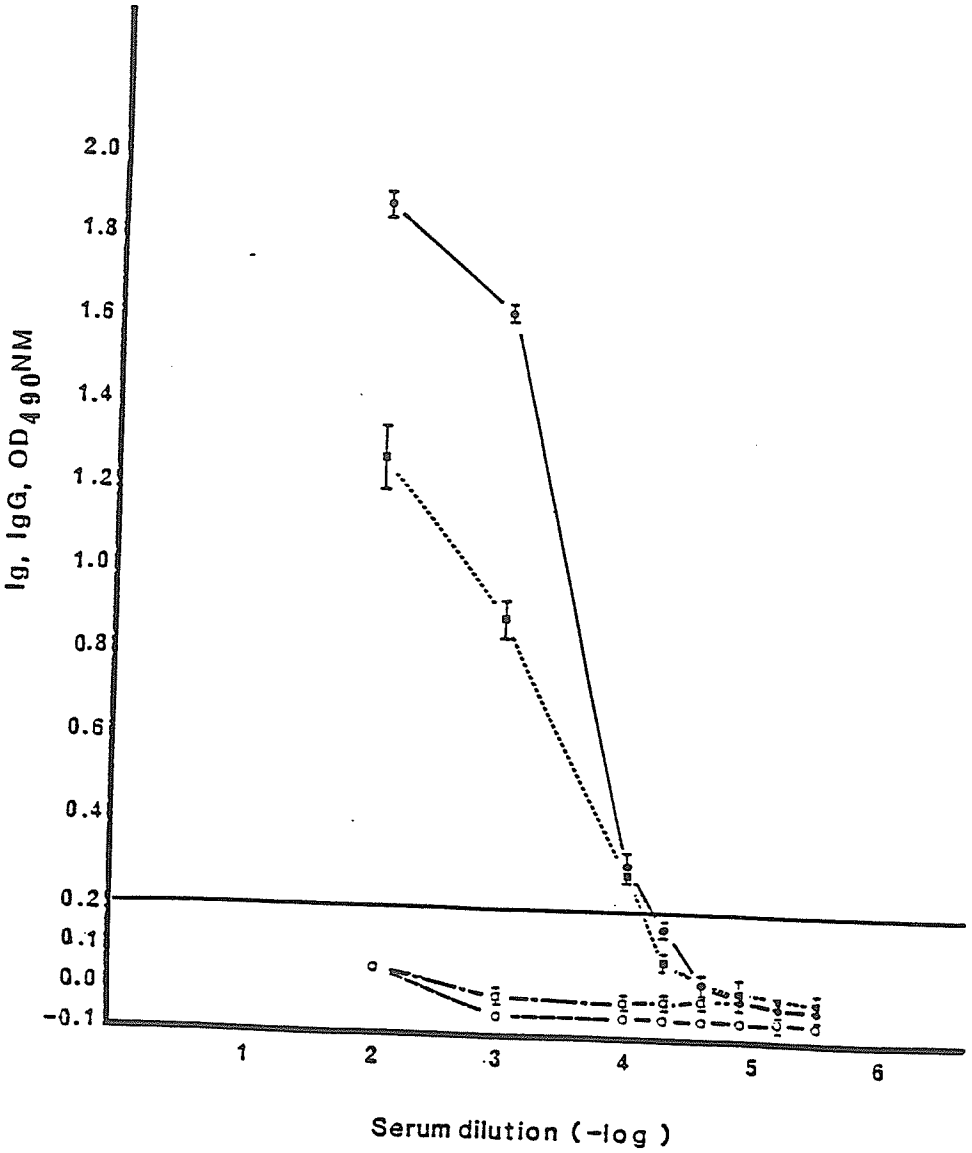


Figure 12. Total Ig and IgG antibodies detected by ELISA in the sera of a rabbit hyperimmunized with intact sporozoites and in pre- immune rabbits. Each serum was diluted serially from 1:100 to 1:320,000 and tested in triplicate for each serum dilution. The mean O.D. ($\pm$ SD) is represented by a point on the graph. Three times the mean O.D. of negative pre- immune rabbit sera is taken as the cut off point (——). Positive sera are those with O.D. $\geq$ 0.2. The end point titer for both total Ig (—●—) and IgG (···■···) antibodies was 1:10,000. Total Ig (-·-○-·-) and IgG (-·-□-·-) antibodies in pre-immune rabbit serum are shown.

IV - SDS- PAGE Pattern of Cryptosporidium Oocysts

The SDS-PAGE protein patterns of Cryptosporidium oocysts purified with CsCl (A) or percoll (B) gradient procedures are compared in Fig. 13. In this 12% silver - stained gel, fewer protein bands are seen in B (the sodium hypochlorite pre-treated oocysts purified with percoll gradient).

A 12% silver - stained gel of 4 calf and 2 human Cryptosporidium oocysts showed 31 bands with MW ranging from 14.4 to 67 Kds (Fig.14). Twenty bands were commom to all calf and human isolates; these were proteins with MW of 58.9, 55, 50.1, 47.3, 45.7, 39.8, 35.9, 33.1, 32.6, 31.3, 28.2, 25.4, 23.2, 21.4, 20.9, 19.5, 17.8, 16.6, 15.5, and 14.8 Kds.

In a 7.5 % gel, only 16 intensely stained bands were observed in all isolates; their MW were 436.5, 330, 266, 199.5, 181.9, 137.1, 128.8, 118.9, 108.4, 94, 89.1, 88.1, 82.2, 80.7, 73.2, and 69.9 Kds (Fig.15)

In the 12 % gel, no differences were observed in the protein patterns of calf oocysts (Fig. 14 A-D) and human oocysts, i.e. the AIDS patient (Fig. 14E) and the immuno-competent child (Fig. 14F).

In the 7.5 % gel, differences were observed between calf and human oocysts (open arrows in Fig.15). Calf isolates had 3 thick protein bands with MW of 146.2, 133.3 and 111.4 Kds while the human isolates showed two faint protein bands with MW of 111.4 and 142.2 Kds and a thick band of 142.9 Kd.

In the 12% SDS-PAGE, the protein patterns of oocysts recovered from one calf infected with calf oocysts (Fig. 16A),

one calf infected with human oocysts (Fig. 16C) , one human isolate (Fig. 16B) and one chicken isolate (Fig. 16D) were compared. The chicken isolate had a pattern different from those of calf and human isolates (Fig.16). The differences are listed in Table 2. Bands of 18.8, 42.6 and 48.4 Kds and a very distinct 58.9 Kd protein were present only in the chicken isolate; bands of 32.6, 33.1, and 45.7 Kds were present only in the calf and human isolates. The chicken isolate had a 16.2 Kd protein instead of the 16.6 Kd seen in calf and human isolates. Finally, the chicken isolate had fainter bands of 15.5 and 39.8 Kds, while a more intensely stained band of 14.8 Kd was seen in human and chicken isolates (Fig.16, hatched arrows).

Fig.17 shows that, in a 7.5% gel, the SDS-PAGE protein pattern of oocysts recovered from the calf experimentally infected with human fecal oocysts (C) is similar to that of the calf experimentally infected with calf oocysts (A) but different from that of the human (B) and chicken (D) isolates. Both above- mentioned calves were not infected with Cryptosporidium prior to oocyst inoculation as shown by a negative Kinyoun acid fast staining of fecal smears. Differences between calf, human and chicken isolates are shown in Fig.17 (hatched arrows) and listed in Table 3. Protein bands of 436.5, 330, 266, 146.2, 137.1, 108.4, 88.1, 82.2, and 69.9 Kds which were present in both calf and human isolates were missing in the chicken isolate. The chicken isolate had more pronounced and intensely stained protein bands of 298, 211.3,

173.8, 105.9, 100 and 83.1 Kds; these bands were either absent or very faint in calf and human isolates. However, a band of 199.5 Kd protein was very intense in calf and human isolates and faint in the chicken isolate. Two protein bands present in the chicken isolate were present only in the human (142.9 Kd) and only in the calf (111.4 Kd) isolates. In contrast, two bands were absent in the chicken isolate; one was present only in the calf (141.2 Kd) and the other only in the human (133.3 Kd) isolates.

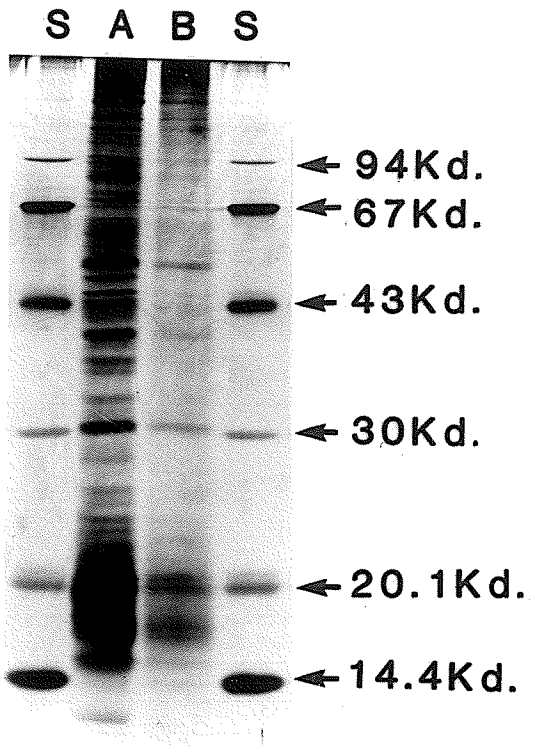


Figure 13. SDS - PAGE protein patterns of CsCl and Percoll gradient purified Cryptosporidium oocysts in 12% gel. Oocysts were purified by two methods; CsCl purified oocysts are shown in lane A and sodium hypochlorite pretreated and Percoll gradient purified oocysts are shown in lane B. Most of the protein bands present in lane A are absent in lane B, as a result of treatment with sodium hypochlorite. Molecular weight standard (S); kilodalton (Kd.).

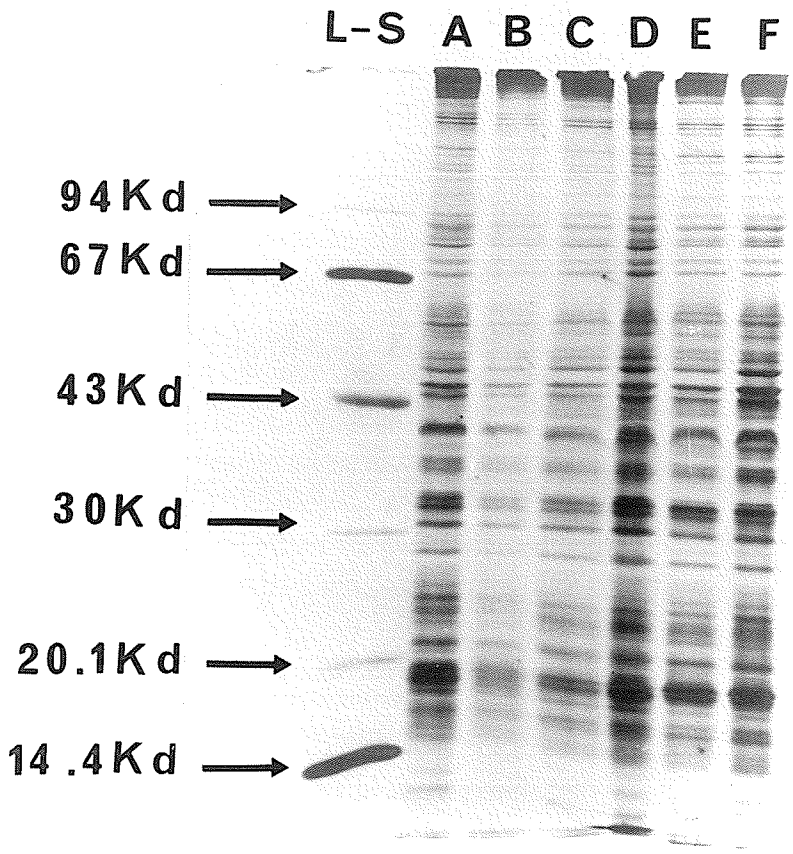


Figure 14. SDS - PAGE protein patterns of calf and human Cryptosporidium isolates in 12% silver - stained gel. Proteins prepared from CsCl purified oocysts ($1x10^6$) were placed in each well. Protein patterns of the 4 calf oocysts are shown in lanes A - D ; those of the AIDS patient oocysts in lane E and those of the immunocompetent child oocysts in lane F. Low molecular weight standards (L-S) of 14.4 - 94 Kd are used as markers.

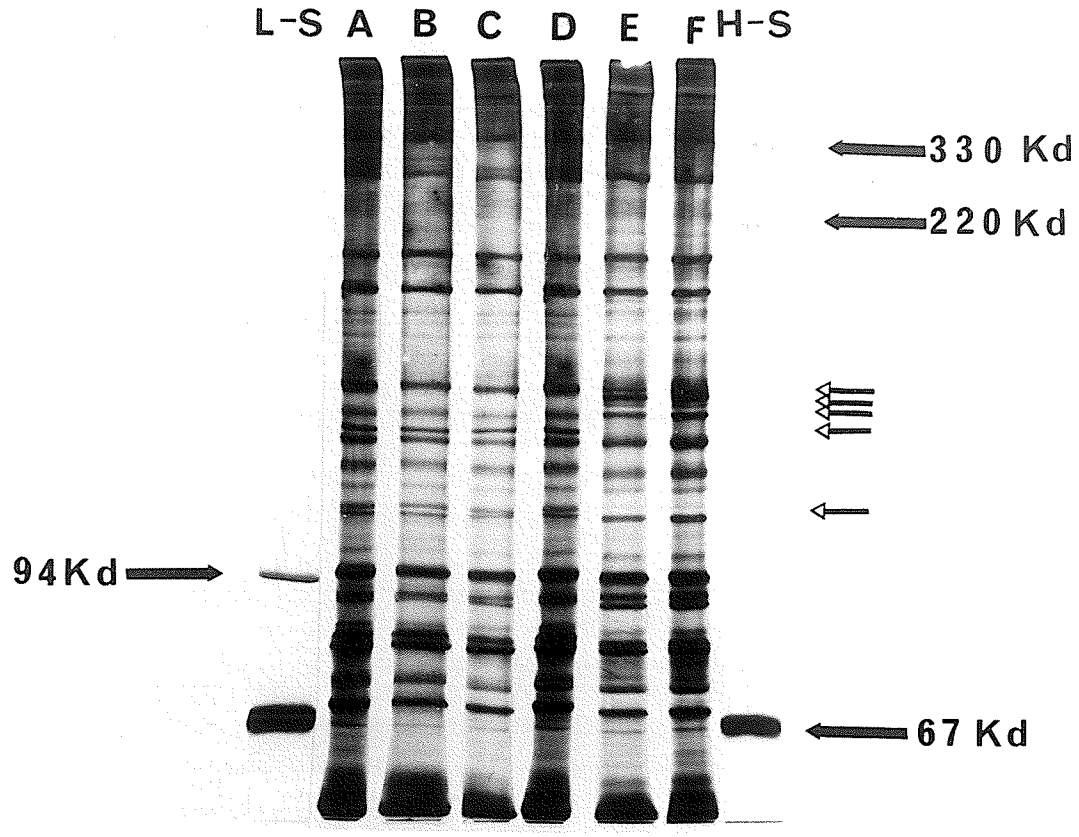


Figure 15. SDS - PAGE protein patterns of calf and human Cryptosporidium isolates in 7.5% silver - stained gel. Proteins from CsCl purified oocysts ($1x10^6$) were placed in each well. Protein patterns of the 4 calf isolates are shown in lanes A - D; those of the AIDS patient oocysts in lane E and those of the immunocompetent child oocysts in lane F. Low (L-S) and high (H-S) molecular weight standards of 67 - 330 Kd are used as markers.

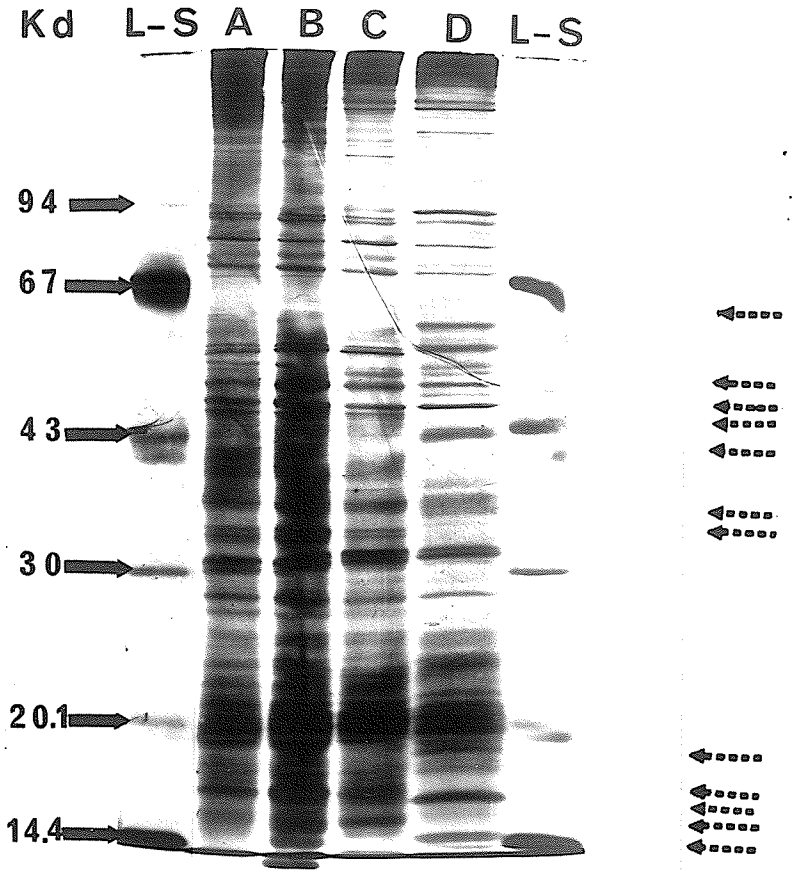


Figure 16. SDS - PAGE protein patterns of calf and human Cryptosporidium isolates in 12% silver - stained gel. Protein preparation from CsCl purified oocysts were placed in each well. Protein banding pattern of oocysts of a calf infected with calf isolate is shown in lane A ; those of a human isolate in lane B; those of a calf infected with human oocysts in lane C; and those of a chicken isolate in lane D. Low molecular weight standards (L-S) of 14.4 - 94 Kd are used as markers.

Table 2. Differences of LMW protein bands in calf, human, and chicken Cryptosporidium oocysts

MW(Kda)	calf(A)	human(B)	calf(C)	chicken(D)
58.9	+	+	+	+*
48.4	-	-	-	+
45.7	+	+	+	-
42.5	-	-	-	+
39.8	+	+	+	+**
33.1	+	+	+	-
32.6	+	+	+	-
18.8	-	-	-	+
16.6	+	+	+	-
16.2	-	-	-	+
15.5	+	+	+	+**
14.8	+	+*	+	+*

Protein bands present in oocysts of a calf infected with a calf isolate are shown in column A; those of a human isolate in column B; those of a calf infected with a human isolate in column C; and those of a chicken isolate in column D. Differences of low molecular weight (LMW) protein bands in calf, human and chicken isolates are listed. Bands detected are marked as (+); bands not detected as (-); bands detected with intensity as +*; those faintly detected as +**

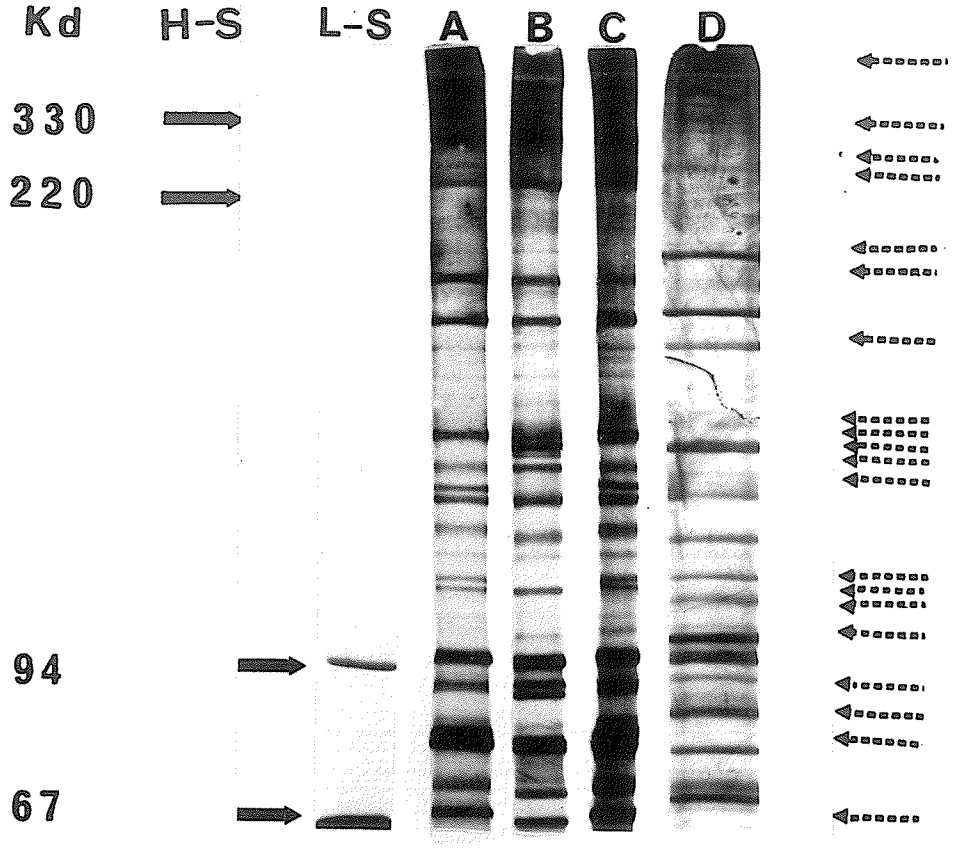


Figure 17. SDS-PAGE protein patterns of calf,human and chicken Cryptosporidium isolates in 7.5% silver - stained gel. Protein preparation from CsCl purified oocysts were placed in each well. Protein banding pattern of oocysts of a calf infected with calf isolate is shown in lane A; those of a human isolate in lane B; those of a calf infected with human isolate in lane C; and those of a chicken isolate in lane D. Low (L - S) and high (H - S) molecular weight standards of 67 - 330 Kd are used as markers.

Table 3. Differences of HMW protein bands in calf, human, and chicken Cryptosporidium isolates

MW (Kda)	calf (A)	Human (B)	calf (C)	chicken (D)
436.5	+	+	+	-
330	+	+	+	-
298	+	+	+	+*
266	+	+	+	-
211.3	+	+	+	+*
199.5	+	+	+	+**
173.8	+	+	+	+*
146.2	+	+	+	-
142.9	-	+	-	+
141.2	-	+	-	-
137.1	+	+	+	-
133.3	+	-	+	-
111.4	+	-	+	+
108.4	+	+	+	-
105.9	-	-	-	+
100	+	+	+	+*
88.1	+	+	+	-
83.1	+	+	+	+*
82.2	+	+	+	-
69.9	+	+	+	-

Protein bands present in oocysts of a calf infected with a calf isolate are shown in column A; those of a human isolate in column B; those of a calf infected with a human isolate in column C; and those of a chicken isolate in column D. Bands detected (+); bands not detected (-); intense band (*); faint band (**).

V- Western Blot Patterns of Cryptosporidium Oocysts

Western blot (WB) was performed on the protein bands of Cryptosporidium oocysts separated by SDS-PAGE by transferring them from gel to nitrocellulose and exposing them to one of the following 3 immune sera: 1) rabbit antisera raised against oocysts (Fig. 18 & 21), 2) rabbit antisera against sporozoites (Fig. 19 & 22), 3) an ELISA positive calf serum (Fig. 20 & 23). The oocysts from two calves infected with calf isolates are shown in lanes A & B, the two humans are shown in lanes C & D, the calf infected with a human isolate is shown in lane E and a chicken isolate is shown in lane F.

A - A 12% gel was used for SDS - PAGE and WB patterns in Fig. 18, 19, and 20.

Fig. 18 shows that the serum of the rabbit immunized with oocysts identified 24 reactive bands in the calf isolates (lanes A,B & E), and 23 bands in the human isolates (lanes C & D), all bands ranging between 11 - 130 Kds. Twenty of these protein bands had MW of 17 to 130 Kds and four had MW of less than 17 Kd. A band having a MW of 11.6 Kd was missing from both human isolates (hatched arrow, Fig.18).

Fig.19 shows that the rabbit IgG antibody against sporozoites identified 18 bands having MW between 17 and 130 Kds in the human isolates (lanes C & D), and only 11 bands in the calf isolates (lanes A, B, & E). The most distinct differences between human and calf oocysts were a band of 113 Kd (arrow 1 in Fig.19) seen only in human isolates and a band of approximately 73 Kd (arrow 2 in Fig. 19) seen only

in the three calf isolates.

Fig. 20 shows that the ELISA IgG antibody positive calf serum recognized 11 bands ranging from 17 - 130 Kds in both human (lanes C & D), and calf (lanes A, B, & E) isolates. One band of approximately 25 Kd was more intense than the others in all isolates (arrow 1 in Fig.20). A protein band of 11.6 Kd was seen in the calf isolates but not in the human ones (arrow 2 in Fig.20).

B - A 7.5 % gel was used for SDS-PAGE and WB shown in Fig.21, 22, and 23.

The rabbit antibody raised against oocysts identified 17 and 18 reactive bands ranging from 50 to 436 Kds in the human (C & D) and calf (A, B, & E) isolates, respectively, and 15 bands in the chicken (F) isolate ranging from 50 to 200 Kds (Fig.21). A highly reactive band of 436 Kd was seen in the human isolates but was less reactive in the calf isolates and was not seen at all in the chicken isolate (arrow 1 in Fig.21). Bands of 70 and 190 Kds were seen in the human isolates but not in the calf and chicken isolates (arrows 3 and 13 in Fig.21). A double band of approximately 200 Kd and bands of 88 and 60 Kds were sharply reactive in the calf isolates, but they were faint in the human isolates and very faint (88 Kd) or absent (200 & 60 Kds) in the chicken isolate (arrows 2, 9, 14 in Fig.21). Bands of 170, 128, 119, and 83 Kds were seen in the chicken isolate but were fainter in the calf and human isolates (arrows 5, 6, 7, 10 in Fig.21). Reactive bands of 181, 108, and 82 Kds were

present in the calf and human isolates but were fainter or absent in the chicken isolate (arrow 4, 7, 11 in Fig. 21). A faint band of 73 Kd seen in the chicken isolate was more reactive in the calf isolates but absent in the human ones (arrow 12 in Fig.21).

Fig.22 shows the bands identified by rabbit antibodies raised against sporozoites in the three calf isolates (A, B, E), and two human isolates (C, D). There were 15 and 13 reactive bands in the range of 60 - 436 Kds, in the calf and human isolates, respectively. A reactive band of 436 Kd was intense in the human isolates, but less reactive in the calf isolates (arrow 1). A double reactive band of 200 Kd and bands of 181, 170, 88 and 73 Kds were seen in the calf isolates, but were not seen or were less reactive in the human isolates (arrows 2, 4, 5, 7, 8 in Fig. 22). Bands of 190 and 70 Kds were seen in the human isolates but not in the calf isolates (arrows 3, 9). A very reactive double band of approximately 150 Kd was seen in the calf isolates but appeared as a single thick band in the human isolates (arrow 6).

Fig.23 shows the reactive bands of the same three calf isolates (A,B,E), and two human isolates (C,D), when incubated with the serum of an experimentally infected calf containing IgG antibody to Cryptosporidium antigens. There were 20 and 19 reactive bands in the range of 60 - 436 Kds in the calf and human isolates, respectively. A highly reactive band of 436 Kd MW in human was fainter in the calf isolates (arrow

1 in Fig. 23). Bands of 266, 190, 77, and 70 Kds were seen in the human isolates but not in the calf isolates (arrows 2, 5, 7, 10). Bands of 240, 200, 181, 75 and 73 Kds were seen in the calf isolates but not in the human isolates (arrows 3, 4, 6, 8, 9).

No bands of high or low MW were observed when Cryptosporidium oocysts extracts were reacted with ELISA negative rabbit or calf sera.

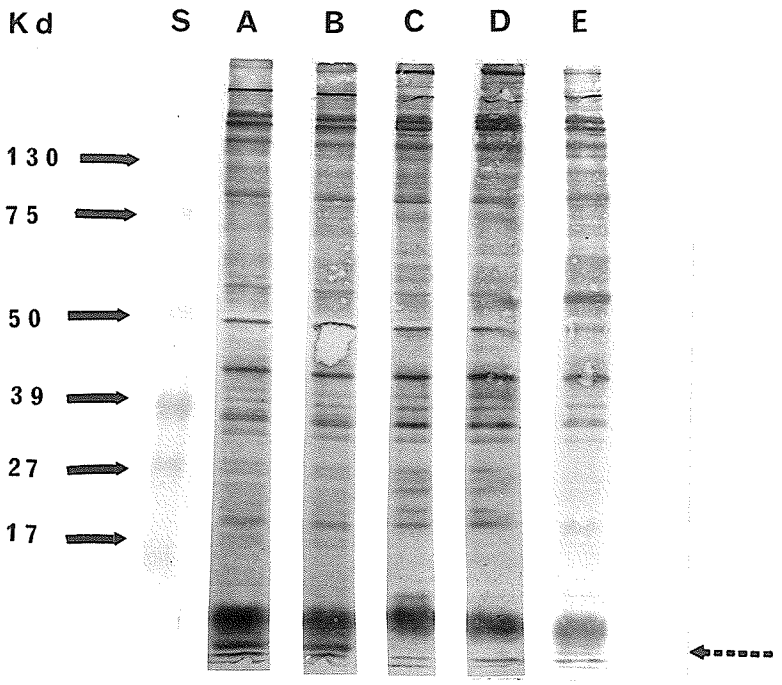


Figure 18. Western blot patterns of oocysts isolated from calves and humans; separated by 12% gel and exposed to the serum of a rabbit hyperimmunized with calf oocysts. Reactive bands of oocysts of two calves infected with calf isolates are shown in lanes A and B; those of an AIDS patient in lane C; those of an immunocompetent child in lane D; and those of a calf infected with human isolate in lane E. Rabbit antiserum used at a dilution of 1:20,000. Solid arrows (➡) show prestained molecular weight protein standards. Hatched arrow (⇦) shows a reactive protein band found only in calf isolate.

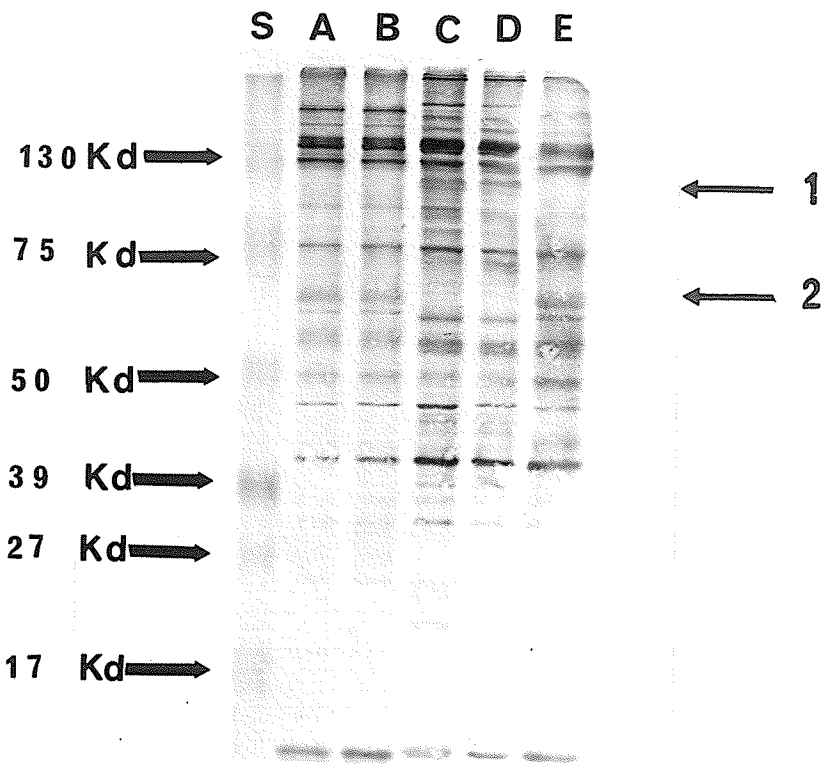


Figure 19. Western blot patterns of oocysts isolated from calves and humans; separated by 12% gel and exposed to the serum of a rabbit hyperimmunized with calf sporozoites. Reactive bands of oocysts of two calves infected with calf isolates are shown in lanes A and B; those of an AIDS patient in lane C; those of an immunocompetent child in lane D; and those of a calf infected with human isolate in lane E. Rabbit antiserum used at a dilution of 1:1000. Solid arrows (➡) show prestained molecular weight protein standards. Arrow 1 shows a reactive protein band present only in human isolates. Arrow 2 shows a reactive protein band found only in calf isolates.

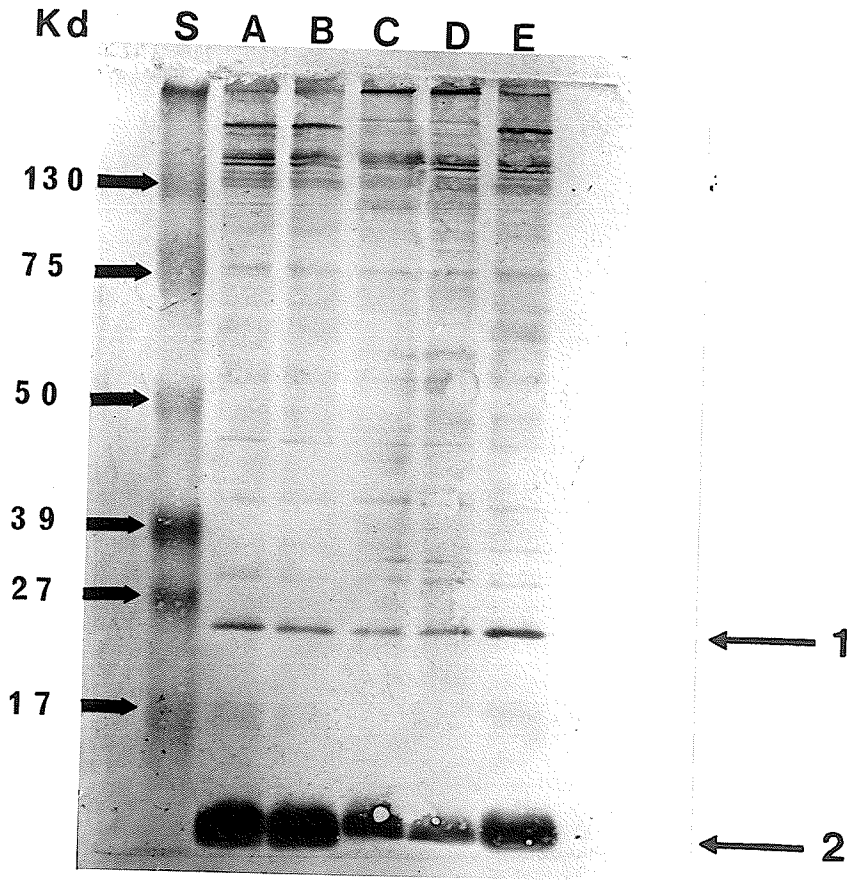


Figure 20. Western blot patterns of oocysts isolated from calves and humans; separated by 12% gel and exposed to the serum of an experimentally infected calf. Reactive bands of oocysts of two calves infected with calf isolates are shown in lanes A and B; those of an AIDS patient in lane C; those of an immunocompetent child in lane D; and those of a calf infected with human isolate in lane E. Calf antiserum used at a dilution of 1:25. Solid arrows (⟹) show prestained molecular weight protein standards. Arrow 1 shows an intensely reacting band of 25 Kd present in all isolates. Arrow 2 shows a reactive band of 11.6 Kd present only in calf isolates.

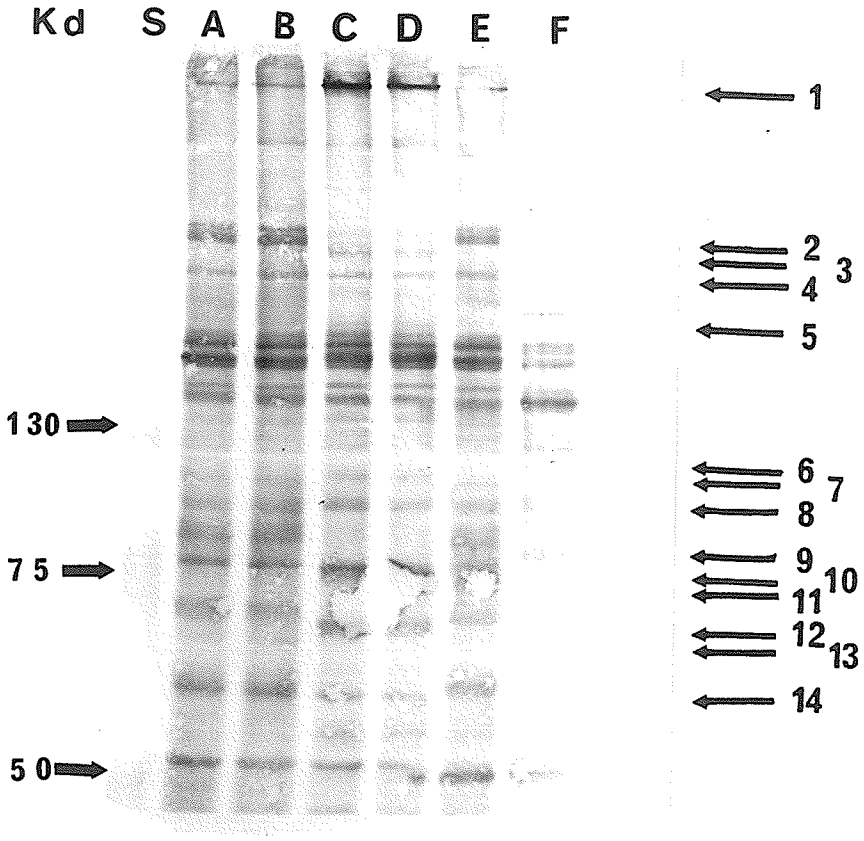


Figure 21. Western blot patterns of oocysts isolated from calves, humans and chicken; separated by 7.5% gel and exposed to the serum of a rabbit hyperimmunized with calf oocysts. Reactive bands of oocysts of two calves infected with calf isolates are shown in lanes A and B; those of an AIDS patient in lane C; those of an immunocompetent child in lane D; those of a calf infected with human isolate in lane E; and those of a chicken in lane F. Rabbit antiserum used at a dilution of 1:20,000. Solid arrows (➡) show prestained molecular weight protein standards. Arrows on the right indicate bands found in some lanes only.

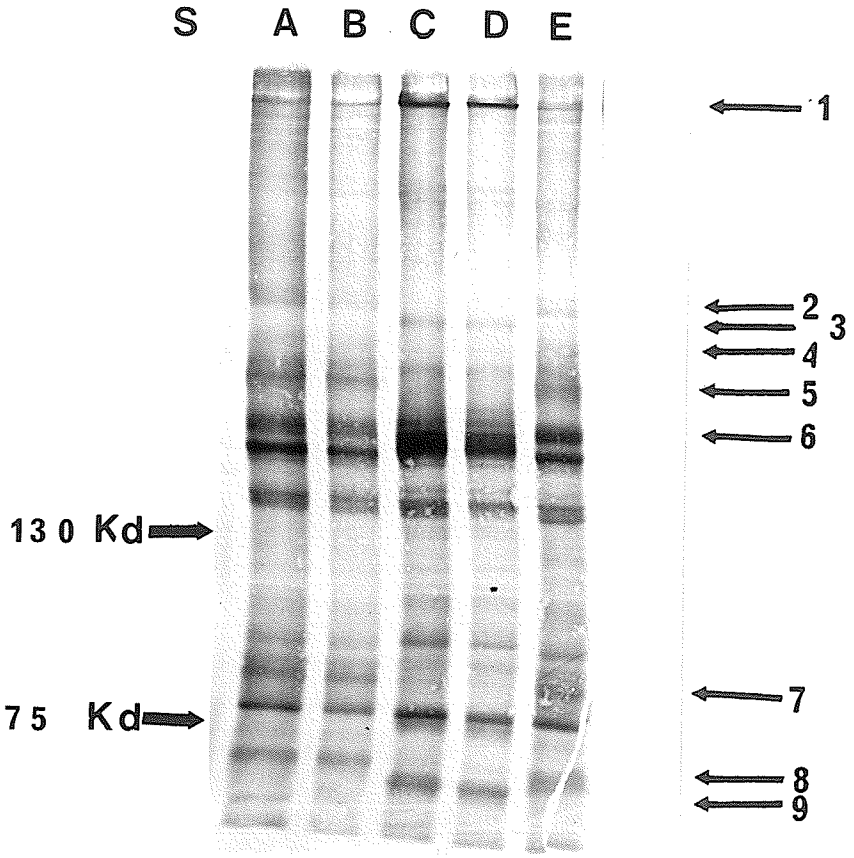


Figure 22. Western blot patterns of oocysts isolated from calves and humans; separated by 7.5% gel and exposed to the serum of a rabbit hyperimmunized with sporozoites. Reactive bands of oocysts of two calves infected with calf isolates are shown in lanes A and B; those of an AIDS patient in lane C; those of an immunocompetent child in lane D; and those of a calf infected with human isolate in lane E. Rabbit antiserum used at a dilution of 1:1000. Solid arrows (➡) show prestained molecular weight standards. Arrows on the right indicate bands found in some lanes only.

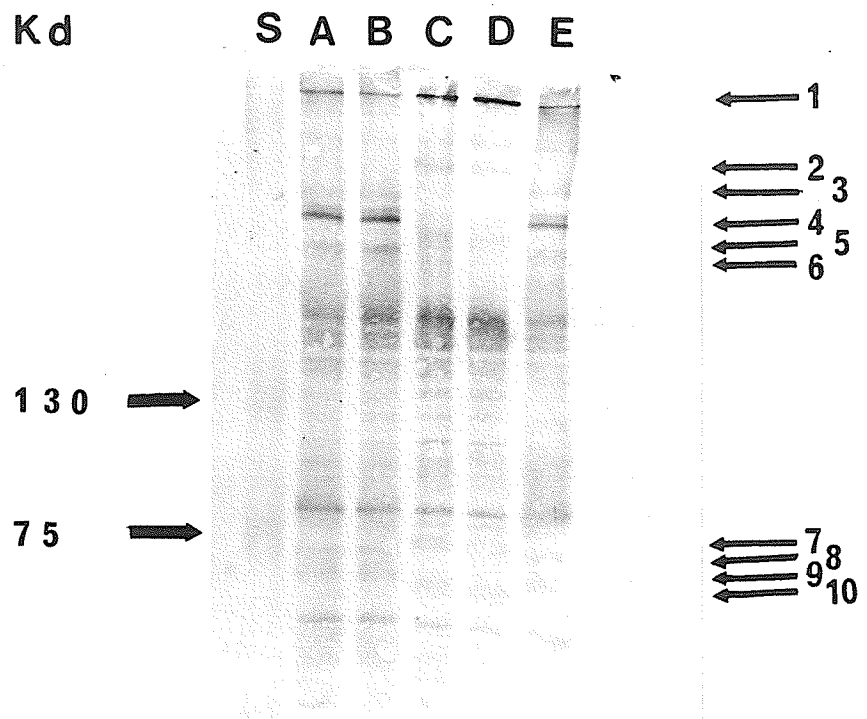


Figure 23. Western blot pattern of oocysts isolated from calves and humans; separated by 7.5% gel and exposed to the serum of an experimentally infected calf. Reactive bands of oocysts of two calves infected with calf isolates are shown in lanes A and B; those of an AIDS patient in lane C; those of an immunocompetent child in lane D; and those of a calf infected with human isolate in lane E. Calf antiserum used at a dilution of 1:25. Solid arrows (➡) show prestained molecular weight standards. Arrows on the right indicate bands found in some lanes only.

PART B

I. Role of Humoral Immunity in *Cryptosporidium* Infection : Studies in B Cell - Deficient Mice

1) Effect of Chronic Anti-u Treatment on Murine B Cell Populations

To evaluate the effects of anti-u treatment on B cell development in neonates, flow cytometry was used to compare the presence of mature, Ig-bearing (sIg^+) B cells in spleen and lymph nodes of individual anti-u treated mice, age-matched untreated (normal) mice, and mice treated with NRIg. The percentage of sIg^+ expressing cells was quantitated by direct staining of spleen and lymph node cells using a FITC - conjugated polyclonal rabbit anti - mouse Ig. The results of a typical experiment are presented in Fig.24. Treatment of mice with anti-u antibodies from the time of birth, resulted in elimination of practically all sIg^+ cells in the spleen or lymph nodes.

As a complementary approach to assessing mature B cell function in normal, NRIg treated and anti-u treated mice, spleen or lymph node cells were examined for their relative capacity to respond to B cell mitogens. Spleen or lymph node (result similar to that of spleen)cells from anti-u treated mice failed to respond to stimulation with the B cell mitogen lipopolysaccharide (LPS), over a wide range of concentrations. Cells obtained from control, age matched mice proliferated vigorously (Fig.25A).

In contrast to B cell responsiveness, responses to the T cell mitogen (Con A) were not significantly impaired. T cells from anti-u treated mice were capable of generating *in vitro* proliferative responses virtually identical to those of normal and NRIg-treated mice (Fig.25B). Experiments were repeated 4 times with 4-9 mice/group, each individual being separately assessed. These anti-u treated mice serve as B cell deficient models for evaluation of the role of humoral immunity in *Cryptosporidium* infection.

2) Infection of Normal and B Cell Deficient Neonatal BALB/c Mice with *Cryptosporidium*: Disease Course

Neonatal (7 day old) BALB/c mice were fed 1.5 $x10^5$ oocysts, leading to severe intestinal cryptosporidial infection as demonstrated by the appearance of large numbers of excreted oocysts in semi-solid or watery stools. Excreted oocysts were found in stools of normal mice 4-5 days after feeding, with the peak of infection 24 - 48 hours later (Fig. 26). In all experiments, *Cryptosporidium* infection of normal neonatal BALB/c mice was self - resolving, with the number of oocysts shed reduced to zero by 11-13 days post infection.

Similarly, anti-u treated and age - matched NRIg-treated neonates developed diarrhea, associated with the shedding of large numbers of *Cryptosporidium* oocysts, 4-5 days after feeding.

In four of four independent experiments, both the intensity and the duration (time of onset, peak, and resolu-

tion) of fecal oocysts excretion were not significantly different when normal (Fig. 26A), NRIg-treated (Fig.26B), and anti-u treated (Fig.26C) mice were compared. Oocysts were seen in stools of NRIg-treated mice as early as day 4, increased in number up to day 7, and then declined to undetectable levels by day 10 post infection. Similarly, Cryptosporidium infected, anti-u treated mice started shedding oocysts by day 5 , the number peaked at levels similar to control groups at day 8 and decreased gradually to zero by day 12 post infection. In all cases, stool collection was continued for 21 days without any reappearance of Cryptosporidium oocysts. No mortality was observed in any of the Cryptosporidium infected mice.

3) Cryptosporidium Specific Antibody Responses

The anti-Cryptosporidium humoral response in individual mice was examined in an ELISA . Following infection, the cumulative anti - Cryptosporidium Ig response (i.e. all Ig isotypes) yielded ELISA titers ranging from 30 to 157 (geometric mean: 61) in 24 normal mice; titers ranging from 41 to 170 (geometric mean: 80) in 12 NRIg - treated mice. The results obtained were below the limits of sensitivity of the assay, in anti-u treated mice prior to, during, or following infection.

The Cryptosporidium - specific antibody responses by isotype in individual mice is presented in table 4. The amount of Cryptosporidium specific antibody was measured in normal,

NRIg-treated and anti-u treated BALB/c mice at various times (14 - 31 days) after infection.

Most infected normal and NRIg- treated mice showed high level of IgM responses. IgM antibodies were not detected in anti-u treated mice. IgG responses were markedly lower than IgM in both normal and NRIg- treated mice. These IgG responses were predominantly made up of IgG1 with much lower IgG2b responses and undetectable IgG2a, and IgG3 Cryptosporidium - specific antibodies (Fig. 27). Anti- u treated groups exhibited very weak or undetectable IgG responses. Specific IgA and IgE levels were very low or not detectable in all groups. Most importantly, no evidence was found for an association between the intensity of Cryptosporidium- specific antibody responses and the intensity of the fecal excretion of oocysts (table 4).

4) Age Dependence of Susceptibility to Cryptosporidium Infection

To investigate the effect of age on susceptibility to Cryptosporidium infection, groups of 7 - 49 day old BALB / c (inbred) mice were fed oocysts varying in numbers from 1.5×10^5 to 1.5×10^6. Table 5 shows the intensity and duration of infection in different age groups; the number of oocysts given to the mice in each group inceased in proportion to age. Typically, groups of normal, NRIg - treated, and anti - u treated neonates (7 - 8 days old) started shedding oocysts in their feces by 4 - 5 days p.i. Oocysts shedding was continued

for a total of 8 - 11 days and was not different among groups of anti - u treated mice and groups of control mice. The intensity of infection was also not significantly different in these three groups ($p > 0.05$). Groups of 11 - 12 day old mice shed oocysts in their stools from day 3 post infection and continued for 6 - 11 days. No oocysts could be detected in stools of groups of 14 - 15, and 21 - 22 day old mice, fed with oocysts. Adult (42- 49) day old BALB / c mice were fed with different amounts of oocysts ranging from 2×10^4 to 1.5×10^6 oocysts / mouse. Even when a very large number of oocysts (i.e. 1.5×10^6 oocysts / mouse) was given to the groups of adults, no oocysts could be seen in their stools. Adult mice stools were checked individually and daily for 21 days, using Kinyoun acid fast staining and a sensitive concentration procedure (Sheather's flotation method) (256).

Attempts to infect adult Swiss (outbred) mice with a maximum of 1.5×10^6 oocysts/mouse were similarly unsuccessful.

5) The Lack of Susceptibility of Adult BALB/c Mice is not Attributable to Humoral Immunity

In order to evaluate the role of the antibody response in the resistance of normal adults to cryptosporidial infection, the susceptibilities of normal and anti-u treated adult BALB/c mice were compared. Adult anti-u treated BALB/c mice failed to show any oocysts in their stools after they were fed with 1.5×10^6 oocysts/mouse (table 5). The infectivity of the oocysts used in these experiments was confirmed by feeding

parallel groups of normal neonates with one order of magnitude fewer oocysts ($1.5x10^5$), a dose which proved infectious for 100% of the young mice.

Examination of anti-u treated adult mice confirmed their deficiency in sIg^+ lymphocytes and the virtual absence of antibody response.

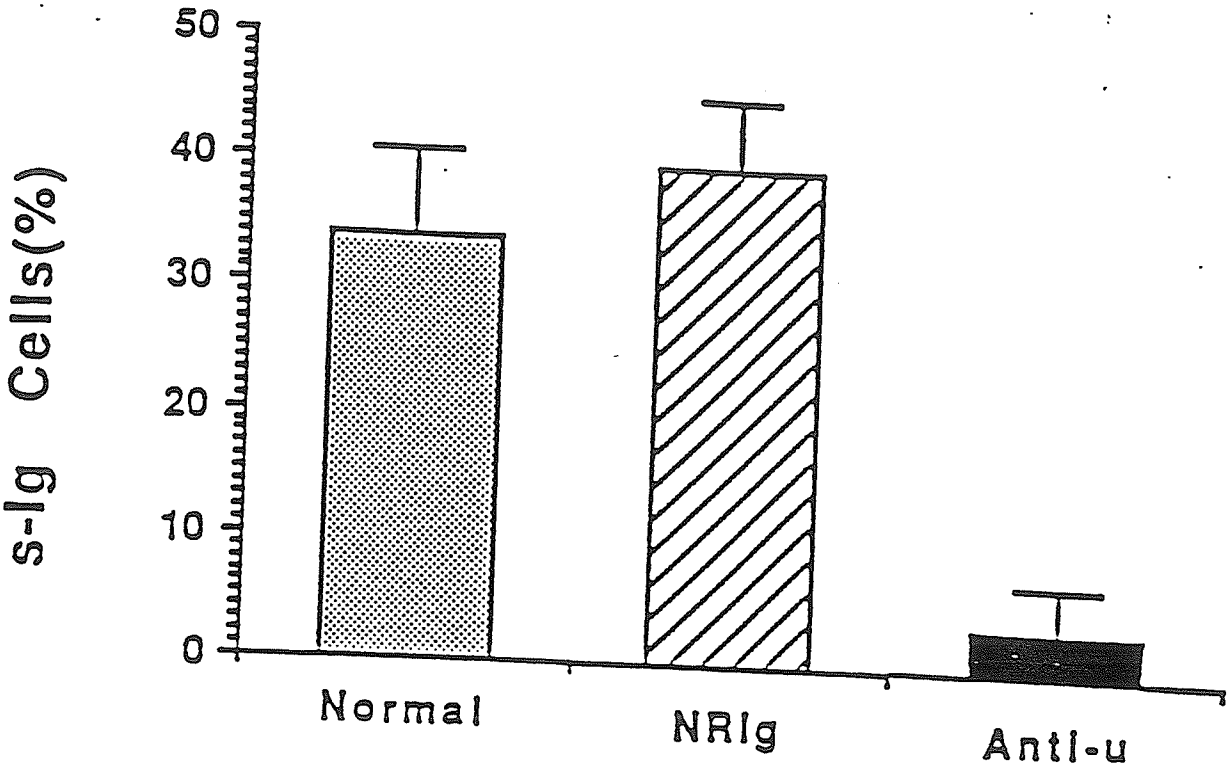


Figure 24. Surface Ig expressing spleen cells of normal, NRIg- treated and anti- u treated newborn BALB/c mice. Spleen cells were stained with FITC conjugate and fluorescence determined using an EPICS V fluorescence activated cell sorter. The mean percentage ($\pm$ SE) of sIg^+ cells in one of 4 experiments is shown for each of the three groups of mice. Anti - u treatment starting at birth eliminates almost all sIg^+ cells.

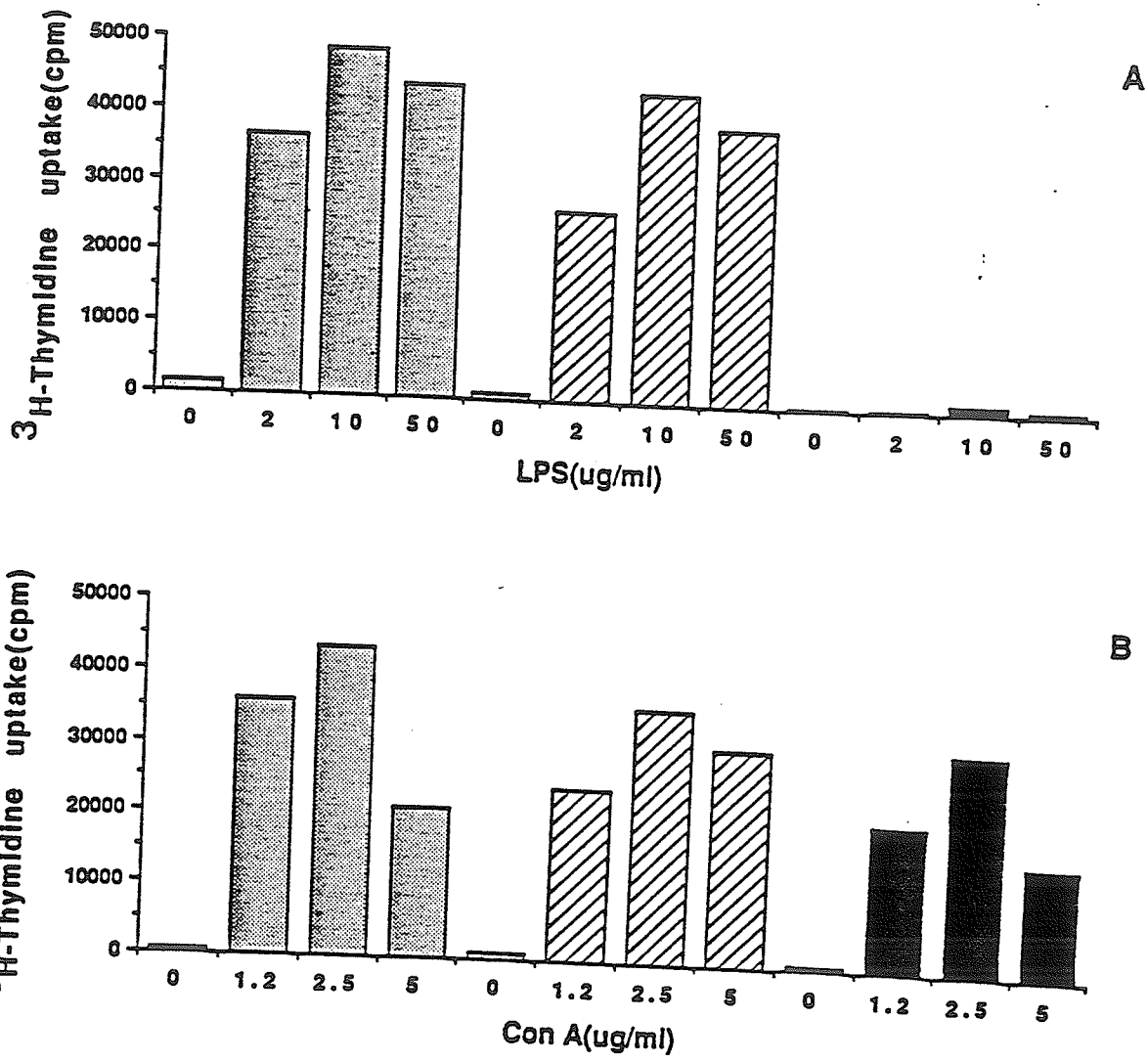


Figure 25. B and T cell responses to mitogens in normal (▩), NRIg - treated (▨), and anti- u treated (■) neonatal BALB/c mice. Proliferation assays were carried out on spleen cell populations. $_3$Spleen cells were cultured, exposed to mitogens, pulsed with [^{3}H] -thymidine and thymidine uptake determined by liquid scintillation counting. The mitogen used to study the response of B cells was lipopolysaccharide (LPS) and T cell mitogen was concanavalin A (Con A). Anti- u treated neonate mice failed to respond to LPS shown in (A). No differences were detected in the three groups of mice exposed to Con A shown in (B).

Figure 26. Cryptosporidium oocysts shedding in infected neonatal normal, NRIg - treated and anti- u treated BALB/c mice. The daily mean of the number of fecal oocysts ($\pm$ SE) seen in 10 microscopic fields is shown, comparing the infected normal (A), NRIg- treated (B), and anti- u treated mice(C). Results of a typical experiment are presented. A minimum of 60 fields / data point was counted. No significant differences were noticed in time of onset, peak and resolution of oocyst shedding.

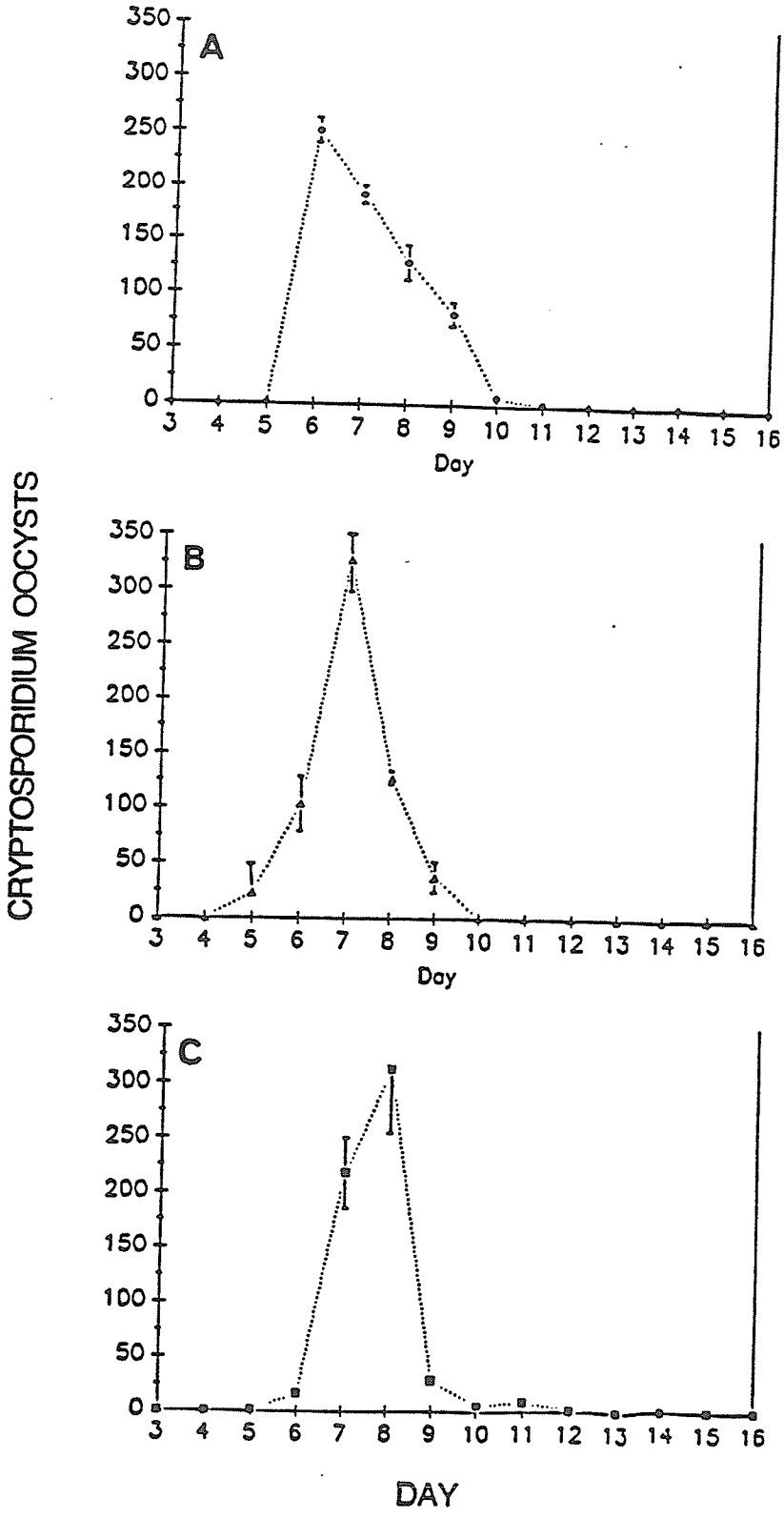

A
B
C
CRYPTOSPORIDIUM OOCYSTS
350
300
250
200
150
100
50
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16
Day
DAY

Table 4. Relationship between fecal oocyst excretion and antibody responses in infected BALB/c mice.

Experiment	Treatment @	Oocysts excretion intensity *	Anti-*Cryptosporidium* response #			
			IgM	IgG	IgA	IgE
1	none	176	269	28	11	<10
2		588	114	19	7	<10
3		519	103	10	5	<10
1	anti- u	711	<5	<5	<5	<10
2		563	<5	8	<5	<10
3		475	<5	ND	<5	<10
1	NRIg	640	167	19	7	<10
2		815	89	10	<5	<10
3		1094	239	16	7	<10

In infected normal, anti- u treated, and NRIg treated BALB/c mice the intensity of fecal oocyst excretion is not related to the magnitude of the antibody response. (@), treatment started within 24 hr of birth and continued 3 times weekly thereafter until the end of the experiment. (*), Average number of oocysts observed / 10 fields / day x number of days *Cryptosporidium* shed. SE was usually <20% and is omitted for clarity. (#), anti- *Cryptosporidium* response measured in groups of infected mice (4-9 / group), using an ELISA for IgM, IgG, IgA and PCA for IgE antibodies. Experiments 1 were done on groups of 17 day old mice, experiments 2 on groups of 19 day old mice and experiments 3 on groups of 31 day old mice. IgM antibodies were not detected in anti- u treated mice. IgG responses were lower than IgM responses in normal and NRIg-treated mice.

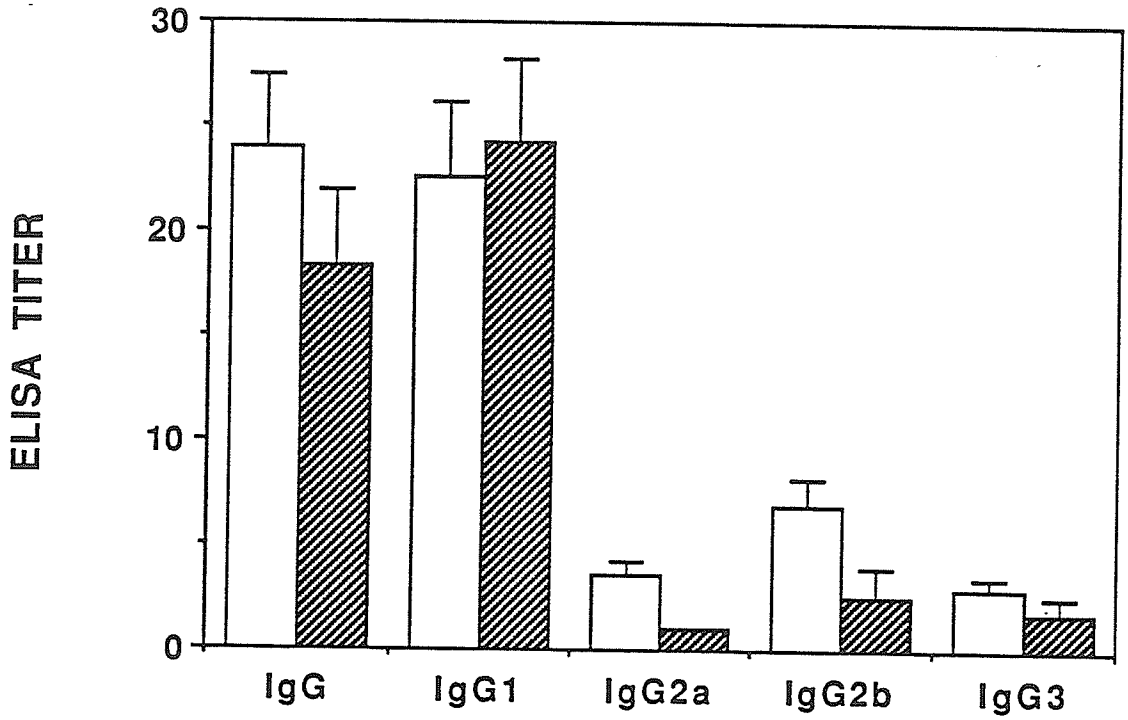


Figure 27. IgG antibody response of infected normal and NRIg-treated BALB/c mice. An ELISA using specific alkaline phosphatase conjugated to anti-IgG1, anti-IgG2a, anti- IgG2b and anti-IgG3 showed that the IgG response of 24 normal (□) and 12 NRIg- treated (▨) mice to Cryptosporidium infection were mainly due to the production of IgG1 antibodies. The mean titer ($\pm$ SE) for each isotype in each group of mice is presented.

Table 5. Effect of age on susceptibility of BALB/c mice to Cryptosporidium infection.

Treatment [1]	Age (days) [2]	Cryptosporidium Oocysts [3]	Intensity [4]	Duration (days) [5]
none	7-8	$1.5x10^5$	389 ± 97	8-11
anti-u	7-8	$1.5x10^5$	624 ± 64	8-11
NRIg	7-8	$1.5x10^5$	727 ± 88	8-11
none	11-12	$3.0x10^5$	206 ± 121	6-11
	14-15	$6.0x10^5$	0	0
	21-22	$1.0x10^6$	0	0
	42-49	$1.5x10^6$	0	0
anti-u	42-49	$1.5x10^6$	0	0

Age dependent loss of susceptibility to Cryptosporidium is not affected by the absense of humoral immunity. (1), Treatment started within 24 hr of birth and continued 3 times weekly thereafter until the end of the experiment. (2), Age at which oocysts inoculation was attempted. (3), Number of Cryptosporidium oocysts fed / mouse. (4), Average number of Cryptosporidium oocysts observed / 10 fields / day x number of days of oocyst shedding (± SE). Comparison of Normal, NRIg-treated and Anti-u treated mice using Student's t test yields $p > 0.05$. (5), Total number of days oocysts detected in stool samples.

II. Role of Specific and Non - specific Cellular Immunity in *Cryptosporidium* Infection

1- T Cell Responses to *Cryptosporidium* Antigens

The role of *in vitro* and *in vivo* T cell responses to *Cryptosporidium* antigens was first evaluated in studies of spleen and lymph node cells of infected normal, anti-u treated and also uninfected control mice.

A- Absence of Detectable *In Vitro* T Cell Proliferation to Cryptosporidial Antigens

The capacity of splenic or lymph node T cells to proliferate upon reexposure to *Cryptosporidium* oocysts was determined following sacrifice of neonatally infected normal, anti- u treated and uninfected normal BALB/c mice (table 6). Although T cells from all groups exhibited strong proliferative responses to the T cell mitogen Con A (Fig. 28A), proliferative responses to *Cryptosporidium* were uniformly weak (stimulation indices < 3, Fig. 28 B&C) in spite of assessing a variety of culture conditions and periods (table 6). Spleen and lymph node cells from normal adult mice injected subcutaneously with different concentrations of oocysts ranging from 3×10^5 to 3×10^6 intact oocysts / mouse at the base of the tail showed strong T cell proliferative responses upon exposure to increasing concentration of intact oocysts (Fig. 29 A,B,C), indicating that this failure is not attributable to the absence of *Cryptosporidium* specific T cells from the murine repertoire.

B- Cytokine Production *In Vitro* in Response to *Cryptosporidium* Oocyst Antigens

T - enriched spleen cells from mice 8 - 14 days p.i. (i.e. at the peak of infection, after the peak, and after recovery) and uninfected controls were cultured in order to evaluate the production of IL- 2, IL- 4 and IFN - gamma in response to cryptosporidial antigens. No significant IL- 2 or IL- 4 production was observed in any of these mice. In contrast, splenic T cells from *Cryptosporidium* infected mice exhibited production of IFN - gamma when exposed to sonicated *Cryptosporidium* oocysts. Culture supernatants from splenic T cells of these mice, but not from uninfected ones, could inhibit growth of WEHI- 279 cells which are sensitive to IFN- gamma. This inhibition could be blocked when antibody to IFN- gamma was used (Fig. 30).

C- Failure to Detect Delayed Hypersensitivity to *Cryptosporidium* Oocyst Antigens

To examine the capacity of normal uninfected, and infected normal, NRIg- treated and anti- u treated BALB / c mice to generate *Cryptosporidium* - specific delayed type hypersensitivity (DTH) responses, these mice were challenged intradermally with intact or sonicated oocysts 12, 14, or 44 days post inoculation. A group of adult BALB /c mice immunized with azobenzenearsonate (ABA) - coupled syngeneic spleen cells and challenged by the id injection of ABA 6 days later, was used as positive control for DTH. A group of unimmunized

mice served as control. Significant DTH responses to cryptosporidial antigens were not observed during or following recovery from Cryptosporidium infection (Fig. 31).

2- Cryptosporidium Infection : Studies in CD4 T Cell-deficient Mice

A- Effect of Continuous In Vivo Anti- CD4 Monoclonal Antibody (mAb) Treatment on CD4 T Cell Populations

To evaluate the role of CD4 T cells in neonatal infections with Cryptosporidium, BALB/c mice were rendered CD4 T cell deficient by continuous administration of anti - CD4 mAb. These mice exhibited no detectable CD4 lymphocytes in spleen or lymph node preparations, as assessed by staining with FITC conjugated rabbit anti- mouse Ig (table 7); however CD8 T cells were elevated ($P< 0.01$).

B- In Vitro Depletion of CD4 T Cells Leads to Marked Increases in the Severity of Cryptosporidium Oocysts Shedding: Disease Course

Oral inoculation of normal, NRIg - treated or anti - CD4 treated BALB /c neonates led to heavy shedding of fecal oocysts in all groups. The results of a typical experiment are presented in Fig. 32. Although the time of onset was similar in all groups, anti - CD4 treated mice exhibited more severe diarrhea and a protracted disease course, with several cycles of infection, an observation never made in normal, NRIg - treated, or B cell deficient mice. However, the

Cryptosporidium infection ultimately self - resolved without mortality in any of the CD4 deficient or normal mice.

Adult anti- CD4 treated mice remained resistant to infection upon inoculation of up to 1.5 x 10^6 oocysts/ mouse.

3- Cryptosporidium Infection: Studies in Irradiated Mice

A- Effect of Irradiation on B and T Cells of Neonate and Adult BALB /c Mice

Groups of neonates (7 days old) and adults (6 weeks old) BALB / c mice were irradiated (550 rad), to abolish B and T cell function. To evaluate the effects of irradiation on neonatal B cell function, spleen cells were examined at different time periods after irradiation for their capacity to respond to B cell mitogen (LPS), over a wide concentration range and compared with that of age matched untreated (normal) mice. The results of a typical experiment are presented in Fig.33. Although, spleen B cells of irradiated young mice did not respond to LPS up to day 10 post- irradiation (p.ir.), they had regained this capacity by day 14 p. ir.

Similarly, spleen T cells did not respond to T cell mitogen (Con A) up to day 10 p.ir., in contrast to age matched control mice, but T cell capacity to respond to Con A stimulation had returned 14 days after irradiation (Fig. 34).

In a parallel series of experiments, adult mice were tested for their spleen B and T cells responses to mitogens at various times after irradiation. Results were similar to those obtained in neonates.

As a complementary means of assessing T cell function in irradiated, unreconstituted mice, the capacity of spleen cells to generate mixed lymphocyte responses (MLR) in irradiated young mice was tested at days 8 and 11 p.ir. Spleen cells from normal (non- irradiated) young mice, whether infected with Cryptosporidium or not, showed good responses, but those of irradiated infected or non- infected age matched mice showed no response at day 8 p. ir., and even at day 11 p.ir. they showed a very weak response (Fig. 35), indicating that only a partial reconstitution had occurred by this point.

B- Infection of Normal and Irradiated BALB /c Mice with Cryptosporidium: Disease Course

Neonate (7 days old) irradiated and age - matched normal controls were infected with Cryptosporidium oocysts. The result of a typical experiment is shown in Fig. 36. The time of onset, peak and clearance of oocysts excretion and intensity of infection were not different in these groups of mice. The results of infection of four experimentally irradiated and two normal groups are presented in table 8. No significant differences could be observed in these groups. No mortality was observed in any of these mice.

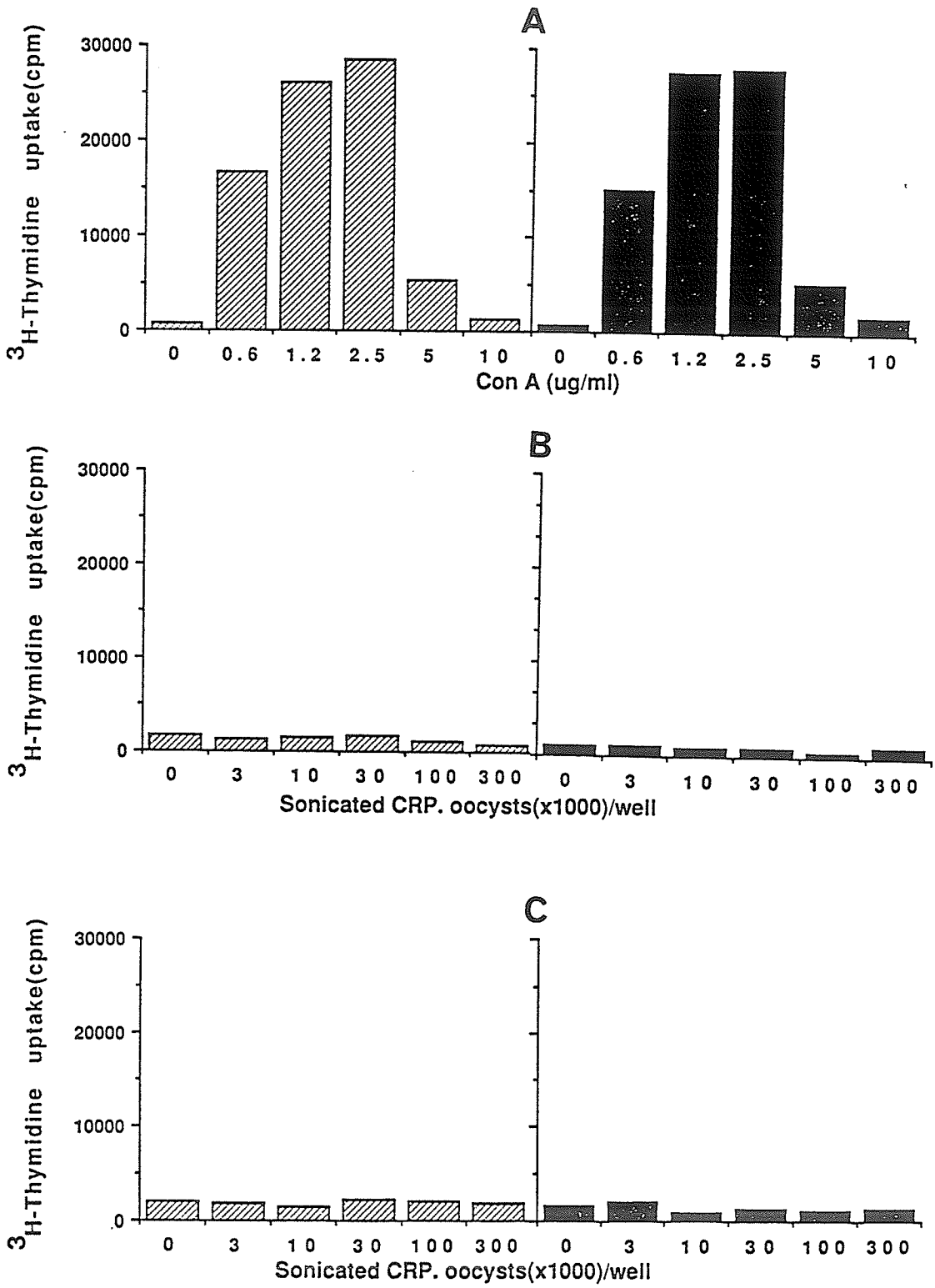
When adult BALB/ c mice were irradiated and inoculated with Cryptosporidium oocysts, they remained resistant and no oocysts were excreted in their stools over a period of 21 days. Irradiated adult mice reconstituted with either B or T cells did not show any infection after they were fed with oocysts.

Table 6. T cell proliferative responses to cryptosporidial oocyst antigens in infected, uninfected and immunized BALB/c mice.

Day of sacrifice	Responder cell population	In vitro culture period (day)	Antigen (oocysts/well)	SI.
13 p.i.	- Spleen @ - T enriched spleen @	3, 5	$0.03\text{-}3x10^{5}$ ⊠	<3
15 p.i.	- Spleen # - T enriched spleen # - Lymph node #	4, 6	$0.03\text{-}3x10^{5}$ ⊠	<3
45 p.i.	- Spleen *	5	$0.25\text{-}4x10^{5}$ ✦	<3
7 ✦	- Spleen ** - Lymph node **	4	$0.25\text{-}4x10^{5}$ ✦	30-51

Proliferation assays on spleen, T - enriched spleen, and lymph node cells were carried out as described in materials and methods. Stimulation index (SI) for all cell populations of infected and uninfected mice, exposed to either sonicated (⊠) or intact (✦) oocysts, was less than 3, but was 30 - 51 in immunized mice at day 7 post immunization (✦). (@), From normal, uninfected; normal, infected mice. (#), From normal, uninfected; normal, NRIg- treated & anti - u treated, infected mice. (*), From normal, uninfected; normal & anti- u treated, infected mice. (**), From normal, unimmunized; Cryptosporidium oocysts immunized mice.

Figure 28. T cell proliferative responses of spleen T cells from Cryptosporidium infected BALB/c mice to mitogen Con A and to cryptosporidial oocyst antigens. Proliferation assays were carried out as described in materials and methods. Spleen cells (▨) and T- enriched spleen cells (■) from infected normal mice showed good response to Con A (A), indicating intact T cell functions, but failed to respond to increasing concentrations of sonicated oocysts, after 3 days (B) and 5 days (C) culture. Responses are presented as mean cpm from duplicate cultures in one of 3 experiments.


A
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3H-Thymidine uptake(cpm)
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Con A (ug/ml)
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10000
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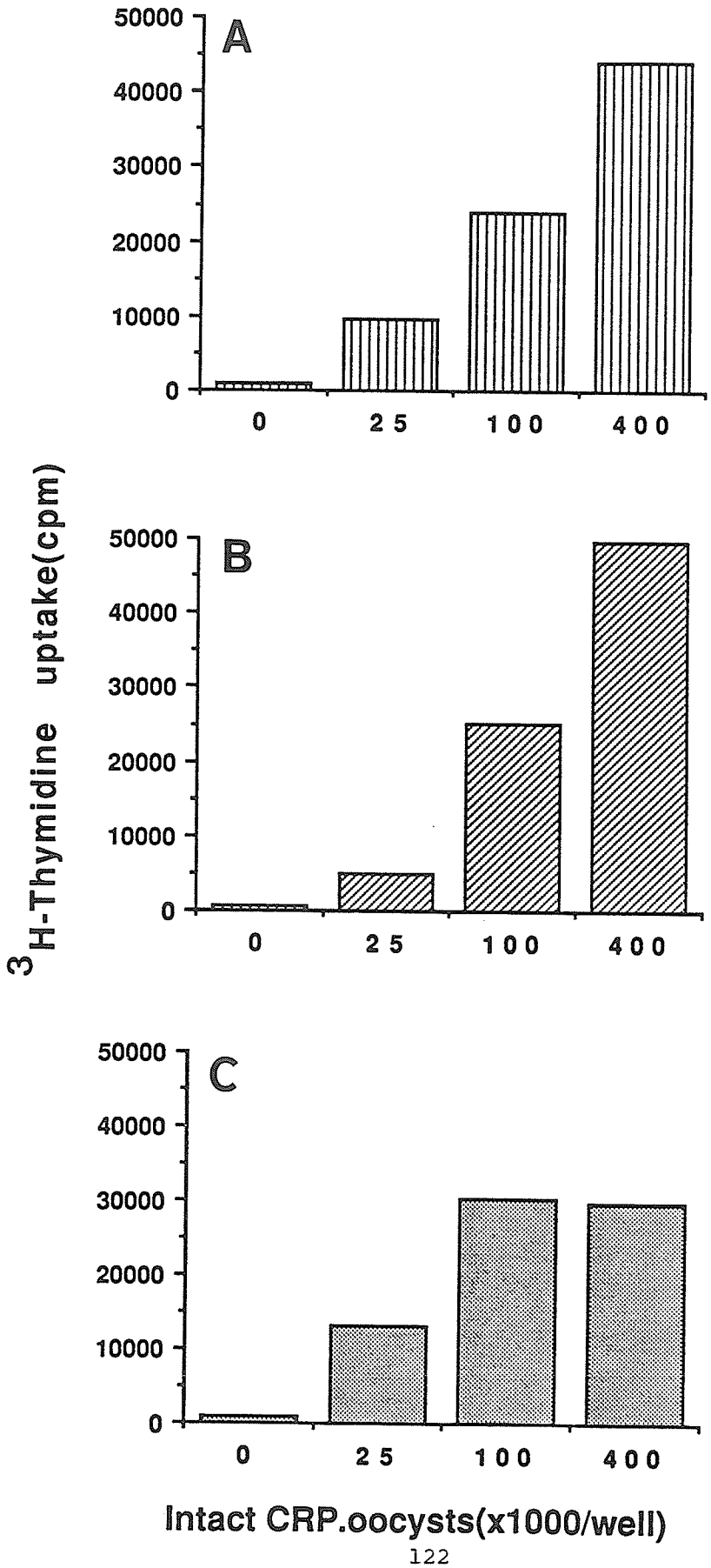
3H-Thymidine uptake(cpm)
0 3 10 30 100 300 0 3 10 30 100 300
Sonicated CRP. oocysts(x1000)/well
C
30000
20000
10000
0
3H-Thymidine uptake(cpm)
0 3 10 30 100 300 0 3 10 30 100 300
Sonicated CRP. oocysts(x1000)/well

Figure 29. T cell proliferative responses to cryptosporidial oocyst antigens in oocyst immunized BALB/c mice. Proliferation assays were carried out as described in materials and methods. Spleen cells from normal mice immunized with $3x10^5$ (A), $1x10^6$ (B), and $3x10^6$ (C) intact oocysts responded to increasing concentrations of intact oocysts; stimulation index (SI) = 30 - 51. Responses are presented as mean cpm from duplicate cultures.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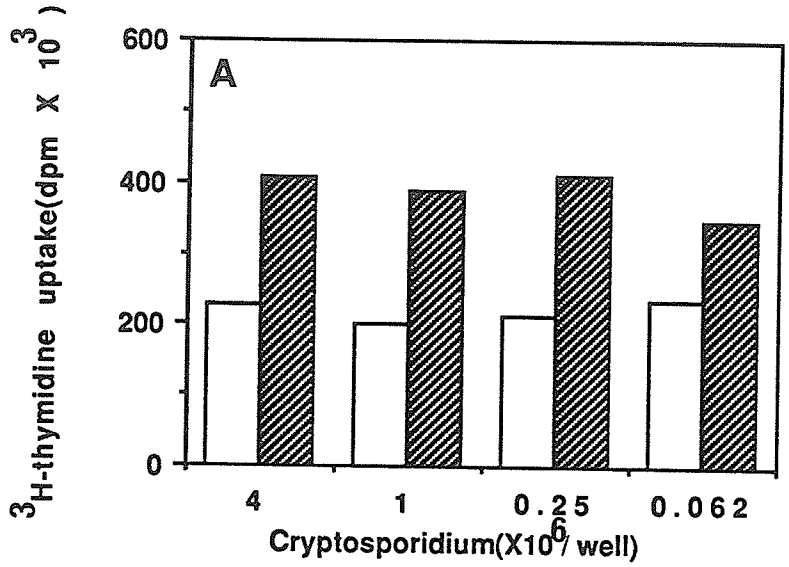
^{3}H-Thymidine uptake(cpm)
Intact CRP.oocysts(x1000/well)

Figure 30. IFN - gamma production by splenic T cells from infected and uninfected BALB/c mice exposed to cryptosporidial oocyst antigens. Spleen T cells from Cryptosporidium infected (A) and uninfected mice (B), were exposed to decreasing concentrations of sonicated oocysts. Supernatants were collected and used to test their ability to inhibit growth of WEHI - 279 cells as described in materials and methods. T cells of infected mice showed a good production of IFN - gamma (□) which was blocked using anti- IFN- gamma mAb (▨), but those of uninfected mice failed to produce this lymphokine. Known concentrations of murine IFN - gamma shown in (C) were used as internal control. Responses are presented as mean dpm from duplicate cultures in one of 4 experiments.


A
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4
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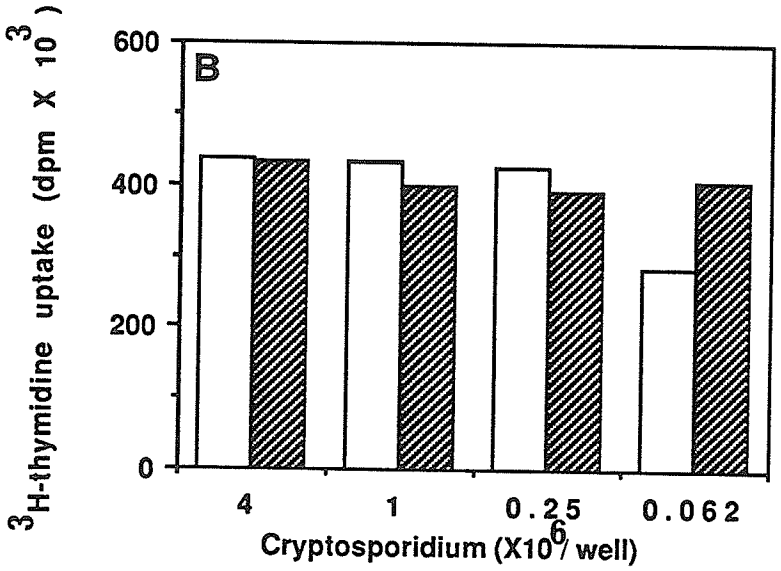
0.062
^{3}H-thymidine uptake(dpm X 10^3)
Cryptosporidium(X10^6/ well)


B
600
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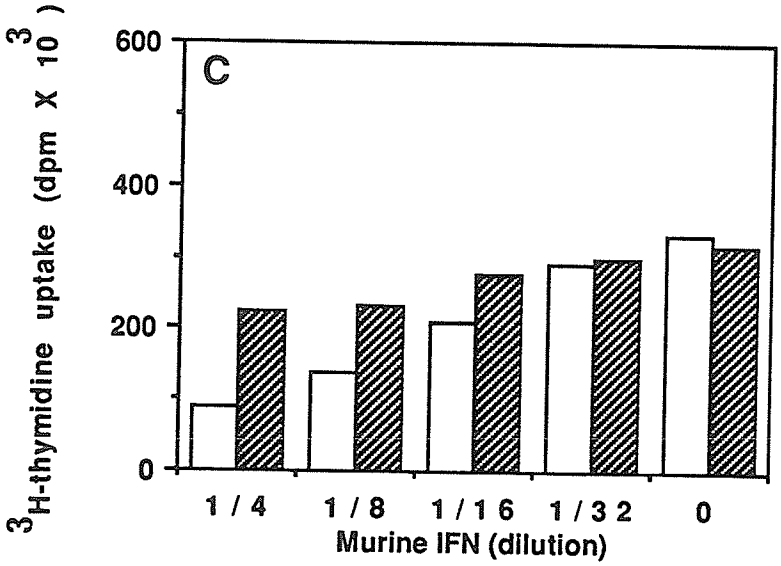
^{3}H-thymidine uptake (dpm X 10^3)
Cryptosporidium (X10^6/ well)


C
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1 / 32
0
^{3}H-thymidine uptake (dpm X 10^3)
Murine IFN (dilution)

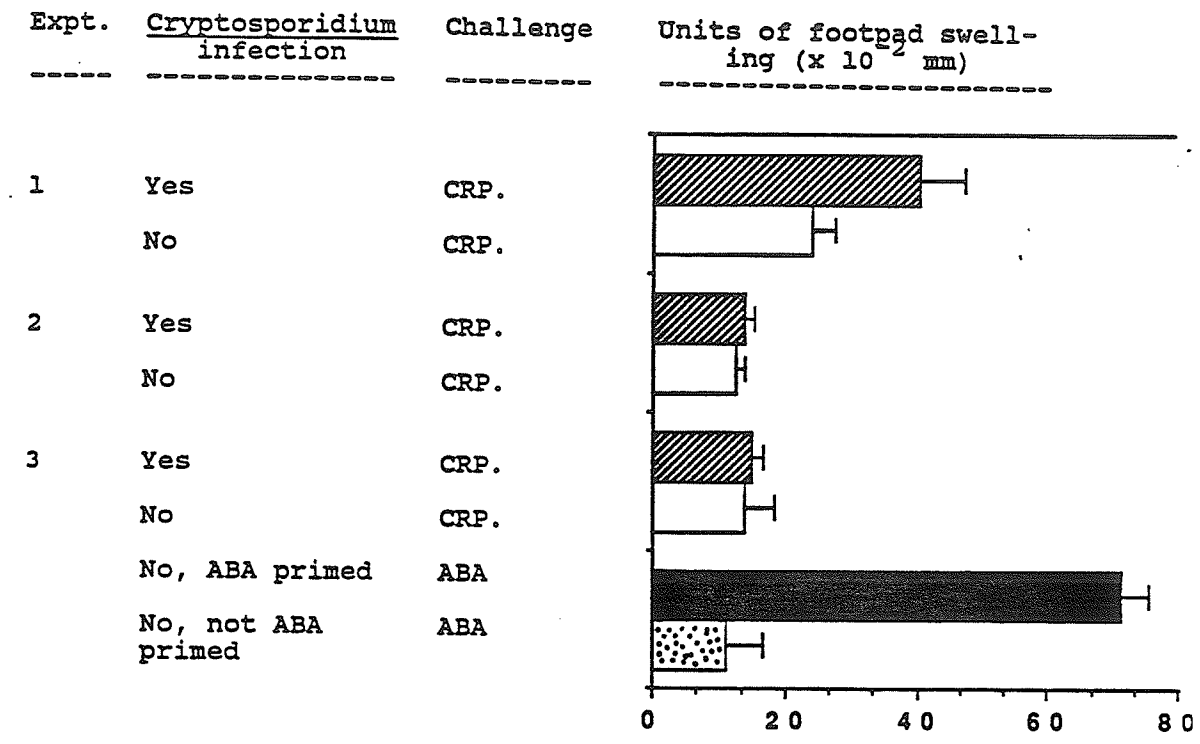


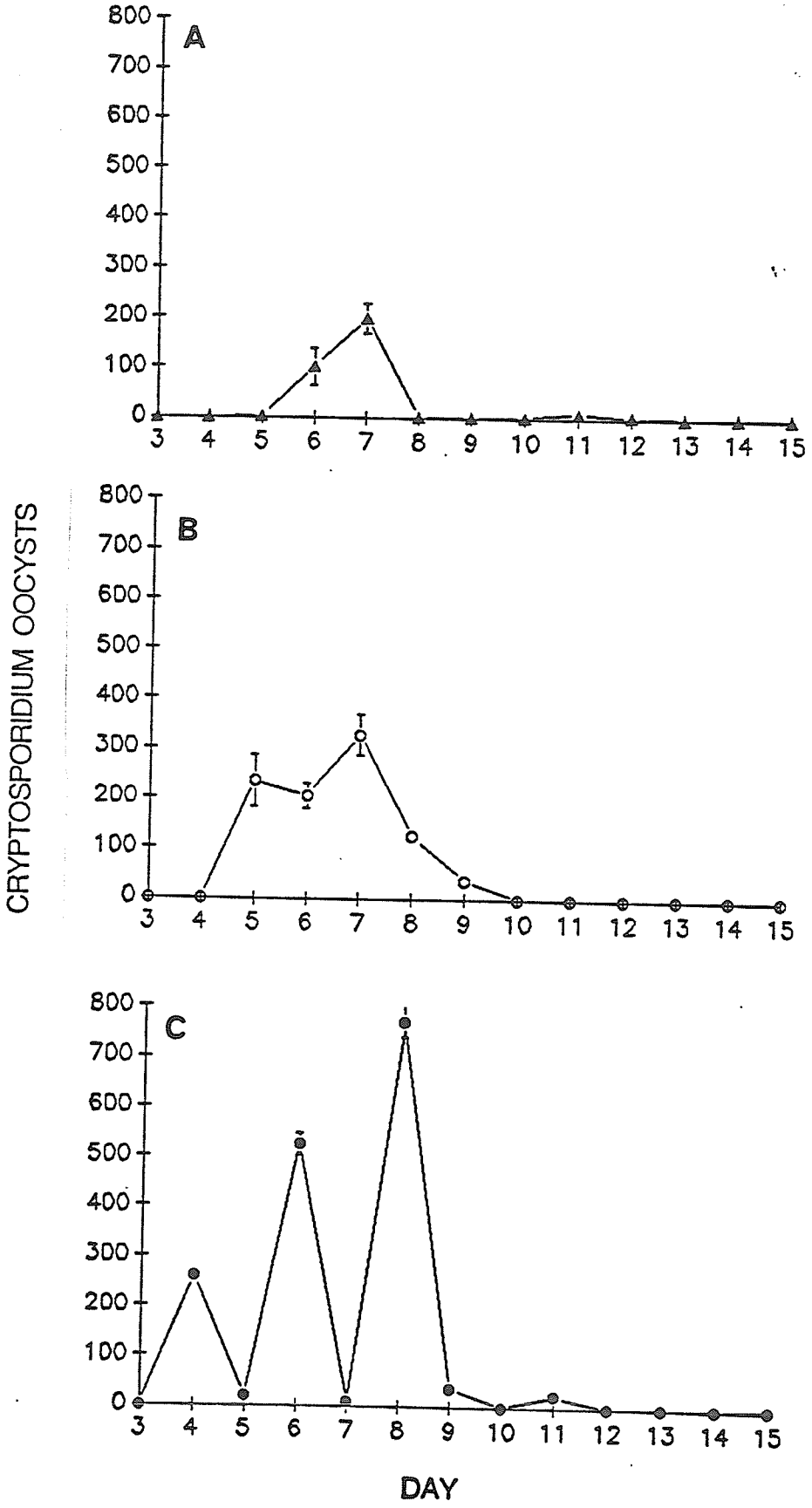
Figure 31. Delayed hypersensitivity (DTH) to Cryptosporidium oocyst antigens in infected and uninfected BALB/c mice. Infected and uninfected mice were challenged intradermally with oocysts as described in materials and methods. Mean of units of footpad swelling ($\pm$ SE) for each group of infected (▨) and uninfected (□), as well as a group of positive control (ABA primed), rechallenged with ABA (■) are presented. No significant differences in DTH responses were detected in infected mice as compared with uninfected mice. Significant ($P< 0.001$) differences in DTH responses were observed in a group of ABA primed mice compared to ABA unprimed mice (▩).

Table 7. Effect of treatment with anti - CD4 mAb on spleen T lymphocytes of BALB/c mice.

BALB/c mice population examined	(% Positive) thy 1.2	CD4 ± (SE)	CD8 ± (SE)
Normal	> 95	35 ± (2.7)	35.6 ± (1.4)
Anti - CD4 mAb treated	> 95	< 5	46.8 ± (2)

Spleen T cells from normal and anti- CD4 mAb treated mice were examined for the percentage of positive thy 1.2^+, $CD4^+$ and $CD8^+$ cells using FITC conjugated rabbit anti - mouse Ig. Fluorescence was determined by an EPICS V fluorescence activated cell sorter. Mean percentage of each subset of lymphocytes (±SE) are presented. Mice treated with anti- CD4 mAb exhibited no detectable $CD4^+$ T lymphocytes. Anti- CD4 mAb treated mice exhibited significantly elevated $CD8^+$ T cells as compared to normal mice ($P< 0.01$).

Figure 32. Cryptosporidium oocysts shedding in infected neonatal normal, NRIg - treated and anti- CD4 treated BALB/c mice. The daily mean of the number of fecal oocysts seen in 10 microscopic fields ($\pm$ SE) is shown, comparing infection in the infected normal (A), NRIg - treated (B) and anti- CD4 treated (C) mice. A minimum of 60 fields / data point were counted. No significant differences were noticed in time of onset, peak and resolution of oocyst shedding, but the intensity of oocysts shedding was significantly higher in anti- CD4 treated mice compared to the control groups ($P<0.001$), in 4 of the 5 experiments. Results of a typical experiment are presented.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14
15
CRYPTOSPORIDIUM OOCYSTS
DAY

Figure 33. Effect of irradiation on proliferative responses to mitogen LPS of spleen B cells from neonate BALB/c mice. Proliferation assays were carried out on spleen cell populations. Spleen cells were cultured, exposed to mitogen, pulsed with [^{3}H]- thymidine and thymidine uptake determined by liquid scintillation counting as described in materials and methods. Spleen cells from irradiated mice (■) failed to respond to B cell mitogen LPS at day 4 p.ir. (A), and at day 8 p.ir. (B); a very weak response was observed at day 10 p.ir. (C); and the ability to respond to mitogen was restored at day 14 p.ir. (D), as compared to spleen cells responses from age - matched normal mice (▨) to mitogen. Responses are presented as mean cpm from duplicate cultures in one of 4 experiments.

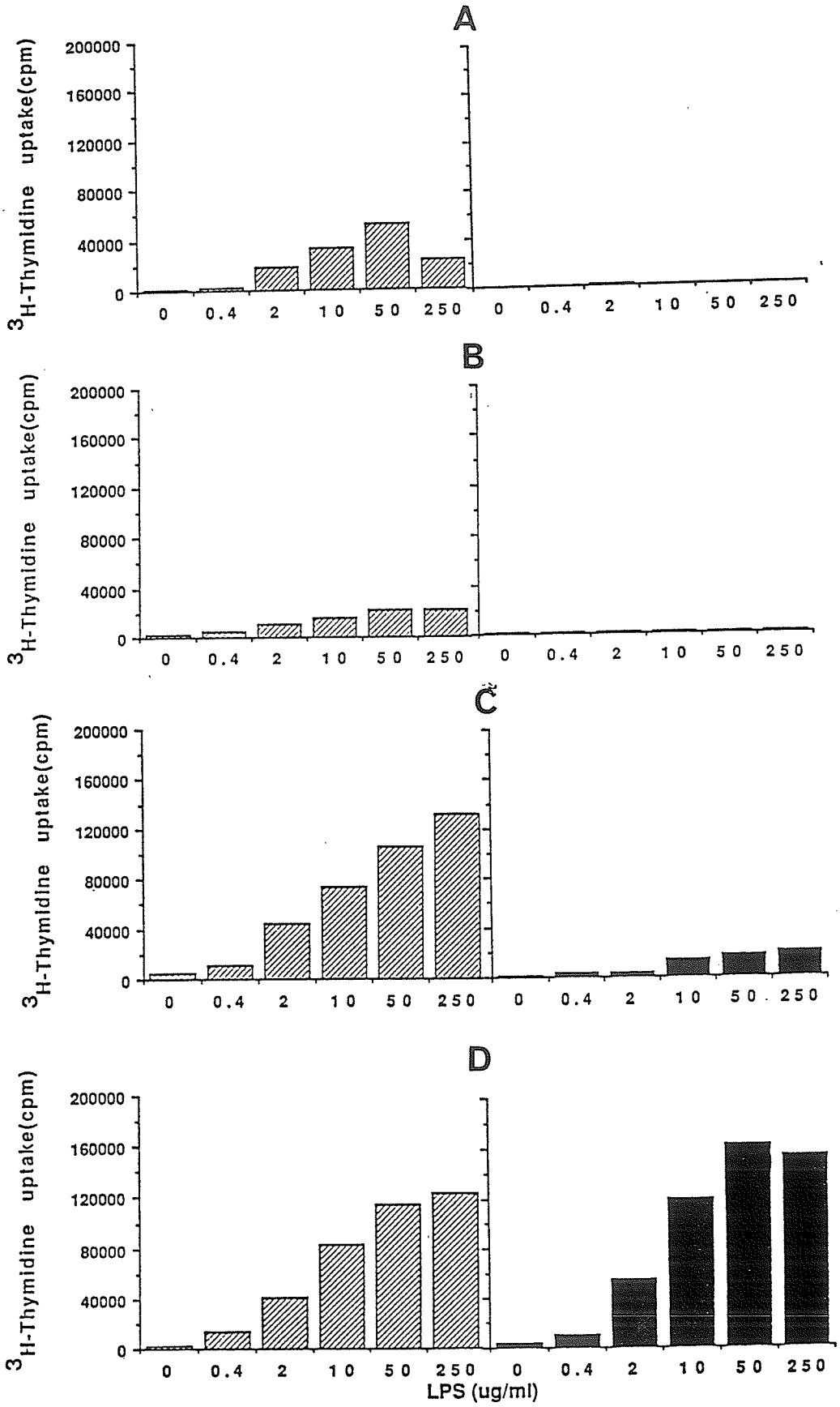
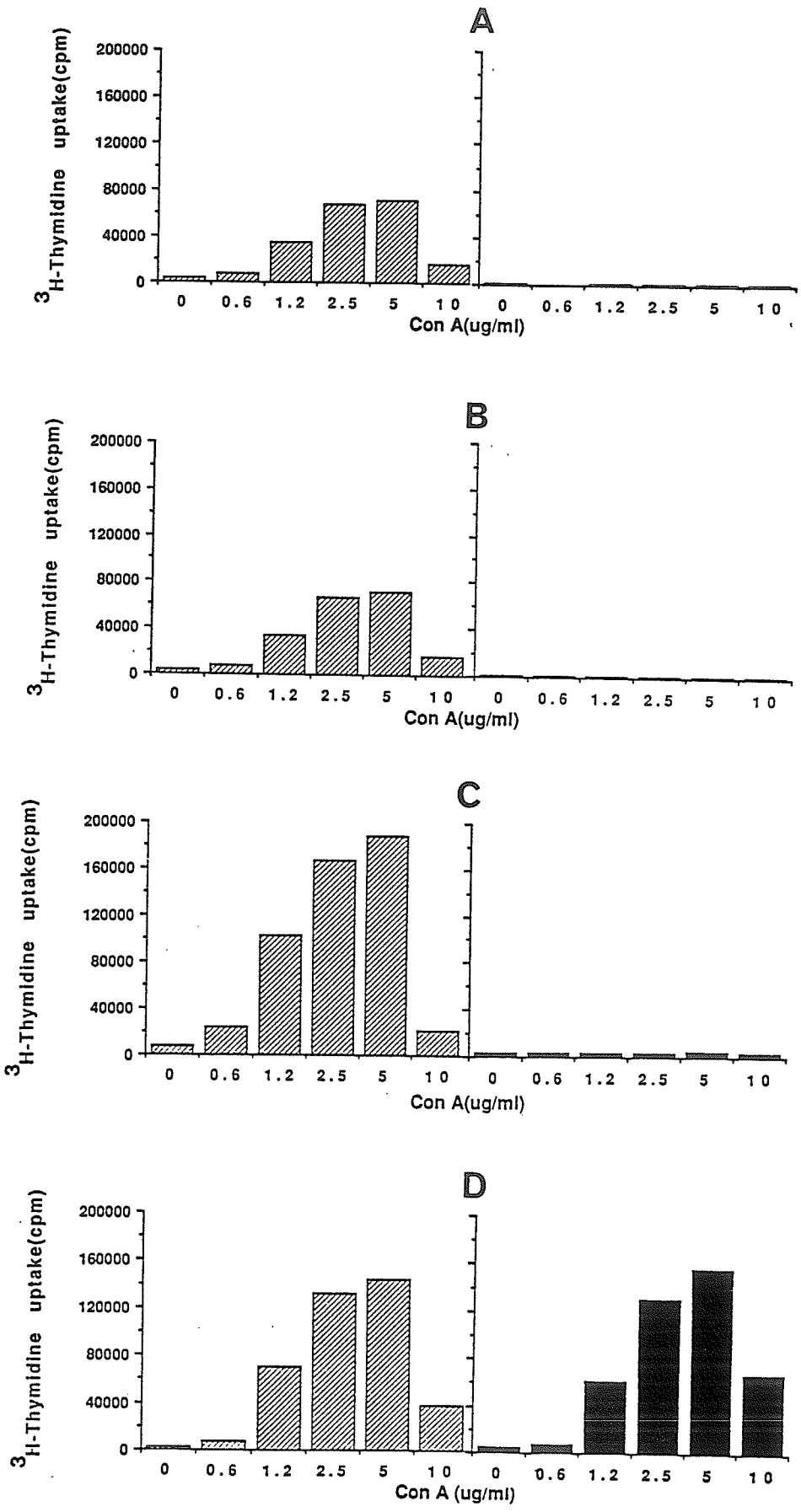

A
B
C
D
^{3}H-Thymidine uptake(cpm)
200000
160000
120000
80000
40000
0
0 0.4 2 10 50 250 0 0.4 2 10 50 250
LPS (ug/ml)

Figure 34. Effect of irradiation on proliferative responses to mitogen Con A of spleen T cells from neonatal BALB/c mice. Proliferation assays were carried out on spleen cell populations. Spleen cells were cultured, exposed to mitogen, pulsed with [^{3}H]- thymidine and thymidine uptake determined by liquid scintillation counting. spleen cells from irradiated mice (■) failed to respond to T cell mitogen Con A at day 4 p.ir. (A), day 8 p.ir. (B), day 10 p.ir. (C), and regained the ability to respond to mitogen at day 14 p.ir. (D), as compared to spleen cells responses of age - matched normal mice (▨) to mitogen. Responses are presented as mean cpm from duplicate cultures in one of 4 experiments.


A
B
C
D
^{3}H-Thymidine uptake(cpm)
200000
160000
120000
80000
40000
0
0 0.6 1.2 2.5 5 10 0 0.6 1.2 2.5 5 10
Con A(ug/ml)

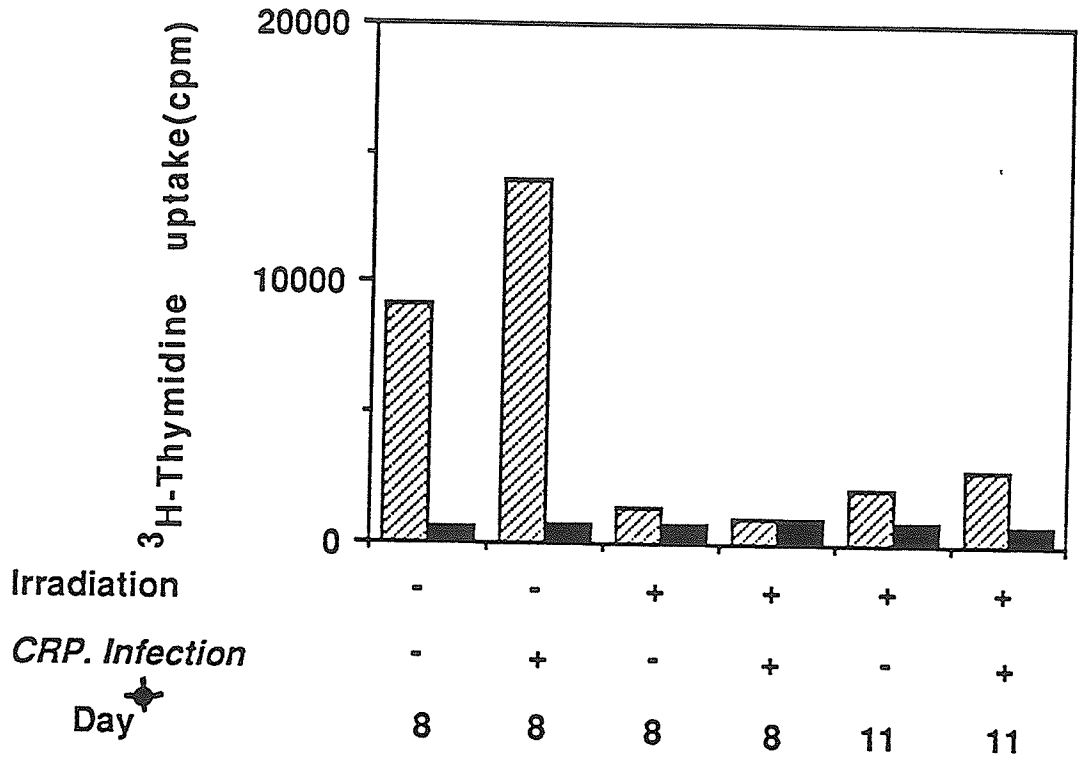
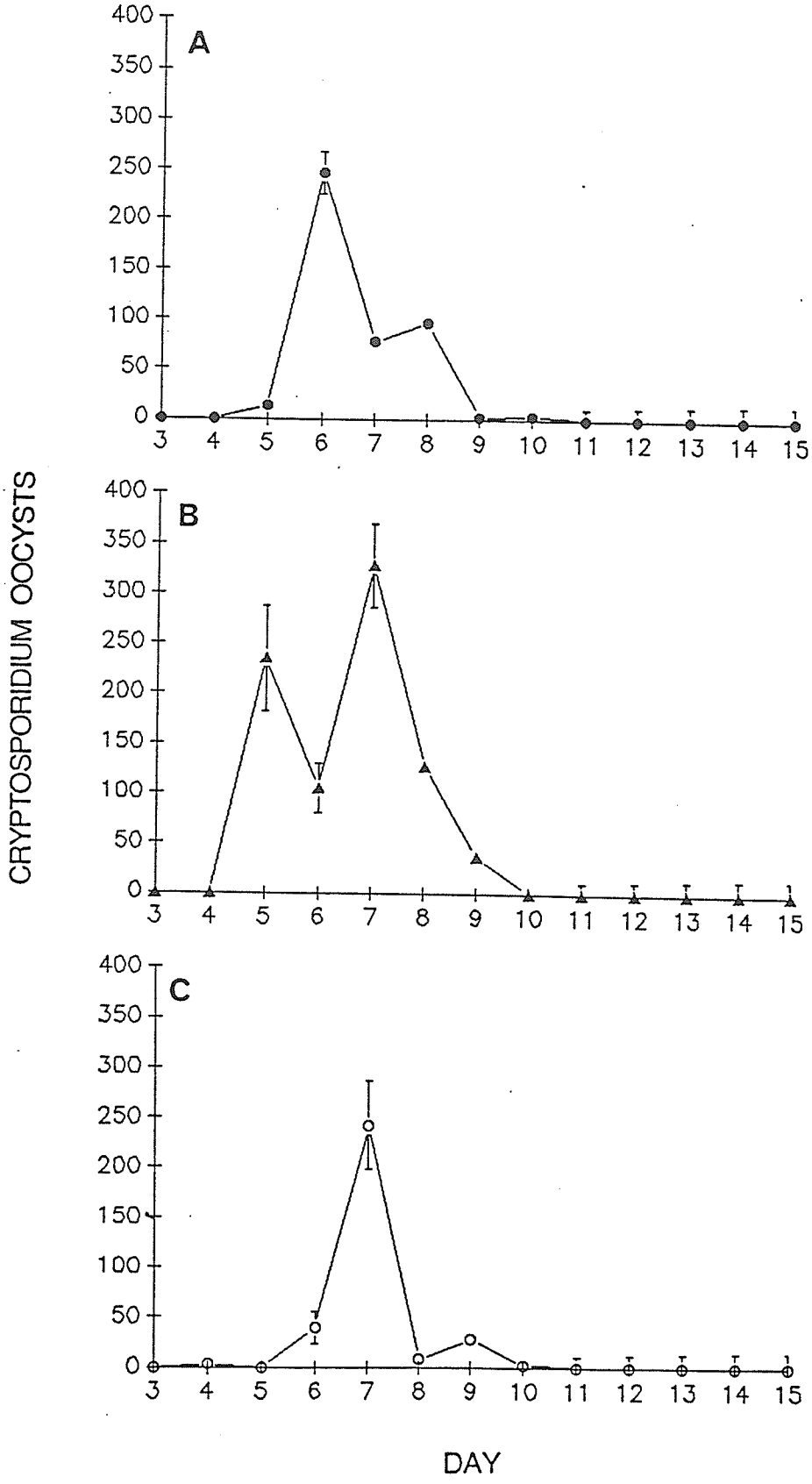


Figure 35. Mixed lymphocyte responses (MLR) in neonatal irradiated and normal BALB/c mice. Splenic T cells from irradiated infected and uninfected mice failed to generate mixed lymphocyte resposes at day 8 post irradiation, and showed very week responses at day 11 p.ir., but normal young mice (infected or uninfected) showed a good response. MLR were carried out as described in materials and methods. Responses are presented as mean cpm from duplicate cultures of responder cells exposed to stimulator cells (▨), and responder cells alone (■), in one of 3 experiments. (✦), days post irradiation.

Figure 36. Cryptosporidium oocysts shedding in infected neonatal normal, NRIg - treated and irradiated BALB/c mice. The daily mean of the number of fecal oocysts ($\pm$ SE) seen in 10 microscopic fields is shown, comparing the infected normal (A), NRIg - treated (B), and irradiated (C) mice. A minimum of 60 fields / data point were counted. Results of a typical experiment are presented. No significant differences were noticed in time of onset, peak and resolution of oocysts shedding.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CRYPTOSPORIDIUM OOCYSTS
DAY

Table 8. Infection profile for groups of irradiated (550 rad) and non- irradiated BALB/c mice.

		Intensity of fecal oocyst shedding (Cryptosporidium oocysts / 10 fields)											
	Day:	3	4	5	6	7	8	9	10	11	12	13	14
	Expt.												
Irradiated mice	1	-	0.6	270	59	7	0.3	0.5	0.3	-	-	-	-
	2	-	2.8	0.1	39	242	8	28	-	2	0.5	2	0.1
	3	-	1	0.3	46	103	40	3	-	14	0.5	1	0.3
	4	-	-	0.5	2.8	32	279	1.5	0.5	0.5	0.3	0.2	-
Normal control mice	1	-	-	13	246	77	97	2	3	1.5	2.5	-	-
	2	-	0.6	109	1.6	8	2.1	0.5	-	1	-	-	-

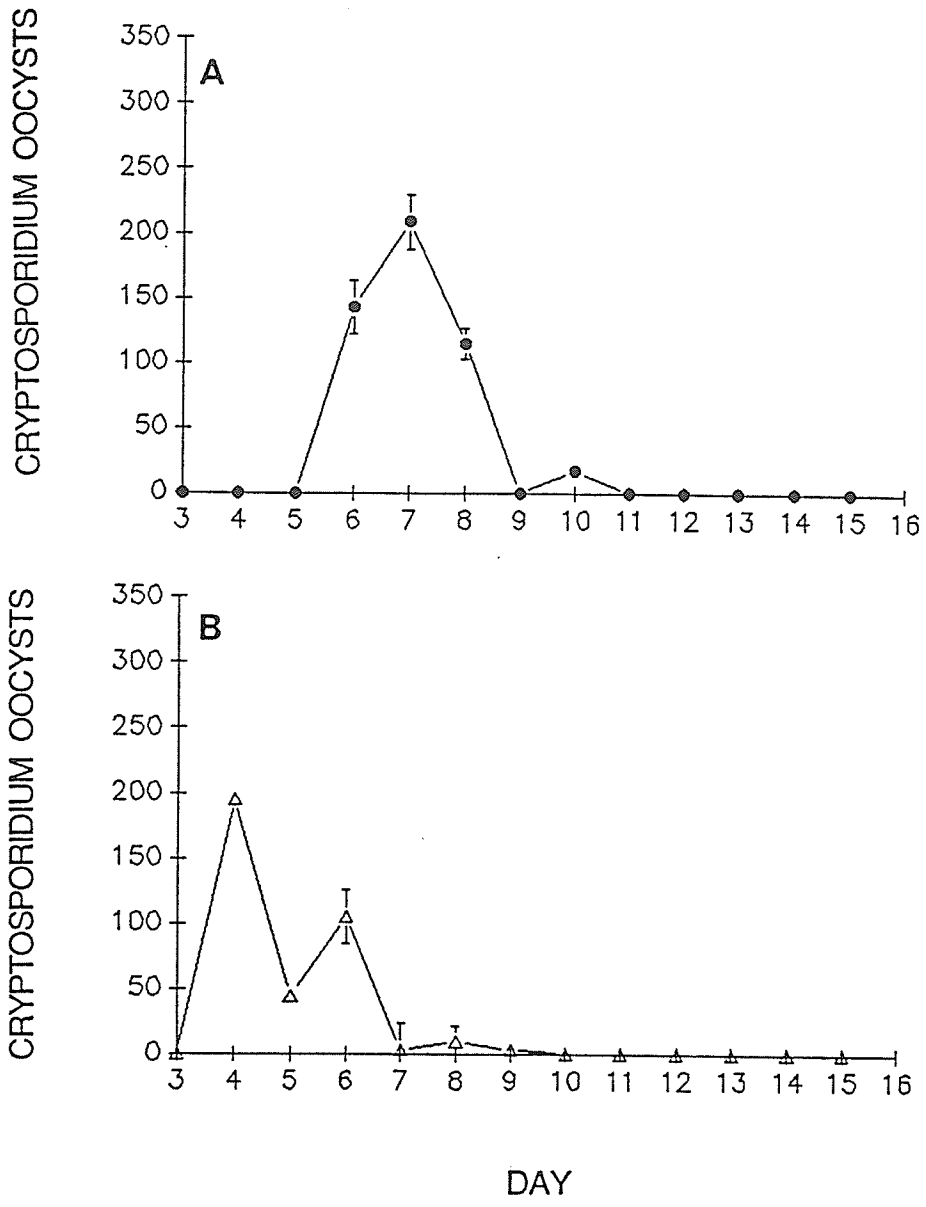
No significant differences were observed, when time of onset, peak and resolution, as well as peak intensity (underlined) of fecal oocysts shedding were compared in four groups of irradiated and two groups of normal infected neonatal mice. In all cases SE was < 20%. In some time points oocysts were not detected and are referred to as (-).

4- Natural Killer (NK) Cell Deficient Mice do not Display Increased Sensitivity to Cryptosporidial Infection

In order to assess the role NK cells play in the resolution of neonatal murine cryptosporidiosis, 7 day old C3H/HeJ- Bg/Bg mice were fed with 1.5×10^5 oocysts / mouse. Neither the time of onset of cryptosporidiosis, the intensity nor the duration of infection were different from those observed in normal mice (Fig.37). No mortality was reported for any of these groups.

A summary data on the intensity of fecal oocysts excretion at peak of infection, as well as time of onset, peak and recovery of oocysts shedding in all experimental and control groups of neonate mice used is presented in table 9.

Figure 37. Cryptosporidium oocysts shedding in infected neonatal normal, and NK cell deficient (Bg/bg) mice. The daily mean of the number of fecal oocysts ($\pm$ SE) seen in 10 microscopic fields is shown, comparing the normal (A), and NK deficient (B) mice. Results of a typical experiment is presented. A minimum of 60 fields / data point were counted. No significant differences were noticed in time of onset, peak, and resolution of oocysts shedding.


CRYPTOSPORIDIUM OOCYSTS
A
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200
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100
50
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16
CRYPTOSPORIDIUM OOCYSTS
B
350
300
250
200
150
100
50
0
3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAY

Table 9. Summary of data on Cryptosporidium infection in immunologically compromised mice.

Experimental group	Intensity of infection *	Excretion of oocysts (day)		
		Start	Peak	Recovery
1- Anti- u treated	360	5	7	14
2- Anti- u treated	313	5	8	17
3- NRIg- treated	437	6	7	16
4- Normal control	252	5	6	15
5- Normal control	192	4	7	15
6- Anti- u treated	266	5	8	13
7- NRIg- treated	328	5	7	13
8- Normal control	158	7	8	16
9- Anti- u treated	631	4	10	15
10- NRIg- treated	727	5	7	17
11- Normal control	389	5	6	13
12- Irradiated	270	4	6	11
13- Irradiated	242	4	7	15
14- Normal control	246	5	6	13
15- Irradiated	103	4	7	15

16- Irradiated	279	5	8	14
17- Normal control	109	4	5	12
18- Anti- CD4 treated	223, 890@	5	7, 8	15
19- Anti- CD4 treated	270, 801@	5	5, 8	15
20- Normal control	64	5	7	14
21- Anti- CD4 treated	283, 539, 669@	3	3, 7, 9	11
22- Anti- CD4 treated	235, 515, 879@	3	3, 7, 9	11
23- NRIg- treated	see # 7			
24- Normal control	197	5	7	15
25- Anti- CD4 treated	820, 817@	4	5, 7	16
26- Normal control	517	4	7	11
27- Bg / Bg	239	4	4	11
28- Bg / Bg	149	4	4	11
29- Normal control	209	4	7	13

Peak intensity, as well as time of onset, peak, and recovery of oocysts shedding in all experimental groups of mice are presented. At least oocysts of 60 microscopic fields were counted and mean number of oocysts / 10 fields at the peak of infection (*) is shown. In all cases SE was < 25% and is omitted for clarity. More than one cycle of infection was observed (@) in anti - CD4 treated infected mice.

DISCUSSION

The study of Cryptosporidium immunology requires antigens which are not available commercially and cannot be obtained in sufficient amounts using cell cultures (65, 287). This fact has forced investigators to use experimentally infected animals and to develop techniques for the concentration and purification of oocysts recovered from their feces.

Experimental Infection of Calves

Cryptosporidial infections have been reported in naturally infected calves and humans in Manitoba (192). Calf feces containing Cryptosporidium oocysts were obtained from the Provincial Veterinary Laboratory. Calf inoculation experiments were conducted to obtain Cryptosporidium isolates in large numbers and to investigate each calf's antibody response over a short period of time.

Our observations on the experimental infection of calves can be summarized as follows: a) For all infected calves, the prepatent period was 4 days and oocysts excretion continued for a mean of 10 days; both findings are in agreement with other reports (215, 247). b) Eight out of ten experimentally infected calves survived the infection while two died of severe diarrhea, 12 days post infection. Cryptosporidium infection appears to have been the cause of death as fecal cultures for bacteria and viruses, as well as transmission electron microscopic studies were all negative. Heine et al. (120) were unable to show an infectious agent other than Cryptosporidium in the feces of the calves in their experiments. Fayer et al. (85) demonstrated that the

intestinal contents of one of their experimental calves which died contained Cryptosporidium oocysts as well as a variety of bacteria including C. perfringens. c) A large number of oocysts were detected in the feces of the two calves which died, as well as in the feces of 75% of the surviving calves. Similar results were reported in primates by Miller et al. (211). By contrast, the report by Casemore et al. (47) indicates that in humans severity of infection was directly related to the number of oocysts excreted in feces.

Purification of Oocysts and Sporozoites

Methods developed for the purification of coccidian parasites (109, 281, 317) were found unsatisfactory for Cryptosporidium. Although, oocysts recoveries were often high using Sheather's flotation techniques, excessive contamination by fecal debris as well as bacteria rendered those oocysts unsuitable for immunological and biochemical studies. Dubey et al. (78) encountered the same problem using Sheather's concentration method to recover Toxoplasma oocysts from cat feces.

A percoll gradient procedure was developed for the concentration of sodium hypochlorite - treated oocysts; it required that all centrifugations be performed at 4^{o} C to avoid excystation. Increasing the centrifugation time from 10 to 60 min did not improve the concentration of oocysts when sodium hypochlorite treatment was omitted. We could not confirm the findings of Waldman et al. (318) on the

purification of oocysts using a percoll discontinous density gradient reported to be able to yield a concentration of $1x10^5$ oocysts / ml after 10 min centrifugation at 250 x g.

Sodium hypochlorite - treated oocysts may be suitable for nucleic acid and other biochemical analysis and for studies on sporozoites; however, they could not be used for immunological studies, due to changes in the oocyst wall caused by the pretreatment. The reduction in fluorescence during IFA test, as well as the loss of most protein bands using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), appear to confirm this speculation.

The cesium chloride gradients produced highly purified oocysts and sporozoites, suitable for biochemical and immunological studies. These oocysts were used to infect two newborn calves and to raise hyperimmune rabbit antisera, as well as being the antigen for IFA and ELISA testing. In addition, they were used for protein analysis by SDS-PAGE and western blotting. Only 50,000 sonicated, cesium chloride purified oocysts were needed to coat each ELISA well rather than the 250,000 reported by Ungar et al. (311) who used sonicated, sodium chloride recovered and sodium hypochlorite pre-treated oocysts.

The availability of a purification technique has enabled us to produce antisera against Cryptosporidium sporozoites, in rabbits.

Enzyme - Linked Immunosorbant Assay (ELISA) Detection of Immunoglobulin M and G Antibodies to Cryptosporidium in Calf Sera

Cryptosporidiosis is considered to be important in the neonatal diarrheal syndrome of calves (9, 120, 219, 250, 257, 267). Mann et al. (192) reported that cryptosporidial oocysts were found in 25.8% of the bovine fecal specimens examined in Manitoba.

Little is known of the immunological responses to the infection of Cryptosporidium species. The indirect fluorescent antibody (IFA) test has been used to detect antibody to Cryptosporidium, in animal and human sera (41, 304), using infected tissues as antigen. Mann et al. (191), using feces - derived oocysts as antigen in an IFA, demonstrated the presence of antibody to Cryptosporidium spp. in 40% of adult bovine sera tested in Manitoba. IFA assays are subjective while ELISA is quantitative and objective. ELISA is technically easy to perform on large numbers of specimens. It can detect smaller amounts of Ab than IFA, and can be standardized without difficulty. Using an ELISA, we demonstrated the presence of specific IgM and IgG antibodies in 10 colostrum-fed experimentally infected calves tested serially for a period of 3-4 months in 4 calves, and for a period of 3 weeks in 6 other calves; all these calves had been inoculated with oocysts at the age of one day. Ungar et al. (311) reported an ELISA procedure for the detection of IgM and IgG antibodies to Cryptosporidium in immunocompromised

persons. They observed no apparent cross-reactivity with other intestinal protozoan parasites. Using an ELISA, Williams (325) measured the level of Cryptosporidium antibody in serum and feces of calves experimentally infected at the age of 7 - 10 weeks. Williams reported variable antibody responses which tended to be directly proportional to the size of the infecting dose. We could not verify Williams' findings since two of our calves received a dose of oocysts lower than that given to the other 8 calves, yet developed higher level of IgM and IgG antibodies. Most calves had IgM and IgG antibodies at zero time (i.e. before oocyst inoculation). The presence of antibodies at zero time is probably due to the presence of Cryptosporidium antibodies in the colostrum they all received at birth. This hypothesis was tested by measuring the level of IgM and IgG antibodies specific to Cryptosporidium in four randomly selected cow's colostrum samples. Both classes of antibodies were detected in all four samples. It should be mentioned that the syndesmochorial type of placentation in ruminants prevents the transplacental passage of immunoglobulin molecules; for survival, newborn of these species are entirely dependent on antibodies received through the colostrum. When young ruminants are fed with colostrum, antibodies are actively taken up by epithelial cells into the lacteals, and possibly the intestinal capillaries, and eventually reach the systemic circulation; in this way newborn animals obtain a massive transfusion of maternal immunoglobulins (295). The level of

antibodies in colostrum may vary from sample to sample. We did not measure the antibody levels of the colostrum given to our experimentally infected calves.

The level of antibodies in Cryptosporidium infected calves decreased one week p.i. and increased again after two weeks. It is known that the young of domestic animals are fully capable of mounting a primary immune response immediately at birth (295).

IgM antibody was detected in all experimental calves, with the exception of one; these antibodies were detected until the end of the study which lasted 3 or 4 months p.i. for four of these calves. Similar results were reported in Philippine children followed up for 6 months (164) and seven patients followed up for a period of one year (309). Ungar et al. speculated that these cases may represent continuous infections (perhaps with production of separate antibodies to different life cycle stages), or new infections or perhaps that the IgM response may persist beyond the expected time. Persistence of IgM antibody has also been described for at least two viruses (29, 83). In contrast to the IgM response, a specific IgG antibody response is expected to persist for a longer period of time. However, the duration of detection of IgG antibodies can vary considerably (46, 153, 304, 309). In our study, the level of IgG antibody was higher than that of IgM in all calves at zero time. IgG is the predominant immunoglobulin in the colostrum of all major domestic animals (295). Compared to IgM, the IgG antibody response was still

higher 2 weeks p.i. and later. Whether the high level of IgG antibodies competed with IgM for available antigens, thus successfully reducing the detection of IgM antibodies, is not clear. According to Cohen et al. (55), high levels of IgG antibodies compete with IgM antibodies for antigen binding, thus resulting in false negative or appreciably reduced IgM detection. On the other hand, false positive IgM antibodies may be detected by IFA, for example due to the presence of rheumatoid factor. Lin et al. (177) used an ELISA to detect IgM antibodies to T. gondii and reported that their results were not affected by either IgG antibodies or rheumatoid factor. They believe that the use of sufficient quantities of adequately purified antigen allows the detection of any IgM or IgG antibodies present. In our ELISA we did not determine the presence of false positive or false negative IgM antibodies.

Despite the presence of Cryptosporidium antibodies in most of our ten calves, none of them was protected from infection. Calves with high titer of serum antibody cleared the infection at the same time as those with low level of antibody. However, two calves with negative or low antibody responses died at day 12 p.i. Similarly, Current (62) found that the presence of colostral antibody in cow's milk failed to exert a protective effect. He stated that antibodies are widely present in cow's milk yet cryptosporidiosis is common in calves. The role of humoral immunity, and in general, the role of B cells in self - resolving cryptosporidiosis will be discussed later.

In calves, individual variations in antibody response were observed. Similarly, Turunen et al. (296) observed individual variations in IgA antibody response in patients with toxoplasmosis. They hypothesized that this could reflect differences in the clinical course of the disease or in the genetically determined immune response, or in both.

We did not look for other immunoglobulin isotypes in calf sera. Using an IFA, Casemore (46) could demonstrate the presence of specific IgG, IgM, IgA, and IgE in the sera of Cryptosporidium infected humans.

SDS-PAGE and Western Blot Pattern of Cryptosporidium Oocysts

Nineteen species of Cryptosporidium have been named since Tyzzer (297) first described C. muris from the gastric glands of laboratory mice. However, the existence of different Cryptosporidium species/strains is still controversial.

Cross-transmission studies by Tzipori et al. (301) demonstrated that Cryptosporidium from calves is not host- specific. They suggested that the parasite is capable of inducing diarrhea in different species of vertebrates and that Cryptosporidium should be regarded as a single-species genus. On the other hand, Levine (168) considered as valid four species of Cryptosporidium, one for each of mammals, birds, reptiles, and fish. However, Upton and Current (313) reported that mammals can be infected by two different species of Cryptosporidium. They found a calf isolate of Cryptosporidium characterized by large oocysts (7.5 x 5.6

um) compared to the previously reported smaller oocysts (4 - 6 um). Based on the differences in oocyst morphology, they suggested that two species of Cryptosporidium (C. muris and C. parvum) can infect mammals.

A comparison of the SDS-PAGE and western blot patterns of different calf and human (mammalian) isolates and one chicken isolate (C. baileyi) was attempted. All these oocysts were morphologically similar.

In this study, the SDS-PAGE patterns of four different calf isolates were similar; as well, the protein patterns of two human isolates (one from an immunocompetent and one from an AIDS patient) were similar. When the protein patterns of calf, human, and chicken isolates were compared, using a 7.5% gel, the patterns were different. Surprisingly, when the human (immunocompetent child) oocysts were given to a calf, the resulting fecal oocysts exhibited the calf pattern, not the human one. The following explanations may be advanced to account for these changes: i) The differences may be due to adsorption of host proteins onto the oocysts either at the point of attachment to the intestinal epithelium, or on the enveloping membrane of the parasitophorous vacuole which is believed to be derived from the host (284). A similar mechanism has been proposed to explain antigenic differences between insect and culture-derived Trypanosoma cruzi metacyclic trypomastigote (278). ii) Another plausible hypothesis is that the host's intestinal environment may affect the protein make up of the parasite. iii) An

alternative hypothesis is that the child was infected with a heterogeneous population of Cryptosporidium species; upon passage into a calf, due to the selection, only the cattle - specific species of Cryptosporidium multiplied extensively.

The protein banding pattern of the chicken isolate was different from that of the human and calf isolates at both low and high molecular weights, using 12% and 7.5% gel respectively. We have not attempted to infect calves using chicken oocysts because of an inadequate supply of the latter. Lack of success in transmiting mammalian isolates to bird and vice versa was reported previously (174, 175, 232). However, Tzipori et al. (302) claimed that, when 1-day-old chickens were inoculated orally with the ileal homogenates of an infected calf, infection occurred in the small intestine, and oocysts were passed for a single day, 7 days after inoculation. Also, Lindsay et al. (175) reported that 1 and 7 - day - old chickens could be infected with C. parvum by intratracheal inoculation of oocysts, but not by oral inoculation.

A comparison of the protein pattern of two species of Cryptosporidium capable of infecting mammals (C. muris and C. parvum) (313) would be of value, but adequate amounts of both large and small oocysts are needed and were not available.

Western blot analysis did not exhibit any difference between human isolates and between calf isolates, even though one of the calves had been infected by oocysts from human feces.

Using a rabbit antisera raised against calf oocysts, a 200 Kd band was very reactive with calf oocysts, faintly reactive with human oocysts and absent with chicken oocysts. Serum from naturally infected calves recognized this 200 Kd band in calf oocysts. This recognition indicates that this antigen probably is not of host origin (tolerance).

The differences between calf and human isolates in 7.5% SDS-PAGE may be explained by differences in host environmental conditions, which may affect the metabolism of the parasite; in other words, environmental conditions may induce phenotypic variation. For example, certain cultural conditions were reported by Mirelman (213) to change the isoenzyme pattern of Entamoeba histolytica from a non-pathogenic pattern to a pathogenic one. The possibility of a mixed population of non-pathogenic and pathogenic zymodemes and selection by modifying the growth conditions was excluded in Mirelman study when a non-pathogenic strain, cloned from a single cell, changed to a pathogenic isoenzyme pattern, under certain cultural conditions. The possibility of involvement of mixed species or strains in Cryptosporidium infection, proposed previously, cannot be easily tested.

EITB revealed a band of 25 Kd in all human and calf isolates recognized by the serum of an experimentally infected calf. This band probably corresponds with the 23 Kd antigen recognized by patients' immune sera in Ungar and Nash study (310). As well, Mead et al. (204) reported that purified sporozoites had a 20 Kd antigen which reacted with immune

sera; they believe that the molecular weight differences between their findings and those of Ungar and Nash are probably due to the differences in the gel used, i.e. 10- 20 % in their report as opposed to 5-15 % in Ungar and Nash study. In our experiment, continuous 12 % gel was used and may be responsible for the slight discrepancy in the molecular weight of this band when compared with the other two studies. As suggested by Ungar and Nash (310) this common low - molecular weight antigen may be useful in the development of diagnostic tests for mammals. The present study showed that either calf or human oocysts could be used equally for this purpose.

When three isolates of Cryptosporidium (one from a naturally infected calf, one from an immunocompetent human, and one from a patient with AIDS) were compared by Current et al. (67) in suckling mice, no differences were detected in the developmental stages in terms of site of infection, time of appearance, duration, or morphology.

Mead et al. (203) compared chromosomal DNA from 5 isolates of C. parvum (three from calf, one from horse and one from human) and 1 of C. baileyi (chicken isolate), using field-inversion gel electrophoresis (FIGE). Their report indicated that the chromosomal DNA migration patterns of C. parvum isolates were indistinguishable, whereas differences were evident between C. baileyi and C. parvum.

All these studies indicate that mammalian isolates may belong to the same species / strain of Cryptosporidium while chicken isolates may belong to a separate species/strain.

Clearly it would be of value to compare isolates from different animal species, especially fish and reptiles. Such studies would be facilitated by the development of techniques for in vitro cultivation of Cryptosporidium.

Role of Humoral Immunity in Self Resolving Cryptosporidium Infection

Relatively little is known about the exact role of humoral or cellular immunity in coccidiosis (173). The role of B cells in determining susceptibility to infection with Cryptosporidium and its resolution was studied. Mice treated with rabbit anti- mouse IgM antibodies (anti- u) from birth exhibit marked inhibition of serum and gut immunoglobulin production resulting from the deletion of mature, surface Ig expressing B cells. Although chronic in vivo treatment with antibody to most isotypes (i.e. anti- γ , anti- ε or anti- α) yields animals which are selectively deficient in that isotype (24, 194), treatment with anti-u results in panspecific abrogation of mature B cells development (58, 115, 137, 163, 193).

In this study, no detectable Cryptosporidium specific antibody could be shown in anti-u treated mice. Anti-u treated mice retain most of their T cell and macrophage functions (115, 137, 196).

Anti- u treated mice were used to investigate the role of B cells and specific antibodies in the elimination of Cryptosporidium infection.

Results indicate that although a significant level of Cryptosporidium specific antibody was produced in normal and NRIg treated mice, neither the incubation period, the intensity nor the duration of shedding of fecal oocysts were dependent on the magnitude of the antibody response in these mice. Abrogation of Cryptosporidium specific antibody responses, brought about by anti-u treatment, had no detectable impact. No mortality was observed in control or B cell deficient mice. In contrast, using the same model Snider et al. (283) showed that B cells and antibody play a role in the development of an effective response to a primary infection with G. muris in mice. Similar to our findings with Cryptosporidium, anti-u treated BALB/c mice infected with influenza virus could recover from infection in the absence of detectable serum and nasal antibody; however, compared with control animals, recovery was delayed by a few days (158).

The possible role of B cells in immunity against sporozoan parasite such as Plasmodium spp. (causing malaria) is controversial, although the protective effects of antibody has been demonstrated. Egan et al. (81) believe that immune effector cells are required for solid protection. However, Chen et al. (54) reported that u - suppressed mice, which lack both B cells and circulating immunoglobulins, can be successfully immunized with sporozoites and they believe that effector T cells are sufficient for natural sporozoite immunity.

The lack of a demonstrable role for antibodies in the

control of cryptosporidial infection was further supported by our observation that inoculation of B cell deficient adult mice with a dose of oocysts ten times greater than that required to infect all neonates, repeatedly failed to infect adult mice. These results suggest that the induction of a humoral immune response is not relevant to the susceptibility of immunologically immature / immunocompromised mice nor to the resistance of healthy adults to infection.

It has recently been reported that murine neonates fed hyperimmune bovine colostrum over the course of cryptosporidial infections exhibited significantly less intense disease than did littermates fed normal bovine colostral whey (87). Similarly, administration of hyperimmune bovine colostrum to infected immunocompromised humans led to marked clinical improvement in their cryptosporidiosis (306). The apparent discrepancy with the findings described above is probabaly attributable to the very high titre of (bovine) antibody in the passively administered colostrum (level of 1: 10,000 to 1: 100,000 by IFA), a level vastly higher than that of the murine antibody synthesized in our studies (IFA titer of 1: 10 in most mice). This interpretation is supported by the recent finding (216) that female mice recovering from enteric cryptosporidiosis (some having been hyperimmunized) did not protect their suckling pups from experimental cryptoporidiosis, inspite of the demonstration of significant levels of passive IgG and IgA in the stomach of the neonatal mice. Thus, while passive antibody (at very high levels)

may prove to be a useful therapeutic tool for the control of enteric cryptosporidiosis in immunoincompetent patients, the role of specific in vivo antibody responses in the resolution of such diseases appears to be minor. Similarly, the likelihood of success for strategies to develop vaccines aimed at enhancing in vivo antibody production may be limited.

All the anti-Cryptosporidium responses measured in this study (and in most of the studies cited) were serum - derived and may not reflect the production of antibodies in the gut. However, antibody responses in serum and gut -lumen of anti-u treated mice are likely to be similar (i.e. virtually absent), as it was previously demonstrated that anti- u treated mice have a severe reduction in IgA producing cells in the gut (163) as well as greatly reduced levels of IgA (and IgM, IgG) antibodies in both serum and gut secretions (283).

Resistance of Adult Mice to Cryptosporidium Infection

Adult mice appear to be resistant to Cryptosporidium infection (12,120). In our study, BALB/c mice were susceptible to Cryptosporidium infection up to the age of 14 days. Heine et al. (119) reported that nude mice, which are deficient in certain subsets of T. cells, are relatively more resistant to cryptosporidiosis at 42 days of age than at 6 days of age. As well, CBA nude mice inoculated at 28 days of age were apparently not susceptible to Cryptosporidium infection (276). Recently Ungar et al. (308) managed to infect adult nude

mice, using gastric gavage to feed them with a large (i.e. $10x10^6$) dose of oocysts; the infection was fatal within 4 months. In our study, irradiated, B cell deficient, and CD4 cell deficient adult BALB/c mice inoculated with $1.5x10^6$ oocysts/mouse, could not be infected. Even Ungar et al. (308) using gastric gavage and an infecting dose of $10x10^6$ oocysts/mouse reported that anti-CD4-antibody treated adult mice were much more resistant to infection than neonates.

Humans exhibit no age immunity to Cryptosporidium (43), although, children appear to be more susceptible than adult (60, 64).

The mechanism of resistance of adult rodents to Cryptosporidium infection is not fully understood.

Since the development of resistance in adult laboratory mice coincides with the development of a mature intestinal flora (73, 268), the role of the latter in the resistance of adult mice to C. parvum was tested by Harp et al. (111). Germfree CD1 and BALB/c mice were colonized at day 7 post Cryptosporidium inoculation, but age matched conventional mice were comparatively resistant to colonization. Conventional mice treated with antibiotics remained resistant to colonization. This indicates that the intestinal microflora is not the sole determinant of resistance or susceptibility to colonization. Harp et al. believe that the increased susceptibility of adult germfree mice to C. parvum colonization may be due to: a) the presence of an intestinal flora competing for receptor sites; b) the production of

anticryptosporidial agents; c) stimulation of gut motility; all three factors may be involved in blocking colonization by the parasite. They suggested that activation of the immune system (e.g. nonspecific macrophage activation), by previous association with the intestinal flora, may contribute to the resistance of adult mice to colonization by C. parvum.

Involvement of intestinal mucus in preventing colonization of Cryptosporidium in adult mice is another possibility. It is believed that mucus contains factor(s) that inhibits attachment and colonization of Giardia lamblia at high concentrations of mucus proteins (1000 ug/ml) (329). As well, colonic mucin glycoproteins can act as an important host defense in amoebiasis by binding to the adherence lectin, thus preventing attachment to and cytolysis of host epithelial cells (51, 241).

T Cell Immunity in Cryptosporidium Infection

Prolonged and severe cryptosporidiosis has been reported in neonatal nude mice thus suggesting that T cells are required for recovery from Cryptosporidium infection (119). Similarly, measles, chickenpox and malnutrition (all known causes of decreased cell mediated immunity), have been implicated as predisposing factors for cryptosporidiosis (75, 189, 289). Of the many immunocompromised individuals reported with Cryptosporidium infection, the vast majority had AIDs (106, 186, 226, 243). The T helper lymphocyte depletion described in patients with AIDS may be the reason for the

severe cryptosporidiosis seen in these patients.

The present studies were undertaken to establish the requirement for the presence of $CD4^+$ T cell subset of cells or a full complement of T cells in inbred BALB/c mice to overcome successfully an infection with Cryptosporidium.

In Vitro T Cell Proliferation and Cytokine Production

Attempts to detect differences in the in vitro proliferative responses of T cells to cryptosporidial antigens in infected versus uninfected mice failed. This cannot rule out the role of T cells in Cryptosporidium immunity, since in this study only oocysts(intact and sonicated) were used to stimulate T cell proliferation in vitro. Other stages of Cryptosporidium were not used since they are difficult to obtain. Lillehoj (173) reported that lymphocytes from chicken infected with different Eimeria spp. showed a proliferative response to sporozoites, merozoites or to the Eimeria soluble antigen (Esa) excreted by cultured parasites; however, merozoites were highly immunogenic, compared to sporozoites. Strong in vitro T cell proliferation was observed when spleen or lymph node cells of oocyst- immunized mice were exposed to increasing concentrations of oocysts. This implies that oocysts are good immunogens in vitro, especially when immune cells were exposed to them previously. On the other hand, the immunological cross-reactivity of oocysts with other stages of the parasite (46) seemed to be sufficient to allow splenic T cells from Cryptosporidium infected mice to produce IFN- gamma

when exposed to sonicated oocysts *in vitro*. In contrast, no significant IL-2 or IL-4 production was observed. The possible role of IFN- gamma in *Cryptosporidium* immunity will be discussed later.

Delayed Type Hypersensitivity Response(DTH)

When infected BALB/c mice were tested during infection or following recovery, along with age matched uninfected mice, for a DTH response upon exposure to *Cryptosporidium* oocyst antigens, no such response was elicited. The lack of *in vivo* DTH response may or may not rule out the role of this subset of T cells, since mice were reexposed only to oocyst antigens (i.e. not to the other stages of *Cryptosporidium*).

$CD4^+$ T Cells Involvement in *Cryptosporidium* Immunity

Utilization of neonatal inbred mice deficient in $CD4^+$ T cells produced by continuous treatment of these mice with anti-CD4 mAb provide an animal model for studying the role of $CD4^+$ cells in *Cryptosporidium* infection of mice. In fact, these anti-CD4 treated mice showed no detectable $CD4^+$ T cells in spleen or lymph node cell populations but experienced a very severe diarrhea with several cycles of infection, as compared to normal and NRIg - treated mice. This may indicate that $CD4^+$ T cells are required during infection and may play a role in decreasing the intensity of infection perhaps due to the production of macrophage - activating factors (i.e. IFN-gamma) (222, 223). TH cells are major producers of IFN-gamma, although other cells of the immune system, notably NK cells,

are able to produce this lymphokine (221). In our study, the production of IFN-gamma was observed when T enriched spleen cells from infected mice were exposed to sonicated oocysts *in vitro*. According to Hughes (131), IFN- gamma is the predominant lymphokine that activates macrophages for enhanced secretion of oxygen-reactive intermediates and oxygen-dependent anti-microbial function.

Ungar et al. (308) reported that depletion of $CD4^+$ T cells in mice prevented the spontaneous clearing of *Cryptosporidium* organisms. However, their results show that oocysts shedding was gradually reduced by 92% after 7 weeks of infection in one experiment, in spite of continued anti-CD4 mAb treatment. In our experiments, CD4 depleted neonatal mice received 0.15×10^6 oocysts/mouse, (approximately 2 logs less than the 10×10^6 oocysts/mouse given by Ungar et al.), and cleared their infection, (which was a severe one), after 15 days, indicating that $CD4^+$ T cells are important but not essential for the resolution of *Cryptosporidium* infection, in this system.

$CD4^+$ T cells are considered as the mediators of resistance to *Eimeria*, an intestinal coccidian parasite related to *Cryptosporidium* (262). Heyworth et al. showed that helper/ inducer T lymphocytes are required for the clearance of *Giardia muris* (124), though anti- trophozoite IgG and secretory IgA antibodies, as well as mouse phagocytic cells (neutrophil and macrophage) were shown to interact, *in vitro*, to promote the clearance of *G. muris* (143).

Cryptosporidium Infection in Irradiated Mice

Irradiated (550 rad) BALB/c neonates were used to further investigate the role of T cells in clearance of *Cryptosporidium* infection. Spleen cells of these irradiated mice exhibited no functional B and T cells even at day 10 post irradiation, while the shedding of fecal oocysts, which started between day 4 to 5, decreased dramatically at day 10 post irradiation - inoculation and disappeared after a maximum of 14 days in all groups of irradiated and control mice. Surprisingly, the intensity of infection was not different from that of non - irradiated age - matched controls. Macrophages are not only radioresistant but are reported to be activated by irradiation; the possible involvement of activated macrophages will be discussed later on.

Role of Non-Specific Immunity in *Cryptosporidium* Infection

To evaluate the possible involvement of non- specific mechanisms in the clearance of *Cryptosporidium* infection, we studied the effect of natural killer (NK) cells in C3H/HeJ-Bg/Bg mice, which are genetically deficient in (NK) cell function. These mice exhibited no significant differences in peak intensity and time of clearance of *Cryptosporidium* infection as compared to normal controls. This may indicate that NK cells are not involved in the recovery from cryptosporidiosis.

The role of radio- resistant, non- specific effector mechanisms (i.e. phagocytic cells, such as macrophages) or

that of non-immunoglobulin products of immune cells (i.e. cytokines, particularly IFN- gamma), in clearing of Cryptosporidium infection, may be supported by the following:

1. Phagocytosis of Cryptosporidium by inflammatory cells was observed in the uterus, intestine, and respiratory tract (172, 188, 195, 199). Cryptosporidial antigen was also found in sub- epithelial tissues, apparently in macrophages, where the parasites seemed to be progressively degraded (161). Infection of the ileum was associated with stunting, blunting and fusion, as well as with infiltration of macrophages, other mononuclear cells, and eosinophils into the lamina propria (301). These data led Liebler et al. (172) to hypothesize that phagocytosis of the parasites is an important mechanism of clearance.

2. Heine et al. (119) have suggested that T cells are required for the recovery of nude mice from a neonatal Cryptosporidium infection. Nude mice also showed a reduction in antibody production to Eimeria falciformis (206); the persistence of Cryptosporidium infection in nude mice may be explained by the absence of other important components. Nude mice have no cytotoxic T cell response, T cell - dependent B cell or macrophage responses (323); and have a reduced capacity to produce the macrophage - activating factor needed to exert cytostasis and delayed footpad reaction (DFR); also nude mice exhibit a reduction of Sendai virus - infected alveolar macrophages (45). Therefore, in nude mice, the lack of clearance of Cryptosporidium infection may be due to

impairment of components of the immune system other than the T cells, and macrophages may be playing a crucial role.

3. Macrophage function is decreased in neonates of all species of animals, as suggested by a number of researchers (15, 26, 275, 295). As a result, neonates have an increased susceptibility to infections.

4. Severe life-threatenning Cryptosporidium infection was reported in patients with AIDS who cannot clear this parasitic infection. This may be attributed to deficiency of TH cells in these patients . However, in our study, mice deficient in this subset of cells could overcome their severe infection either few days later, or at the same time, as control mice.

There are several reports of functional defects of macrophages in HIV -infected subjects (27, 28, 79, 122, 214, 264, 288). For example, the failure of macrophage phagocytosis in AIDS patients was clearly shown (27, 28). Bender et al. (27) believe that this may contribute to their frequent infections with opportunistic pathogens and their inappropriate immune responses to these microorganisms. Whether macrophage defects are due to infection of mononuclear phagocytes with HIV or are secondary to other events, such as cytokines production, is not known yet. Deficiency of macrophages in AIDS patients may cause inadequate phagocytosis of the parasite and inability of the patients to clear Cryptosporidium infection.

5. The possible involvement of activated macrophages in the

resolution of Cryptosporidium infection of neonate mice and the resistance of adult mice may be supported by the recent findings of Ungar et al. (308). They reported that chronic cryptosporidial infections in adult nude mice were resolved soon after the transfer of spleen cells from normal BALB /c mice that had previously recovered from Cryptosporidium infection.

6. The possibility of involvment of activated macrophages in Cryptosporidium infection clearance may further be supported by the fact that irradiated mice which were depleted of radiosensitive cells (lymphocytes) could clear their infection. Similar doses of irradiation have been demonstrated to cause activation of macrophages in the complete absence of any detectable T cell function (260). The precise mechanism of macrophage activation in these mice is not clear at the present.

The role of gamma-IFN in Cryptosporidium infection may be a direct one as it was suggested by Wisseman et al. for Rickettsia prowazekii (327). They reported that interferon like factors from antigen and mitogen-stimulated leukocytes have an antirickettsial and cytolytic effects on infected endothelial cells, fibroblasts and macrophages.

CONCLUSION

Experimental infection of calves was necessary for the production of fecal oocysts in amounts adequate for all the immunological studies intended.

Results indicate that calves can be experimentally infected by the oral route. The infecting dose is unrelated to the pattern of oocyst shedding and to the level of antibodies produced. Antibodies were detected prior to infection, possibly transferred through the colostrum given to all calves at birth, and therefore, they appear to be not protective.

Procedures had to be developed for the concentration and purification of fecal oocysts and sporozoites to be used in ELISA, SDS-PAGE, WB and cell culture assays. Oocysts purified by CsCl gradient were found to be suitable for immunological studies.

Using SDS-PAGE and western blotting, it appears that mammalian isolates belong to the same species / strain of Cryptosporidium while the chicken isolate appears to be a separate species. The reactive protein band of 25 Kd detected in human and calf oocysts, can be used as antigen for serodiagnosis and serosurveys.

In our studies of the immunological bases of resistance to, and recovery from Cryptosporidium infection, no significant differences in the frequency, intensity or duration of infection were detected in B cell deficient neonatal BALB/c mice, compared to control groups. Therefore, B

cells and specific humoral immunity appear to play a very minor role in the protection of mice against Cryptosporidium infection. This finding suggests that the development of vaccines to stimulate antibody production may be of limited value.

Studies of the role of T cell immunity in normal and CD4 deficient BALB/c mice demonstrated that they can clear the Cryptosporidium infection in a similar period of time. However, CD4 deficient mice exhibited a markedly more severe diarrhea, significantly more fecal oocyst shedding and, in contrast to age-matched normal mice, went through several cycles of severe diarrhea before ultimately resolving the infection. A lack of IFN-gamma production by CD4 deficient mice is suggested, as we were able to demonstrate that Cryptosporidium infected (but not the non-infected) normal BALB/c mice produce IFN-gamma, in response to in vitro restimulation with oocysts. The absence of IFN-gamma needed for the activation of radioresistant macrophages, may explain the severe cryptosporidiosis reported in CD4 cell deficient murine, and perhaps human hosts.

Compared to normal unirradiated mice, irradiation of neonatal BALB/c mice, at doses sufficient to totally abrogate mitogen stimulated T and B lymphocytes responses, did not significantly affect the disease intensity or duration. This finding may be explained by the non-specific activation of macrophages previously observed in vivo, following lethal irradiation.

Attempts to show involvement of non-specific cytotoxic effector cells, such as natural killer cells, failed to prove a role for NK cells in the recovery from Cryptosporidium infection.

All normal, anti-u treated, anti-CD4 treated and irradiated adult BALB/c mice remained resistant to attempts at infecting them, even when 10 fold higher doses of oocysts than those infective for neonates were used. It appears that in mice, resistance to Cryptosporidium is related to age. Macrophages are reported to be deficient in neonates, which may help explain the susceptibility of these young mice to infection.

Although, the precise mechanism(s) involved in recovery from Cryptosporidium infection remained to be resolved, the apparent involvement of macrophages and T cell derived IFN-gamma open unexpected avenues, requiring further investigation, to explain the mechanism(s) of clearance of infection, as well as their potential use in the control of life-threatening Cryptosporidium infections, in the young and in the immunodeficient hosts.

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