PROTECTIVE IMMUNITY IN *CRYPTOSPORIDIUM* INFECTION: ROLE OF $CD4^+$ T LYMPHOCYTES AND IFN-GAMMA IN MURINE CRYPTOSPORIDIOSIS

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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TO

MY WONDERFUL PARENTS,

SISTER

AND

MY DEAR UNCLE

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Abbreviations

AIDS	acquired immune deficiency syndrome
anti-u	rabbit anti-mouse IgM antibody
BSA	bovine serum albumin
C	centigrade
CFA	Complete Freund's adjuvant
CMI	cell mediated immunity
Con A	concanavalin A
CO_2	carbon dioxide
$CsCl_2$	cesium chloride
dH_2O	distilled water
dpm	disintegrations per minute
DTAF	Dichlorotriazinyl Amino Fluorescein
EDTA	disodium ethylene diamine tetra-acetate
ELISA	enzyme linked immunosorbent assay
FACS	florescence activated cell sorter
FCS	fetal calf serum
Fig	figure
FITC	florescein isothiocyanate
g	gram
xg	gravitational force
>	greater than
hr	hour
HIV	human immunodeficiency virus
IEL	intra-epithelial leukocyte
IFA	indirect immunofluorescent antibody test

IFN-gamma	gamma interferon
Ig	immunoglobulin
IgA	immunoglobulin A
IgE	immunoglobulin E
IgM	immunoglobulin M
IgG	immunoglobulin G
IL	interleukin
i.p.	intra-peritoneal
i.v.	intra-vascular
LK	lymphokine
<	less than
log	logarithm
LPS	lipopolysaccharide
mAb	monoclonal antibody
M cells	microfold cells
uCi	microcurie
ug	microgram
min	minute
ml	millimeter
um	micrometer
MHC	major histocompatibility complex
MLN	mesentric lymph node
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
NK	natural killer
NRIg	normal rat immunoglobulin

nm	nanometer
PBS	phosphate buffer saline
PSF	penicillin, streptomycin and fungizone
p.i.	post-infection
p.ir.	post-irradiation
rad	radiation
/	per
+	positive
r-IL	recombinant interleukin
rpm	rotation per minute
S.D	standard deviation
SEM	standard error of the mean
sec	second
S.I	stimulation index
sIg	surface immunoglobulin
scid	severe combine immune deficiency
TE	Tris-EDTA
^{3}H-thymidine	tritiated thymidine
U	unit
vs	versus
wk	week

ABSTRACT

Cryptosporidium is a protozoan parasite that can cause an intestinal infection with symptoms that range from moderate, self-limiting diarrhea in normal adults/children to potentially life threatening, cholera-like illness in the very young, malnourished or immunocompromised. The frequency with which Cryptosporidium infection is reported has grown substantially over the last decade. Host immune responses are poorly understood, an impediment to development of effective therapy.

This study has focused on the role of specific T cell mediated immunity in cryptosporidiosis. Cryptosporidium driven cytokine production (IFN-gamma in particular) elicited in response to infection was investigated. Neonatal mice were fed with Cryptosporidium oocysts in order to obtain an experimental animal model of the disease cryptosporidiosis. For the study of cellular immune respnses to Cryptosporidium, normal, NR-Ig treated, anti-CD4 mAb treated and irradiated BALB/c mice were used. We established a short term in vitro culture system in which Cryptosporidium driven cytokine production (IFN-gamma, IL-2, IL-4) by lymphocytes from spleen and mesenteric lymph nodes (MLN) was evaluated by cytokine specific bioassays.

Our results have revealed that in vivo CD4 T cell depletion causes more intense and protracted infection in neonates but fails to alter the resistance of adults to

infection. In vitro restimulation with purified oocysts, elicit marked IFN-gamma but not IL-2 or IL-4 responses coincident with peak neonatal infection. In vitro culture of spleen or MLN lymphocytes of Cryptosporidium fed but not unfed control adults elicits pronounced IFN-gamma production. This suggested a significant role for IFN-gamma synthesis in protection from and resolution of cryptosporidiosis (in normal hosts). We hypothesized that CD4 T cell deficient mice did not produce IFN-gamma. The absence of IFN-gamma needed for activation of macrophages would explain the severe cryptosporidiosis seen in CD4 T cell deficient neonates.

Suprisingly, irradiated (550 rads), unresistant neonates resolve cryptosporidiosis with the same kinetics as do CD4 or CD8 T cell reconstituted irradiated recepients or normal non-irradiated controls. Collectively, these results suggest that CD4 T cell IFN-gamma production is an important but not necessary effector mechanism in the resolution of experimental cryptosporidiosis.

From these data, we conclude that two immunological processes may be responsible for recovery from and resolution of cryptosporidiosis in the model studied: A)Activation of Cryptosporidium specific CD4 positive T lymphocytes to synthesize IFN-gamma. B)Activation of phagocytic cells in the small intestine by IFN-gamma. IFN-gamma activates the gut macrophages, resulting in enhanced phagocytic capacity, elimination of Cryptosporidium and resolution of infection.

Adult mice (normal and CD4 deficient) were found to be not susceptible to high doses of Cryptosporidium (3 x 10^6 oocysts/mouse). This may reflect the fact that these mice have a mature immune system which is capable of responding immediately to the parasite challenge. The capacity of neonatal and adult BALB/c mice to generate IFN-gamma responses were characterized in this study. We observe that with increase in age these mice acquire the capacity to produce IFN-gamma and there is loss of susceptibility to Cryptosporidium infection. This suggested an alternative explanation for the resistance of adults to cryptosporidiosis.

INTRODUCTION

INTRODUCTION

Organisms of the genus Cryptosporidium are small (4-6u), widely distributed coccidian parasites which invade and replicate within the epithelial cells lining the respiratory or gastrointestinal organs of vertebrates. Cryptosporidium is now recognized as an important cause of diarrhea in animals and as a common enteric parasite in humans, representing one of the most important enteropathogens causing diarrheal disease worldwide (1, 2, 3, 4, 5, 6, 7).

Discovered in 1907, Cryptosporidium predates AIDS by many decades. Cryptosporidium infection is now recognized as one of the two major causes of death due to parasitic infection in AIDS patients, the other being Toxoplasma gondii. Long considered to be a widespread animal pathogen, Cryptosporidium is now of particular relevance as a pathogen in both immunocompetent and immunodeficient human populations. In immunocompetent individuals, Cryptosporidium can cause a short term, flu-like, self-limiting gastrointestinal disease. However, in immunocompromised patients (especially HIV-positive individuals) cryptosporidiosis is often prolonged, life-threatening and untreatable; the patients exhibiting severe diarrhea, dehydration and ultimately death (6, 8, 9, 10).

The effector mechanisms by which this parasite is eliminated in vivo are unknown. Extensive research towards the developement of therapeutic agents for cryptosporidial

infections has failed and attention is now turning to immunological approaches. We have made an attempt to understand the host responses which control the proliferation of Cryptosporidium in vivo. We propose to determine the immunological mechanisms by which Cryptosporidium infection is resolved in experimental animal models of normal healthy individuals and immunocompromised patients. Using a combination of traditional techniques of monitoring disease progression in intact animals and current techniques in cellular biology, we hope to improve our understanding of the disease cryptosporidiosis.

HISTORY

J.J. Clarke in 1895 may have been the first to observe a species of Cryptosporidium which he described as "swarm of spores" lying upon the gastric epithelium of mice (1). In retrospect these minute organisms were probably the motile merozoites of Cryptosporidium muris described by Tyzzer in 1907 as a protozoan parasite infecting the gastric epithelium of asymtomatic laboratory mice. This protozoan parasite was placed in a new genus called Cryptosporidium or "hidden sporocysts". Unlike the oocysts of previously known coccidia, the oocysts of this parasite did not have sporocysts surrounding the sporozoites.

In 1910, Tyzzer described many of the life cycle stages of Cryptosporidium muris and in 1912 he described the

morphology and life cycle of a second species, Cryptosporidium parvum found in the small intestine of laboratory mice (11). Using a microscope with limited resolution, Tyzzer saw stages of asexual development, sexual development and sporogony and he also noted that Cryptosporidium oocysts left the body via the feces.

During the ensuing 70 years, approximately 19 additional species of Cryptosporidium were named from a variety of vertebrate hosts. Morbidity and mortality in avian cryptosporidiosis was described by Slavin in 1955 in turkeys (12). Interest in Cryptosporidium species by the veterinary medical profession has increased significantly since 1971 when Panciera and his colleagues first reported its association with bovine diarrhea (13). The clinical syndrome was chronic diarrhea and debilitation, histological lessions of villous atrophy and Cryptosporidium seen in the striated border of epithelial cells. Between 1968 and 1981, species of Cryptosporidium in fish, reptiles, birds and mammals were named on the assumption that each host harboured a seperate species of Cryptosporidium (5).

The first recognized case in human being was reported in 1976 by Nime et al, when Cryptosporidium were seen on a rectal biopsy of a three year old child with enterocolitis (14). A review of three cases between 1976-1979 focused attention on opportunistic cryptosporidial infections in immunocompromised patients (15). Each patient had fever with chronic watery

diarrhea ranging from 10 days-3 years duration. In every case, histological examination of jejunal biopsy specimens showed the presence of Cryptosporidium either as the sole suspected pathogen or in association with Giardia lamblia.

Prior to the description of AIDS in 1981, there were only 7 case reports of human cryptosporidiosis in the literature (16). However, over the last 7 years, the number of cases has increased by several orders of magnitude (6). In Scotland, it ranks third in adults after Campylobacter and Salmonella infections and is the most common cause of gastrointestinal disease in children after viruses (17). In Canada, the exact prevalence of Cryptosporidium infection is not known as it is not a reportable disease, except in Alberta. In Manitoba, the Cryptosporidium infection rate is 25/100,000 for children under five years of age and about 7 times higher in northern communities than those in southern Manitoba (18).

Between 1980 and 1983, more than 80 cases of cryptosporidial gastroenteritis in humans were reported either as a self-limiting gastroenteritis in normal patients (14) or as the severe symptoms produced in immunocompromised patients, especially those with AIDS (19). Over the last 6-7 years, Cryptosporidium infection has been documented to cause a persistant cholera-like illness in infants (20), and immunocompromised adults in particular those suffering from AIDS (21).

Although, recent numerical data are not available on the

prevalance of Cryptosporidium infection in Canadian patients with AIDS (due to its non-reportable nature), its importance can be seen from the observation that 19% of AIDS death in the United Kingdom in 1988 were due to Cryptosporidium infection (22). There is also much data available on prevalence and incidence in both the United States and Africa.

TAXONOMY

1) Classification

Cryptosporidium is a protozoan parasite assigned to phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, suborder Eimeriorina and family Cryptosporidiidae (23). Membership in phylum Apicomplexa is determined by the possession of an apical complex located at the anterior end of the parasite, this assists the parasite in host-cell penetration (24). Organisms in class Sporozoasida exhibit locomotion. In subclass Coccidiasina, the life cycle includes merogony (asexual multiplication), gametogony (gamete formation) and sporogony (sporozoite formation).

In order Eucoccidiorida, merogony is present in vertebrate hosts. Independent development of male and female gametes occur in sub-order Eimeriorina. The family Cryptosporidiidae is characterized by parasites with one host in their life cycle, development just under the surface membrane of the host cell in a parasitophorous vacuole, a form of replication termed "intracellular-extracytoplasmic",

microgametes without flagella and oocysts with 4 sporozoites but no sporocysts (25). Oocysts exhibit marked variability in the thickness and resistance of the cell wall, an observation of considerable importance to the normal life cycle . Although most oocysts have a thick, highly impermeable cell wall (i.e. resistant to 25% bleach), a portion have thin walls that are easily lysed within the gut, a phenomenon that may contribute to the unique potential of this organism for autoinfection.

2) Number of Species and Hosts

Morphological criteria alone may not be helpful in speciating Cryptosporidium. Antigenic and chromosomal analysis suggest a minimum of two species. Data are inadequate to futher speciate the organism.

About 20 species of cryptosporidiae were named based upon the host in which they were discovered (5). The hosts include human, monkeys, rodents, carnivores, rabbits, horses, cattle, sheep, goats, pigs and deer. Other hosts include fish, reptiles, chickens, turkeys, parrots, ducks and geese. Cross-tranmission studies proved the absence of host specificity for many species (5, 26). Investigators are now divided between the belief that Cryptosporidium is one species (26) or there are 4 valid species, one for each vertebrate class: Cryptosporidium muris in mammals, Cryptosporidium meleagridris in birds, Cryptosporidium crotali in reptiles and Cryptosporidium nasorum in fish (5). The former is more prevalent. The species causing disease in man is considered to

be Cryptosporidium parvum (27).

Upton and Current (28) have suggested that two different species infect mammals, Cryptosporidium muris the less common form with large oocysts, and Cryptosporidium parvum with smaller oocysts responsible for most mammalian cryptosporidiosis. There may be two valid species infecting birds, Cryptosporidium meleagridis and Cryptosporidium baileyi (7). According to Upton et al, statistical evaluation of oocysts structure suggests that multiple species of Cryptosporidium may exist among the reptiles examined. There is need for clear documentation regarding how many or which species infect fish, reptiles, birds and mammals.

LIFE CYCLE

Several investigators have observed the different developmental stages of the coccidian parasite Cryptosporidium (4, 5, 16, 23, 29, 30). The life cycle of Cryptosporidium involves three main stages: merogony, gametogony and sporogony.

Sporulation: The sporozoites excyst from the oocysts through an opening in the oocyst wall opposite the oocysts residiuum (feeder organelle), caused by the dissolution of a single sutural junction and partial inward collapse of the oocyst wall. Living sporozoites are comma shaped, with a rounded posterior end that tapers to a pointed anterior end.

It has a compact nucleus located in the posterior third of the parasite. It exhibits gliding motility usually in the direction of the curvature, often accompanied by a flexion into a sigmoid configuration. Sporozoites probe the brush border of the enterocytes (of intestine) by extension and contraction of the anterior end. Short, rapid forward and backward gliding movements occurs during the initial period of host cell contact. These movements result in the indentation of the microvillous region.

Sporozoites differentiate into trophozoites, 1.5-6.0 mm in diameter. Trophozoites are uninucleated with 6 or 8 merozoites. Each trophozoite has a single prominent nucleus which has a large nucleolus. It derives nutrition from the host cell or gut contents. Trophozoites are spherical to subspherical and contain an eccentrically located nucleus within a cytoplasm that is more homogenous than that of the host cell.

As the sporozoite rounds up to form the trophozoite, its nucleus migrates posteriorly and a vacuole appears at the anterior end called as the "Parasitophorous Vacuole". All other developmental stages occur within this resultant vacuole.

Merogony: Asexual multiplication results from the asynchronous nuclear division of trophozoites to form two types of meronts (or schizonts, therefore the process is also

called schizogony). Type 1 meronts contain 6-8 nuclei. On maturation these nuclei are incorporated into 6-8 merozoites (type 1 merozoites). Type 1 merozoites are banana shaped with a slight curvature. It has a spherical to subspherical nucleus located in the central third of the parasite. Its nucleus is anucleolar and is surrounded by a dialated envolope. It contains prominent dense granules. Type 1 merozoites display gliding and flexing movements and probe the host cells with their anterior ends which results in the attachment of the parasite to the microvillous border. These type 1 merozoites bud from a distinctive region known as the "attachement zone" or "feeder organelle", attached to the basillar region of the parasitophorous vacoule.

Type 2 meronts; each merozoite after leaving the type 1 meront may invade a new host cell by membrane invagination where it develops into type 1 meront (recycle) or type 2 meront. On maturation, type 2 meronts contain 4 merozoites. Mature, spherical type 2 meronts contain 4 free merozoites and a residiuum attached to the basillar region of a parasitophorous vacuole. Type 2 merozoites are frequently arranged in parallel within this parasitophorous vacuole. Type 2 merozoites are banana shaped, blunt, slightly curved with a spherical nucleus near the center of the cell. Type 2 merozoites freed by rupture of the vacuole display flexing and gliding movements and penetrate into the microvillous border of enterocytes adjacent to the meront from which they emerge.

Sporozoites are distinguished from merozoites in that the sporozoites contain amylopectin granules near the center of the cell and a nucleus near the posterior end. The nucleus of type 1 and 2 merozoites is always near the middle of the cell.

Gametogony: Merozoites from type 2 meronts invade new host cells where they initiate sexual multiplication by differentiating into microgamonts and macrogamonts.

Mature microgamonts contain 16 microgametes, some attached firmly to the residuum and some free within the vacuole. Microgametes are bullet shaped, sperm like, non flagellated, have a slightly expanded anterior end and a nucleus that occupies most of the center of the cell. Few free microgametes display jerky forward gliding movements. Microgametes also contain mitochondria and a polar ring of microtubules surrounding the compact, fusiform nucleus. Numerous granules of the size and staining characteristics of glycogen particles are present in the cytoplasm of microgametes.

Macrogamonts contain a single macrogamete which has numerous small refractile granules and 1-2 prominent refractile globules scattered in the cytoplasm. Macrogametes show the presence of wall forming bodies near the periphery.

Fertilization of the microgamete and macrogamete results in the formation of a zygote (4.5-6.0 um in diameter).

Sporogony: The fertilized macrogamont develops into an oocyst which undergoes sporulation to produce 4 potentially infective sporozoites. The zygote which is formed may follow several sporogenous developmental routes:

a) The zygote may transform into an oocyst by secretion of a thick wall and the development of sporozoites. The oocyst is attached to the host cell, it then detaches and passes out of the gut.

b) The zygote may secrete a thick wall and the resultant oocyst may become detached from the host cell before the development of sporozoites.

c) The zygote may develop into a thin-walled oocyst containing sporozoites. It has been suggested that these thin-walled oocysts may be responsible for autoinfection.

The cytoplasm of an unsporulated oocyst contains a single nucleus, numerous amylopectin granules, lipid bodies and aggregates of ribosomes. The oocyst wall varies in thickness (180-600 nm), the inner wall limited on either side by a unit membrane that ranges in thickness from 210-255 nm. Within the inner oocyst wall and opposite the region of amylopectin accumulation is a suture-3 um in length, which is the site of oocyst wall collapse during excystation.

Most of the thick-walled oocysts are released from the host enterocytes and pass through the gut unaltered and are found in the feces.

Sporulation of the thin-walled oocysts occurs rapidly

after it is released from the vacuole of enterocytes. The naked infectious sporozoites released from the thin-walled oocysts penetrate into the microvillous border of enterocytes (autoinfection). This phenomenon of autoinfection is likely to be highly relevant to the pathogenesis of persistant infection in immunocompromised hosts. Thin-walled oocysts are not normally detected in the feces. These thin-walled oocysts also contain 4 sporozoites and granular residuum. Some thick-walled oocysts may also undergo excystation and release sporozoites but the majority pass through the gut. The net contribution of oocysts versus merozoites in autoinfection remains unknown.

Epidemiological Considerations

Cryptosporidium appears to be transmitted by a variety of mechanisms. Zoonotic tranmission was initially considered to be the principle source of human infection, because, a) this organism is commonly associated with diarrhea in animals, particularly calves (31). b) Interspecies transmission to lambs, pigs, and birds has been documented (32). c) The first and second documented human cases had farm animal exposure (14, 15). d) Symptomatic calf handlers and their families in Bangladesh and U.S.A. (33) were found to have high rates of excretion of Cryptosporidium species. This was due to fecal-oral contamination with oocysts. Recent evidence has highlighted Cryptosporidium infections without evidence of zoonotic transmission (20). Casemore and Jackson supported the

proposal that cryptosporidiosis need not be viewed as a zoonosis. (34).

Person-to-person transmission is likely (1). This may occur by fecal-oral spread related to sexual practices (35, 36) or hand-to-mouth spread as seen with children in day care centers (4, 20, 37). The occurence of infections in household contacts of infected patients (26, 38, 39), healthy homosexual men (40), infections in health care personnel (18, 39), and nosocomial infections among hospitalized patients (18, 23) indicate that Cryptosporidium is highly infectious and transmissible from person to person.

Asymptomatic carriers of Cryptosporidium can possibly act as important reservoirs (16).

Contaminated water has been suggested as a vehicle of infection with Cryptosporidium species in travellers to Leningrad (41) and the Carribean (42). Cryptosporidium oocysts were found in secondary sewage effluent in Arizona (43). D'Antonio et al (44) reported a recent water borne outbreak of gastroenteritis in San Antonio, Texas. Cryptosporidiosis has been described in travellers (41), particularly those returning from developing countries (18). Richardson et al (45) investigated an outbreak of waterborne cryptosporidiosis in Swindon, Oxfordshire concluding that cryptosporidiosis is a risk in conventionally treated public water supplies.

Another potential source of transmission is the respiratory tract. The rate of tracheal infection among

immunologically normal humans, which may contribute to aerosol spread is unknown. So far there has been one report of a laryngo-tracheal infection in a child (46). Air transmission may explain the colonization of the respiratory tract in animals with Cryptosporidium species and presence of organisms in the respiratory epithelium and in the sputum of patients with AIDS (47, 48).

Transmission by fomites was suggested in 1984 Philadelphia day-care center diarrhea outbreak (20).

Investigators have considered the possibility that house flies (Musca domestica) might play a role in Cryptosporidium transmission, based on the association of flies with feces in areas of poor sanitation (49).

Laboratory Diagnosis

From 1976-1981, the diagnosis of cryptosporidiosis was based primarily on the electron microscopic examination of intestinal or rectal biopsies (50). Drawbacks to diagnosis by biopsy include invasiveness, restricted sampling which may miss an affected area, necessity for immediate careful specimen processing to avoid autolysis or removal of organisms from the surface membrane.

Non-invasive diagnosis was first reported in 1978 for calves (51) and in 1980 for humans (52) when oocysts were detected in fecal smears stained with Giemsa stain. Subsequently, numerous techniques to concentrate stool

specimens and to stain oocysts have been applied to the detection of Cryptosporidium species, but there is little consensus on which methods are most satisfactory.

Staining Techniques.

A large number of staining techniques have been used to detect Cryptosporidium oocysts. The most widely used have been the modified acid-fast procedures, which differentiate red-stained oocysts from similarly sized and shaped green stained yeast forms (16). A comparison of 13 currently used staining techniques found the modified Ziehl-Neelsen hot acid-fast stain to be the best overall for sensitivity and morphology when used in conjunction with a 10% potassium hydroxide initial digestion (53). A dimethyl sulfoxide-carbolfuchsin stain (54) and the modified Kinyoun acid-fast stain (42) eliminate the need for heat or steam and show similar staining and sensitivity. Auramine-phenol can be used as an alternative to Ziehl-Neelsen stain, it is found to be more sensitive (55). Yeasts, which are often confused with Cryptosporidium oocysts because of their size and morphology, stain green with acid-fast procedures and brown with iodine, whereas oocysts do not stain with iodine.

A Giemsa stain may show clear oocysts with pale blue internal structures but does not differentiate yeasts from oocysts (53). The safranin-methylene blue stain reported to be both sensitive and rapid (56) may be taxing when large numbers

of samples are screened. Negative stains, such as methenamine silver, modified periodic acid-schiff and iodine may be less reliable than the others listed (53).

Flourescent stains also have been used to detect Cryptosporidium. These include acridine orange, which causes fluorescence in both oocysts and yeast forms (57), auramine-rhodamine, which stains only oocysts but not always uniformly (23), and auramine-carbol fuchsin which also distinguishes between yeasts and Cryptosporidium species (58) and which was significantly better than modified acid-fast staining in one report (59).

Monoclonal antibody staining techniques are also widely used (29, 60, 61). With the help of monoclonal antibodies it would be possible to use ELISA and other immunologically based tests. Familiarity with two staining techniques and use of control slides are essential to assure diagnostic accuracy.

Concentration and Purification Techniques.

Concentration of stool specimens is important in epidemiologic surveys, in evaluation of household or other contacts of infected individuals and in non-acute illness with small number of oocysts. The Sheather's sucrose flotation technique (21) is found to be equal to (62) or better than (57) the Formalin-ethyl acetate technique for oocysts concentration. Sheather's sugar flotation employs a dense sugar solution to leviate the oocysts. Oocysts rise to the top

of the solution because the solutions specific gravity (1.27) is greater than the specific gravity of the oocysts. A risk of aerosolization or leakage of specimen is involved in this procedure. A modification of the traditional Sheather's sugar flotation is recommended by using a screw-cap centrifuge tube (21).

The three step process for detection of Cryptosporidium oocysts recommended by Ma and Soave (57) include: 1) Iodine wet mount of fecal material, which will leave oocysts unstained while yeasts stain brown 2) A modified Kinyoun acid-fast stain to be used as the permanent record, and 3) Concentration with Sheather's sugar coverslip flotation.

A modification of Formalin ethyl acetate concentration, using a disposable parasite concentrator was used by Zierdt in 1984 (63). This procedure offers the advantage of shorter processing time and less chance of direct contact with fecal specimens.

Purification of Cryptosporidium oocysts is of importance for biochemical and immunological studies. Decontamination procedures such as use of 60% ethanol or antibiotics were found to be adequate for pathogenesis studies in animals. More elaborate purification procedures are required for propagation in cell culture and chick embryo preparations. Seperation of oocysts from fecal material by formalin-ether sedimentation, followed by discontinous density gradient centrifugation was used by Waldman et al (64) to obtain purified oocysts for use

in immunological studies, as well as for propagation in cell culture and chick embryo preparations. Sterling and Arrowood in 1986 described a purification procedure that included flotation followed by passage of oocysts through Whatman (F-11 and DE 52) cellulose columns, providing a yield of 5 x 10^6 intact oocysts (65). They also suggested isolation of oocysts and sporozoites by discontinous sucrose and isopycnic percoll gradients in 1987 (66). Heyman et al developed a method in which oocysts were separated by sucrose density gradient centrifugation and then passaged through glass bead columns. These purified oocysts were antigenically active and of use in immunological studies (67).

Casemore obtained purified oocysts suitable for IFT (Indirect immunofluorescence test) (60). His purification method involved preliminary cleaning and concentration of specimens by the modified formol-ether method but with omission of formalin, followed by use of percoll density gradient medium.

Ungar et al (68) obtained purified oocysts by using distilled water saturated with sodium chloride and several washes with water, followed by treatment of pellet containing oocysts with 1% sodium hypochlorite solution. By using antigen from disrupted oocysts, they were able to demonstrate IgG and IgM antibodies in diagnostic and epidemiological studies.

Clinical Features

Cryptosporidiosis in humans is perceived as two distinct disease entities; a short and self-limited diarrheal illness in immunocompetent humans, and a persistent, often life-threatening, diarrhea in immunodeficient patients. Cryptosporidium has been recognized as a frequent cause of illness in immunocompetent individuals, including children in day-care (20), travellers (18, 69), health care workers (18, 39), household contacts of infected individuals (18, 39), animal handlers (21), laboratory personnel (70), hospitalized patients who acquire nosocomial infections (18, 23), children in tropical and third world countries (69), and male homosexuals even without HIV infection (39).

Symptoms in Immunocompetent Patients:

Over 600 cases of cryptosporidiosis in immunocompetent hosts have been described, the majority of cases are identified in children (71). The incubation period has been reported to range from 1 day to 2 weeks (33). Watery diarrhea is reported in 67 to 100% of infected contacts. Diarrhea is often characterised by 2 to 10 watery stools a day, often beginning on the first or second day of illness. Abdominal pain, cramping and vomiting are also seen. Anorexia, malaise and weight loss may also be observed. Bloating and gas are less common symptoms than with Giardia lamblia infection, and 10-70% of patients experience a low grade fever (69, 71). Symptoms last for 1 to 2 weeks on the average (69) but may continue for more than 1 to 2 months (71). In all reported

cases to date in immunocompetent hosts, the symptoms and infections ultimately resolved spontaneously.

Symptoms in Immunocompromised Hosts:

In contrast to the short term, flu-like gastrointestinal illness in immunocompetent patients, Cryptosporidium may cause severe protracted diarrhea in immunodeficient patients. T-lymphocyte dysfunction in humans, resulting from infection with HIV virus, is the single most important disease syndrome predisposing to chronic diarrhea due to cryptosporidiosis (72). Most of the immunocompromised patients in 71 published records had AIDS (6, 8, 9, 10, 40), but others had hypogammaglobulinaemia (21, 73), or were receiving immunosuppressive treatment (36, 74). Watery diarrhea of many months duration with anorexia, abdominal pain and weight loss is common (40, 75). Vomiting, weakness, malaise and low-grade fever are often noted. Malabsorption (75) and stool volumes of up to 20 liters/day are well documented as are fatalities caused by dehydration and cachexia. Cough is occasionally seen in association with Cryptosporidium infection and the organism has been identified in patients with progressive pulmonary disease (48). A report of asymptomatic carriage of Cryptosporidium in one adult patient with AIDS (76) and spontaneous resolution of infection in one child (77), suggests, that in exceptional circumstances, the infection can be tolerated, either with or without drug therapy. In individuals with severe combined immune deficiency;

disseminated fatal cryptosporidiosis, affecting intestinal and pulmonary systems has been reported (78).

Treatment

The primary therapeutic intervention available for humans with cryptosporidiosis is supportive care with oral or intravenous hydration (5). Development of effective treatment is limited by lack of simple in vitro cultivation systems to study biochemical and metabolic requirements of Cryptosporidium species and by the lack of a good small animal model of clinical disease for screening efficacy of drug compounds.

The efficacy of 15 anticoccidial prophylactic regimens was tested in suckling mice by dosing before and after infection (79). Even at high concentrations, none prevented infection, although amprolium, arprinocin, dinitolmide, salinomycin and sulfaquinoxaline reduced oocyst excretion.

In calves, 9 drugs were tested and only lasalocid was found to be effective, but at a toxic dosage (80). However, Kimata et al (81) indicated that both azithromycin and lasalocid have prophylactic or therapeutic activity against Cryptosporidium in prednisolone-immunosuppressed mice at non-toxic doses.

Tzipori et al (82) tried 16 antimicrobial agents in mice without any success. Almost 100 different drugs have been tried out but none of them have played an important role in

improving the condition of Cryptosporidium infected patients consistently (4, 5). The macrolide antibiotic Spiramycin, which is similar to erythromycin and clindamycin appears promising in AIDS patients (83, 84). Treatment with a-difluoromethly-ornithine, an inhibitor of polyamine synthesis, with activity against a variety of protozoans has been associated with clinical improvement (85). Toxicity to the bone marrow and gastrointestinal tract are, however, significant impediments to the use of this drug. Eflornithine may induce complete responses or palliation of diarrhea in some AIDS patients with intestinal cryptosporidiosis (86). Diclazuril treatment was carried out in AIDS patients suffering from severe cryptosporidial diarrhea by Connolly et al (87).

Oral bovine transfer factor which is believed to confer cell-mediated immunity to humans, may be of importance in treating cryptosporidiosis in AIDS patients (88).
Remission of cryptosporidiosis in a child with congenital agammaglobulinaemia, following treatment with hyperimmune bovine colostrum was reported by Tzipori et al (89). However in individuals with hypogammaglobulinaemia (90) and in AIDS patients (1) with cryptosporidiosis, the oral administration of bovine colostrum containing Cryptosporidium specific IgG antibodies was of use. Also, Nord et al (91) concluded that hyperimmune colostrum with high titres of specific anti-Cryptosporidium antibody could be effective in treating

patients with cryptosporidiosis. Thus, therapeutic use of anti-Cryptosporidium antibody can be considered to be of potential value in clinical management of infection. It's general utility remains to be determined.

All classes of nonspecific antiperistaltic, antidiarrheal agents, including opiates, loperamide and kaopectate have been used extensively for cryptosporidial enteritis but none of them are of any advantage. Recently, Mead et al developed an adult animal model for Cryptosporidium parvum infections with features similar to the infections observed in AIDS patients. A variety of therapeutic agents (hydroxyurea, 1,2-dimethyl-3-hydroxypyrid-4-one (DHPO) and azithromycin) were administered in these mice but were not found to be effective in controlling the infection (92).

There are no vaccines available for prevention of cryptosporidiosis in either animals or humans (5).

Immunity

In the struggle between humans and parasites, the various host protective mechanisms are important determinants of the final outcome: disease or survival. Little is known about the immune mechanisms involved in cryptosporidiosis.

Cryptosporidium infects immunologically healthy individuals causing symptoms of variable severity; there is usually a violent attack of diarrhea with abdominal pain, vomiting and fever. The illness is self-limiting and symptoms may be short

lived or persist for many weeks (69, 71, 93). In contrast, immunologically incompetent individuals (patients receiving immunosuppressive therapy, AIDS patients, very young children, the malnourished) exhibit a prolonged, debilitating and often fatal illness. (1, 2, 4, 15, 72, 74, 94, 95).

Based on observations that Cryptosporidium species have been identified in hypogammaglobulinemic patients with normal T-cell functions (21, 90, 96), as well as in patients with high titers of Cryptosporidium antibodies and low T-cell counts (97), it has been suggested that both humoral and cellular immunity may be required to eliminate cryptosporidial infection (98).

Over 100 anti-protozoal and anti-bacterial agents have been found ineffective in patients suffering from cryptosporidial infection (4, 5). As a consequence of this failure, and because of the rapidly growing magnitude of this disease in both developing and developed countries, immunological approaches are being evaluated.

The hypothesis that control of Cryptosporidium parvum infection by the immune system is supported by the following observations: a) The disease is self-limiting in immunocompetent hosts in contrast to the persistent infection in immunocompromised hosts. b) Recovered immunocompetent humans and calves are resistant to reinfection, and c) Administration of anti-Cryptosporidium specific bovine colostrum to a hypogammaglobulinemic child with persistant

cryptosporidiosis, resolved clinical signs associated with the disease and terminated oocyst shedding (89). Thus the immunological approaches for control of disease are of importance.

1) Humoral Immunity.

A potentially promising approach for the prevention of cryptosporidiosis may lie in the development of vaccines which stimulate humoral immunity. Many of the early case reports of human cryptosporidiosis were from patients exhibiting Ig deficiencies (73, 96, 99), such as congenital hypogammaglobulinemia. Cryptosporidium specific antibody production is commonly noted in normal humans and animals (60, 68, 100) over the course of self-resolving infections. The potential therapeutic role of high concentrations of passively administered anti-Cryptosporidium antibodies from serum (101), hyperimmune xenogenic colostrum (102), or purified monoclonal antibodies (103) was evaluated. In vitro treatment with immune bovine sera at an IFA titre of 1:10,000 were reported to be able to neutralize the infectivity of sporozoites (103).

To study the protective role of antibody responses in protection from and resolution of cryptosporidiosis, an experimental animal model of the disease (B-cell deficient mice) can be used. Chronic treatment of mice, begining at birth, with rabbit anti-mouse IgM antibodies (anti-u) yields mice which are virtually B cell deficient (104). These mice

display very few (< 5%) surface-Ig expressing B cells in spleen or lymph nodes and have severely reduced levels of serum and gut immunoglobulins of all isotypes. The majority of T cell and macrophage functions are intact in these anti-u treated mice (104, 105).

2) Cellular Immunity (T Cell Immunity):

Studies carried out in congenitally nude mice (Nu/Nu) suggest that T cell immunity may play the major role in resolution of cryptosporidiosis (106). Cryptosporidial infection was persistant and more severe in the congenitally athymic mice than their counterparts (Nu/+) with functional T cell repertoires. The persistant cryptosporidial infection seen in these nude mice (T cell deficient) had many characteristics in common with those observed in immunocompromised individuals (AIDS patients). However these nude mice also exhibit low antibody levels and poor macrophage function (107), making it difficult to conclude that depletion of T cell immunity is specifically responsible for the increased severity of cryptosporidiosis in nude mice.

The CD4 helper lymphocyte depletion described in AIDS patients appears to involve the small-bowel mucosa where the immunological reaction to this intracellular protozoan parasite occurs (108). Similarly, measles infection, another cause of cell-mediated immunodeficiency, has been implicated as a predisposing factor in developing countries (109).

Ungar et al (110) suggest that redundant immunological mechanisms limit Cryptosporidium infection such that both CD4 + T cells and IFN-gamma are required to prevent initiation of cryptosporidial infection whereas either alone can limit the extent (IFN-gamma) or duration (T cells) of infection.

3) Mucosal Immune Responses.

Main components of mucosal immune responses are mucosal lymphocytes, IgA antibody, mucosal mast cells and IEL (intraepithelial leukocytes).

IgA Antibody: During intestinal parasitic infections, homing of T cells and B lymphoblasts destined to become IgA plasma cells is enhanced (61). IgA antibody over mucosal surfaces may prevent the attachment of infectious agents to the epithelial cells and impair antigen absorbtion. Local IgA antibodies protect mice against infection with Taenia taeniaformis (111). The role of secretory IgA and IgM antibodies in Cryptosporidium infection is not known (60). Children with HIV and Cryptosporidium parvum infections showed no anti-Cryptosporidium parvum IgA antibodies (112). However, Kassa et al (113) demonstrated high levels of anti-cryptosporidial IgA antibodies in HIV patients with clinical cryptosporidiosis. Their data also suggest that elevated IgA serum antibodies to Cryptosporidium are not associated with protection in HIV patients. Also, Hill.B.D. (114) showed that titres of secretory IgM and IgA antibodies rise in association

with declining oocyst output in ruminants suffering from cryptosporidiosis. The role of passive lacteal immunity in cryptosporidiosis thus deserves investigation.

The role of mucosal mast cells and IEL in Cryptosporidium immunity is not known.

4) Role of Innate Immunity.

Natural immunity is strongly modulated by genetic factors (61). Susceptibility to infection by Leishmania donovani in mice is governed by alleles at a single or closely linked locus, mapping away from the major histocompatibility locus and situated on the same chromosome as the locus governing susceptibility to Salmonella typhimurium (115). Innate immunity may also depend upon the environmental conditions within the potential host. The role of innate resistance in Cryptosporidium infection is not known.

5) Role of Non-specific Factors of Immune System.

Natural killer cells (NK cells), polymorphonuclear phagocytes (neutrophils and eosinophils) and serum factors other than antibodies participate in the non-specific immune responses against parasites (61).

NK cells: NK cells are large granular lymphocytes that lyse certain virus infected cells, tumor cells and some normal cells (116). A role for NK cells in protozoan infections is suggested by the observation that mouse strains vulnerable to Plasmodium chabaudi and Babesia microti infections are those

with lowest NK activity (61). Moreover, an increase in the activity of NK cells was observed in the spleens of those infected animals who survive the parasite challenge. Also, inoculation of infected nude mice with spleen cells from syngenic, recovered normal littermates permitted the recipients to eliminate the parasites rapidly. However, nude mice have higher levels of NK activity but cannot clear Cryptosporidium infection (117, 118), thus suggesting that NK cells may not play an important role in clearance of Cryptosporidium infections.

NK cells express natural cytotoxicity i.e. exhibit MHC unrestricted killing of variety of target cells in the absence of antigenic stimulation. NK cells have been demonstrated to be a major producer of IFN-gamma, almost equivalent to that produced by CD4 + T cells under some conditions (119). Also, Ungar et al (110) suggest that production of IFN-gamma by a non-T cell contributes to host immunity against cryptosporidiosis. NK cells may thus have a potential role in Cryptosporidium infection.

Thus, the relative capacity of NK cells to prevent cryptosporidiosis, resolve Cryptosporidium infection and to generate Cryptosporidium specific cytokine responses need to be futher examined.

Macrophages: Macrophages are present in significant numbers in the connective tissues, skin and gastrointestinal tract (120). They play an important role in antigen specific

responses. The interaction of parasite antigen and T cells induces activation of macrophage or lymphokine (LK) release, resulting in parasite damage. IFN-gamma appears to be the principal lymphokine responsible for both intracellular and extracellular killing of parasites (120).

Hughes suggested that the growth of Trypanosoma, Eimeria, Toxoplasma and Leishmania may be inhibited by activated macrophages probably via activation with IFN-gamma (121). Eimeria and Toxoplasma are coccidian parasites closely related to Cryptosporidium. Canessa et al (122) suggested that IFN-gamma activates macrophages to kill intracellular Toxoplasma gondii trophozoites as well as an unrelated pathogen Listeria monocytogenes.

Another cytokine which may mediate some of the significant changes in cellular homeostasis which accompany the invasion of mammalian hosts by viruses, bacteria and parasites is TNF-alpha (Tumor necrosis factor-alpha). Clark et al (123) suggest that cell-mediated immunity to the protozoan parasite Plasmodium chabaudi may involve IFN-gamma activated macrophages which release reactive oxygen species and TNF-alpha which help in the induction of leukocytes to secrete reactive oxygen. Green et al (124) concluded that TNF-alpha was produced by Leishmania major amastigote-infected macrophages and that IFN-gamma dramatically enhanced secretion of this cytokine. Endogenous TNF-alpha produced during infection of macrophages with Leishmania major acted in an

autocrine fashion to trigger the production of toxic nitrogen metabolites (primarily nitric oxide) that killed the intracellular protozoan parasite. Also, Bogdan et al (125) concluded that the presence of IFN-gamma is crucial for TNF-alpha mediated killing of Leishmania major parasites by macrophages.

A possibility thus exists that TNF-alpha very effectively synergizes with IFN-gamma for the induction of anti-cryptosporidial activity by activated macrophages.

Liebler et al (122) demonstrated the presence of Cryptosporidium species in the phagocytic cells in the uterine lumen and the vagina of adult BALB/c mice. They suggest that phagocytosis may be an important immune response and a mechanism of parasite clearance.

M cells (microfold cells) are specialized cells located over each Payer's patch of the small intestine and border on the intestinal lumen. M cells have been shown to endocytose and transport microorganisms (including viruses and bacteria) from the lumen to underlying phagocytic lymphoid cells. This path of entry presumably permits antigenic sampling and may represent an essential component of mucosal immunity. Cryptosporidium was the first example of a parasite to have been located deep within the cytoplasm of M cells overlying Peyer's patches (4, 126). Also, partially digested and intact parasites have been seen in Peyer's patch among macrophages beneath the M cells (126). The authors suggest that,

competence of the intestinal immune system and its ability to respond to the stimulus presented via M-cell endocytotic transport is responsible for the self-limited cryptosporidial disease in immunocompetent hosts.

OBJECTIVES

OBJECTIVES

Little is resolved about the mechanisms of resistance or susceptibility to Cryptosporidium infection in humans or experimental animals. Cryptosporidiosis is reported with increasing frequency in both the developed and developing countries causing a potential problem in public health. Due to the profound effects of Cryptosporidium on HIV infected patients, attention has been focused on this organism that was previously an unrecognized pathogen in the general population. Lack of in vitro or in vivo model systems have limited the progress in understanding the biology and pathophysiology of Cryptosporidium. Thus development of these systems is essential for speeding discovery of chemotherapeutic and immunotherapeutic regimens. A study of the immunological mechanisms by which Cryptosporidium infection is prevented or resolved, can be carried out in experimental animal models that mimic normal healthy individuals and immunocompromised patients.

The Specific Objectives and Approach Used in my Study are:

1) To establish an experimental animal model of the disease: Cryptosporidiosis was elicited by feeding of oocysts to neonatal mice.

2) To examine the role of specific T cell immunity in cryptosporidiosis (in vivo) : We selectively depleted single components of the immune system (lymphocyte subpopulations) in

order to determine which arms of the immune response may play a role in the establishment and the control of infection. The contribution of CD4 + T cell responses in protection from and in resolution of Cryptosporidium infection was determined in experimentally infected, immunocompetent and immunocompromised mice models.

3) Characterization of antigen-driven cytokine production elicited in response to infection: We established a short term in vitro culture system in which Cryptosporidium driven cytokine production by lymphocytes from spleen and mesenteric lymph nodes was evaluated. We studied the role of IL-2, IL-4 and IFN-gamma in resistance to and resolution of cryptosporidiosis.

4) Evaluation of the role played by non-specific immune responses (phagocytes) in cryptosporidiosis.

MATERIALS AND METHODS

MATERIALS

Source of Cryptosporidium Oocysts: Feces containing Cryptosporidium oocysts were initially obtained from naturally infected Canadian Holstein male calves. These fecal samples were kindly provided by Dr. L. Sekla (Associate Director, Cadham Provincial Laboratory, Winnipeg) and Dr. G. Nayar (Chief Microbiologist, Provincial Veterinary Laboratory, Winnipeg).

Animals Used: BALB/c mice were obtained from breeding colonies mantained at the University of Manitoba Medical School. BALB/c scid/scid mice were obtained from JAX, BH, ME. These animals were kept and used in strict accordance with the guidelines of the Canadian Council on Animal Care. Mice were routinely monitored for antibodies to Mycoplasm, Sendai virus and rodent Coronaviruses by ELISA (Murine Immunocomb, Charles River). Mice were mantained in the absence of antibiotics.

Chemicals: Potassium dichromate and diethanolamine were products of Fisher Scientific (Edmonton, Alberta). Methylene blue, hydrochloric acid, ethyl alcohol, sodium azide, sodium phosphate monobasic, sodium carbonate, sodium bicarbonate, magnesium chloride, paraformaldehyde and glacial acetic acid were obtained from Can Lab (Winnipeg, MB). Ethylene diamine tetra acetic acid, bovine serum albumin and para-nitrophenyl phosphate disodium were purchased from Sigma Chemical Co.(St.

Louis, MO). Sucrose, phenol, sodium dodecyl sulphate, sodium chloride, Tris, sodium phosphate dibasic, boric acid, Tween 20 and sodium hydroxide were products of Mallinckrodt Canada Inc. (Mississauga, Ontario). Basic fuchsin was obtained from Anachemia (Winnipeg, MB); sodium hypochlorite from Marrin Brothers Ltd.(Winnipeg, MB); 2-mercaptoethanol from Kodak (Mississauga, Ontario); HEPES (N-2-hydroxyethylpiperazine-N-2 ethane sulphonic acid) from Research Organic Inc.(Cleveland, Ohio); Fetal bovine serum from Intergen company (N.Y., USA); NewBorn Bovine Serum from ICN Biomedical (Mississauga, Ontario); cesium chloride from Bethesda Research Laboratories (MD, USA) and trypan blue from Matheson Coleman and Bell (Ohio, USA). L-glutamine, penicillin, streptomycin and amphotericin B were products of Flow Laboratories (Mississauga, Ontario). Alkaline phosphatase conjugated strepavidin and fluorescein (DTAF - dichlorotriazinyl amino fluorescein) conjugated, affinity purified goat anti-rat IgG (H+L) were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Microscope immersion oil (high viscosity) was purchased from Stephens Scientific (NJ, USA). Readysafe scintillation fluid was obtained from Beckman (Mississauga, Ontario). Hybridoma XMG 1.2 (anti IFN-gamma antibody) was kindly provided by Dr. T.Mosmman (University of Alberta, Edmonton), recombinant IL-4 by Dr. W. Paul (NIAID, NIH Bethsda, MD), HT-2 cells by Dr. K. Rock (Harvard Medical School, Boston, USA), CT.4S cell line by Dr.W.Paul (NIH,

Bethesda, MD) and anti-CD3 mAb by Dr. J. Bluestone (University of Chicago). Recombinant IL-2 was a product of Genzyme, Boston, USA. Anti-CD4 monoclonal antibody YTS 191.1 (a hybridoma) and anti-CD8 mAb YTS 169.4.2.1 was kindly provided by Dr. H. Waldman (Dept.of Pathology, University of Cambridge, UK). Anti-CD4 (GK 1.5) ATCC (Rockville, MD) and anti-CD8 HO2.2 ATCC tissue culture supernatant were used for flow microfluorimetry analysis. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was a product of Sigma Chemical Co.(St.Louis, MO). WHO-NIAID IFN-gamma international reference reagent Gg 02-901-533 was provided by Dr. C. Laughlin (NIAID, NIH, Bethesda, MD).

Equipment: A microscope was purchased from Carsen Medical Scientific Co.Ltd. (Winnipeg, MB); a Haemocytometer was obtained from CanLab (Winnipeg, MB) and oocyst disruptor used was a Branson sonifier. An Amicon centriprep concentrator (Mississauga, Ontario) was used. 25x89 mm ultraclear centrifuge tubes were obtained from Beckman Instrument Inc (Mississauga, Ontario); 96 well ELISA plates, polysterene flat-bottom plates from Corning Science Products (Mississauga, Ontario); Falcon graduated conical tubes and superfrost microscope slides were obtained from CanLab (Winnipeg, MB). The ELISA microplate reader was a Dynatech Intruments Inc. product (Virginia, USA) and a Nunc immuno washer was used. Centrifuges used were Beckman ultracentrifuge L8-70M model, Sorval superspeed RC-2B and Sorval RT6000B refrigerated

centrifuge. Irradiation was performed at the Cancer Institute, Health Science Center, University of Manitoba, using a Cobalt 60 source. FACS analysis was kindly performed by Dr. Rector at the University of Manitoba using an EPICS V FACS (Coulter Electronics, Inc; Hialeah,FL). A PHD cell Harvester (Cambridge Technology Inc. Massechusetts, USA) and a Liquid Scintillating Counter (Packard; Ontario) was used.

METHODS

Preparation of Oocysts.

Cryptosporidium oocysts were obtained from the feces of experimentally infected calves. Feces containing oocysts were stored at 4°C in 2.5% potassium dichromate solution. Depending on experimental approach, oocysts were purified in different ways:

A) For Experimental Infection of Mice :

An appropriate volume of the fecal sample (typically 100-1000 ul) was washed with PBS and centrifuged at 5000 rpm for 10 min; washing was repeated two times and oocysts were counted using a hemocytometer (at 400X magnification). The pellet containing Cryptosporidium oocysts was resuspended in PBS and used as an innoculum to feed mice (20ul/neonate, 50ul/adult).

B) As a Source for In vitro Immunological Assays :

A widely reported method of oocyst concentration is that of sucrose floatation as described by Sheather in 1983 (127). This method has several drawbacks,including poor recovery and purity, interference with staining properties and adhesion of samples to the slide. Purification of Cryptosporidium oocysts by cesium chloride density gradient centrifugation was tried (20). In preliminary experiments we found such methods to be inadequate for in vitro culture assays. Consequently, with the generous assistance of Dr. N. Simonsen (University of

Manitoba, Winnipeg), the oocysts-containing feces,stored at 4°C in 2.5% potassium dichromate solution were first concentrated by a modification of the dichromate sucrose flotation procedure of Willson and Acres (128). A solution consisting of 9ml of 2.5% potassium dichromate preserved feces, 24ml cold sucrose (825g in 500ml dH_2O) solution and 6ml of cold $d.H_2O$ was placed in 50ml tube, inverted 10 times and centrifuged at 800 x g for 15 min. The thick viscous surface layer (about 10ml) was decanted and washed with 0.85% saline and centrifuged at 1200 x g for 20 min; washing was repeated 2 times, and the oocysts were counted using a hemocytometer. To confirm their identity, all counts were stained by the modified Kinyoun's acid-fast staining procedure (42). In 25 x 89 mm ultra clear tubes, a cesium chloride gradient was prepared, consisting of 10ml layers of cesium chloride in TE buffer (50mM Tris, 10mM EDTA) with a density of 1.40, 1.10 and 1.05 g/ml in the bottom, middle and top layers, respectively. 5ml of sterile 0.85% saline containing oocysts was layered on top of the cesium chloride gradient and centrifuged at 16000 x g for 1hr at 4°C. The top white band and the band below it were collected by using a syringe and 18 gauge needle. The bands were washed seperately with sterile 0.85% saline and centrifuged at 1200 x g for 15 min at 4°C.(x 2). The pellet was stained using the acid-fast method. The pellets obtained from the above two bands was resuspended in 5ml sterile 0.85% saline and layered on top of the cesium chloride gradient and

centrifuged at 16000 x g for 1hr at 4°C. The white band obtained from the second cesium chloride spin was stained and counted. About 70% of the oocysts were recovered from the white band (obtained from the second cesium chloride spin), when 189 x 10^6 oocysts were passed through the cesium chloride gradient. Clean, bacteria free oocysts (shown by lack of bacterial growth on blood agar and nutrient agar) were recovered from the white band. Viability of these purified oocysts was demonstrated by using the oocysts to successfully infect four BALB/c neonates. The purified oocysts were stored in 10% culture media (x4 PSF) at 4°C until used. These oocysts were stored at 4°C and used for in-vitro assays.

Administration of Inoccula to Mice.

Neonatal BALB/c mice were fed orally with a variable number of oocysts (5 x 10^3-1.5 x 10^5 /mouse) in appropriate volume of saline (typically 20ul). The minimum dosage required to infect neonatal BALB/c mice was found to be 5,000 oocysts/mouse (data not shown). However, in this study neonates were fed 1.5 x 10^5 oocysts/mouse in 20ul of saline in order to retain consistency with previous studies done in our laboratory (129). Adult BALB/c mice were fed 4 x 10^4 to 3 x 10^6 oocysts/mouse in 50ul of saline. Control mice received saline alone or were left unfed. Experimental and control groups of mice were caged separately in filter top cages in a laminar flow hood, in an isolated infection room.

Stool Collection, Cryptosporidium Oocyst Detection and Quantitation.

Stools were collected from each individual mouse or group of mice (neonates, adults) every day for a minimum of 2-3 weeks. Stools were preserved in 10% formalin at 4°C until analysed. A modified Kinyoun's acid-fast stain was used to stain Cryptosporidium oocysts and record their numbers (semi-quantitative estimation). The staining procedure involved washing the fecal sample with PBS at 5000 rpm for 10 min (x2), resuspending the pellet in 200ul of PBS and using about 100ul of above sample to prepare a thick smear on a glass slide. The smear was air-dried, fixed with methanol for 10 min and stained with Kinyoun's carbol fuchsin for 2 min. After a brief wash with tap water, the slide was decolourized with acid alcohol for 20-40 seconds, again washed with tap water, counterstained with methylene blue for 20-30 sec, washed with tap water and air-dried. Upon examination under high power (400 X) and oil immersion, Cryptosporidium oocyst stained bright red against a blue background. Two slides were prepared for each sample, a minimum of 60 fields (at 400 X magnification) were counted. Data are recorded as the mean number of oocysts observed/10 fields (+/- SEM), based on analysis of 120 fields.

Cryptosporidium Infection in Adult Mice.

1. Animals

Adult (6-7 wks old) BALB/c and adult BALB/c scid/scid mice (6-7 wks old) were used. Prior to infection with Cryptosporidium, BALB/c scid/scid mice were maintained in Class II biological safety cabinets. Adult mice after infection were maintained in an isolated infection room in a laminar flow hood. BALB/c scid/scid mice were caged seperately in sterile cages with sterile filter tops and were fed sterile food and water.

2. Innoculation of Mice with Cryptosporidium Oocysts.

Normal adult BALB/c mice (4-6/group) and BALB/c scid/scid adult mice (4-6/group) were fed 50ul saline containing Cryptosporidium oocysts varying in number from 4×10^4 to 3×10^6/mouse. In addition, age matched controls received saline alone.

3. Stool Collection and Oocysts Enumeration.

Stools were collected daily from each group of adult mice everyday for a period of at least three weeks. The presence of oocysts in their stools and their numbers were determined using Kinyoun's acid-fast staining.

All experimental adults were euthanized (with an over dose of ether) 21 days post infection (p.i), after failing to exhibit signs of infection.

4. T Cell Proliferative Responses.

Cytokine production in response to Cryptosporidium

specific oocyst antigen:

Spleen and MLN (mesenteric lymph node) cells from normal, uninfected BALB/c adult mice and Cryptosporidium fed (1.5 x 10^5-1 x 10^6/mouse) BALB/c adult mice (age of mice used-1 week, 2, 3, 4, 6, 9, 12 and 28 week old) were cultured in vitro with anti-CD3 mAb coated plates and increasing concentrations of purified oocysts, to monitor T cell activity. Animals were sacrificed on day 5 and day 8 p.i., spleen and MLNs were removed aseptically and single cell suspensions were made. The washed single cell suspensions were prepared in 10% FCS (fetal calf serum) and the number of viable cells were determined as follows - 50 ul cells + 50 ul trypan blue + 50 ul 1% glacial acetic acid + 50 ul counting cell suspension were mixed and the number of viable cells was determined by using a hemocytometer. Glacial acetic acid causes lysis of the red blood cells while trypan blue is excluded only by viable cells. Spleen and MLN cells were cultured in duplicate cultures at 20 x 10^6 cells/ml and 6 x 10^6 cells/ml respectively in 96 well flat bottom plates, in the presence of increasing concentrations of $CsCl_2$ purified Cryptosporidium oocysts (0,10^3,10^4 oocysts/well) and immobilized anti-CD3 (serum free 145-2C11) as a positive control.

Culture medium consisted of RPMI 1640 supplemented with 100 ug/ml penicillin, 100 ug/ml streptomycin, 0.25 ug/ml fungizone, 2mM glutamine, 2 mercaptoethanol and 10% heat inactivated fetal calf serum. Cultures were incubated at 37°C

for 24 hours in 5% CO_2. For the assessment of cytokine production by cells from these mice, the culture supernatants were harvested and kept at - 20°C for later evaluation of IFN-gamma, IL-2 and IL-4 production.

5. Bioassays of IFN-gamma, IL-2 and IL-4 Production.

IFN-gamma was determined by ELISA using 100 ul of purified anti-IFN-gamma antibody (XMG 1.2, at 10 ug/ml) added to each well (except control well) as a capture reagent. ELISA plates were incubated at 4°C overnight. The plates were then emptied and 200 ul of blocking buffer (1% bovine serum albumin in PBS, pH 7.4) was added to each well and incubated at 37°C for 2 hours. After washing the wells six times (PBS pH 7.4, 0.05% Tween 20), each well received 100 ul of 1:2 or 1:4 dilutions of the harvested tissue culture supernatant samples or serial dilutions of mouse Con-A supernatant as a standard (120 U/ml IFN-gamma as calibrated against WHO-NIAID international reference reagent Gg-02-901-533). Plates were incubated at 37°C for 2 hours, following which they were again washed six times and filled with 100 ul of biotinylated R4.6A2 anti-IFN-gamma antibody (1:500 dilution) and were incubated overnight at 4°C. The next day the wells were washed 6 times and 100 ul of strepavidin alkaline phosphatase (1:2000 dilution) was added prior to incubation (37°C for 2 hours). The wells were then washed and each well received 100 ul of p-nitrophenyl phosphate substrate after which the plates were incubated at room temperature for 30, 60, 90, 120 mins.

Results were obtained using an ELISA microplate reader at absorbance of 405 nm. IFN-gamma in tissue culture supernatants was determined by comparison with the standard curve constructed from the IFN-gamma standard (120 U/ml) run in each assay.

IL-2 and IL-4 production were assessed by Dr. Xi Yang, University of Manitoba using HT-2 and CT4-S bioassays respectively. IL-2 production was assessed by using HT-2 cell line (5000 cells/well in 50 ul) which is IL-2 responsive. These cells were added in 50 ul medium to 150 ul of twofold dilutions of tissue culture supernatant to give a final concentration of 5 x 10^3 cells/well in 96 well flat bottomed plates. The standard used was r-IL-2 (40 units/ml). Cultured cells were incubated at 37°C for 24 hours in 5% CO_2 and 10 ul of MTT (3-(4,5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) at 5mg/ml concentration was added to the wells. Plates were incubated at 37°C for 6 hours in 5% CO_2. Acid propanol-2-ol, 190 ul/well was used as the stop solution. Plates were incubated at 37°C for 10 minutes. Absorbance was read at 570 nm within 1 hour of addition of the stop solution. This calorimetric assay was used for the measurement of cell viability and proliferation. Only the viable cells have the ability to reduce the tetrazolium salt MTT to formazan that can be easily measured by an ELISA reader. The specificity of this bioassay for IL-2 was assured by analysis of replicate samples in the presence or absence of a cocktail of tissue

culture supernatant containing anti-IL-2 mAb S4B6 and anti-IL-2 receptor mAb 7D4. Addition of this cocktail blocks cell activation (proliferation or MTT conversion to formazan) by 90-97%.

IL-4 production was assessed in a similar assay by using CT4-S cells which are highly responsive to murine IL-4 in a dose dependent fashion. The standard used was r-IL-4 (64 units/ml). The specificity of the CT4-S bioassay for IL4 was confirmed by inclusion of anti-IL4 mAb 11B11 in parallel assays .

Cryptosporidium Infection in Neonatal Mice.

1. Animals

Neonatal (7-8 days old) normal BALB/c and BALB/c scid/scid (7-8 days old) mice were used.

2. Innoculation of Mice with Cryptosporidium Oocysts

Neonatal normal BALB/c (5-6/group) and neonatal BALB/c scid/scid (5-6/group) were fed 20 ul saline containing Cryptosporidium oocysts varying in number from 5 x 10^3 to 1.5 x 10^5 /mouse, the standard innoculum being the latter. Age matched controls received saline alone. Each group of mice was caged separately in a laminar flow hood. BALB/c scid/scid neonates were maintained under sterile conditions.

3. Stool Collection and Enumeration

Stools were collected from each group of neonates every day for at least two weeks. The presence of oocysts in fecal

samples and their numbers were recorded using Kinyoun's acid-fast staining technique. The pattern of disease progression in the two groups of mice was compared.

All experimental neonates were usually euthanized 15 days p.i.

4. T Cell Proliferative Responses

A) Antigen Specific T Cell Proliferative Responses to Cryptosporidium Oocyst Antigen.

Neonatal (8 day old) BALB/c mice were infected with 1.5 x 10^5 oocysts/mouse. Mice were sacrificed on day 8 and day 15 p.i.; spleen and MLN cells obtained were cultured at concentrations of 24 x 10^6 cells/ml and 6 x 10^6 cells/ml (100 ul/well) respectively, with increasing concentrations of purified oocysts (0, 10^3, 10^4, 10^5 oocysts/well) (in duplicate). Cultures were examined microscopically from day 2 to day 5. Plates were cultured for the 4 days at 37°C for 24 hours in 5% CO_2; pulsed with ^{3}H-thymidine (1uCi) and plates were further incubated at 37°C for 24 hours in 5% CO_2. To the harvested supernatant, 2ml scintillation cocktail was added and thymidine uptake was determined by liquid scintillation counting.

B) Polyclonal T Cell Stimulation.

Spleen and MLN cells from normal, uninfected neonatal BALB/c mice and Cryptosporidium infected neonatal BALB/c mice (fed 1 x 10^5-1.5 x 10^5 oocysts / mouse) were cultured in vitro

with anti-CD3 pre-coated wells and increasing concentrations of purified Cryptosporidium oocyst (0, 10^2, 10^3, 10^4/well). Spleen cells were cultured at 6 x 10^6 cells/ml while MLN cells at 6 x 10^6 cells/ml, in a 24 well flat bottom plate at the peak of infection (day 8) and following infection (day 15). Plates were incubated at 37°C for 24 hours in 5% CO_2. Supernatants were harvested and production of IFN-gamma was determined by ELISA. IL-2 and IL-4 assays were carried out using bioassays as described above.

Intensity of IFN-gamma Production by Cryptosporidium Infected Neonates:

Spleen cells and MLN cells from normal BALB/c neonates and Cryptosporidium infected BALB/c neonates (fed 1.5 x 10^5 oocysts/mouse), (5-6/group) were cultured in vitro with increasing concentrations of purified Cryptosporidium oocysts (0, 10^2, 10^3, 10^4 oocysts/well (100 ul), in duplicate). Spleen cells were cultured at 24 x 10^6 cells/ml (100 ul/well) while MLN cells at 6 x 10^6cells/ml (100 ul/well) in 24 well plates at day 5 post- infection, day 8 post-infection (peak of infection) and day 15 post-infection (post-recovery). Plates were incubated at 37°C for 24 hours in 5% CO_2. Supernatants were harvested and the dose-response kinetics of IFN-gamma response was determined by ELISA.

Phenotype of Cells Responsible for IFN-gamma Production in Cryptosporidium Infected Mice:

Spleen cells and MLN cells from normal, uninfected

neonatal BALB/c mice and Cryptosporidium infected neonatal BALB/c mice (fed 1.5 x 10^5/mouse) were cultured in 96 well flat bottom plates with purified Cryptosporidium oocysts at increasing concentrations of 0, 10^3, 10^4 oocysts/well in the presence and absence of anti-CD4, anti-CD8 and anti-CD4 + anti-CD8 monoclonal antibodies. Anti-CD4 mAb-YTS 191.1 and anti-CD8 mAb-YTS 169.4.2.1 (IgG2b rat antibodies) were purified by ion exchange chromatography using the Pharmacia Biopilot system. Briefly, two litres of exhausted tissue culture supernatant was dialyzed against citrate buffer (0.01 M, pH 5.3) and applied to the Biopilot S-Sepharose column. The eluted rat mAb was used for in vivo intraperitoneal injection as well as for in vitro use. Spleen cells were cultured at 20 x 10^6 cells/ml (100 ul/well) final concentration and MLN cells at 6 x 10^6 cells/ml (100 ul/well) final concentration at day 8 post-infection. Cultures were incubated at 37°C for 20 hours in 5% CO_2. Supernatants were harvested and tested for IFN-gamma production by ELISA.

Cryptosporidium Infection in Immunocompromised Models.

1. Preparation of Anti-CD4, Anti-CD8, and Anti-CD4 + Anti-CD8 Treated Mice.

Neonatal BALB/c mice, 7 days old (6-8/group) were injected intraperitoneally with 0.2 ml of concentrated anti-CD4 mAb, anti-CD8 mAb and 0.1 + 0.1 ml of anti-CD4 + anti-CD8

mAb, three times a week for the duration of the experiment. These concentrations (approximately 200 ug/injection) were demonstrated to deplete CD4 or CD8 T+ cells in vivo by 97 % as assessed by flow cytometry (see below). By a similar procedure CD4 T cell deficient adult (8 wk old) BALB/c mice (4/group) were prepared. The mice were infected with 1.5 x 10^5 oocysts per mouse 24 hours after the first antibody treatment. Untreated infected mice or normal rat immunoglobulin (NR-Ig) treated mice served as controls. Each group of mice was caged separately, kept in an isolated infection room in filter top cages with a lid in a laminar flow hood. Infection was monitored for three weeks.

2. Stool Collection and Enumeration of Oocysts.

Stools were collected from each group of mice daily for at least three weeks. The presence of oocysts in stools and their numbers were recorded using Kinyoun's acid fast staining technique.

3. FACS Analysis

To determine the effectiveness of monoclonal antibody treatment in vivo, T enriched spleen cells were stained independently with anti-CD4 (GK 1.5) or anti-CD8 (HO2.2) tissue culture supernatants followed by affinity purified FITC-conjugated goat anti-rat Ig. After extensive washings these cells and controls stained with FITC-anti-Ig alone were analysed immediately or fixed and stored in a solution of 2% paraformaldehyde, 1% BSA until analysed. The percentage of CD4

and CD8 positive T cells and their relative fluorescence intensity was determined using an EPICS V FACS. Duplicate preparations of each sample were independently stained and analysed.

4. Phenotype of Cell Producing IFN-gamma in Cryptosporidium Infected Neonates.

Spleen cells and MLN cells from untreated, Cryptosporidium infected neonates (fed 1.5 x 10^6 oocysts/mouse); anti-CD4 mAb, anti-CD8 mAb or anti-CD4 + anti-CD8 mAb treated, Cryptosporidium infected neonates (mAb treatment as above, fed 1.5 x 10^6 oocysts/mouse,4 mice/group) were cultured with increasing concentrations of purified Cryptosporidium oocysts (0, 10, 10^2, 10^3, 10^4 oocysts/well in duplicate). Spleen cells were cultured at 24 x 10^6 cells/ml (100 ul/well) while MLN cells at 6 x 10^6 cells/ml (100 ul/well) in 24 well flat bottom plates at the peak of infection (day 8 post infection). Plates were incubated at 37°C for 20 hours in 5% CO_2. Supernatants were harvested and tested for IFN-gamma production by ELISA.

Cryptosporidium Infection in Irradiated Mice.

Adult BALB/c mice (6-8 weeks old) and neonates (8 days old), (6-7/group) were irradiated with 550 rads, for 3.5 minutes using a cobalt 60 source. Irradiation was followed by reconstitution with CD4 enriched or CD8 enriched spleen cell populations or the mice were left unreconstituted. Later the

same day, these and non-irradiated normal control mice were innoculated with 1.5 x 10^5 oocysts /mouse. Each group of mice was caged separately, kept in an isolated infection room in filter top cages with a lid in a laminar flow hood. Stools were collected from each group of mice daily for at least two weeks. The presence of oocysts in stools and their numbers were recorded using Kinyoun's acid-fast staining technique. Spleen and MLN cells of irradiated and non-irradiated mice were cultured on day 8 post-infection with increasing concentration of purified oocysts (0, 10^3, 10^4 oocysts/well). Spleen cells were cultured at 20 x 10^6 cells/ml while MLN cells at 6 x 10^6 cells/ml. Plates were incubated at 37°C in 5% CO_2 for 24 hours. Supernatants were harvested and IFN-gamma production was determined by ELISA. In order to evaluate the extent of spontaneous B and T cell reconstitution following irradiation, spleen cells of irradiated and non-irradiated mice were tested for their capacity to respond to the B and T cell mitogens LPS and Con- A respectively, at different time periods (day 4, 8, 10, 14 post-irradiation) as follows:

Spleen cell preparations were cultured in duplicate at 20 x 10^6 cells/ml in the presence of the B cell mitogen LPS (final concentrations of 0, 0.4, 2, 10, 50 and 250 ug/ml) and in the presence of the T cell mitogen Con-A (final concentrations of 0, 2.5, 5, 10, 20 and 40 ug/ml). Cultures were incubated at 37°C for 24 hours for Con-A and 48 hours for LPS in 5% CO_2, pulsed with ^{3}H-thymidine and harvested 18 hours

later. Thymidine uptake was determined by liquid scintillation counting.

Disease course of cryptosporidial infection in neonatal and adult irradiated mice and age matched non-irradiated normal controls was monitored as before.

RESULTS

RESULTS

1. Purification of Oocysts.

Several procedures for oocyst purification have been used by different investigators. Fecal samples containing Cryptosporidium oocysts, on microscopic examination revealed a lot of debris (amorphous material, intestinal cells, cellular material) and washings with PBS did not separate the oocysts from the debris. The $CsCl_2$ density gradient centrifugation technique recommended by Dr.Kilani (University of Manitoba) was attempted. However the results obtained were not suitable for our experiments. A modification of the $CsCl_2$ density gradient centrifugation technique was then carried out with the kind help of Dr.Simonsen (University of Manitoba). The modified technique (as in methods) used gave three bands and a distinct pellet in the $CsCl_2$ gradient. Microscopic examination of the middle band (white band) demonstrated lack of debris. This was further confirmed by plating on blood agar and nutrient agar plates which were incubated at 37°C for 24 hours and observed for contamination. Absence of bacterial growth confirmed the cleanliness of the oocysts. The oocysts obtained from the white band were counted using a hemocytometer; recovery was 70% when 189 x 10^6 oocysts were passed through the $CsCl_2$ gradient. Kinyoun's acid-fast stained smears confirmed the identity of the cryptosporidium oocysts. The oocysts stained bright pink against a blue background.

The band below the white band contained very few oocysts contaminated with debris; while the pellet was filled with debris. These were shown by direct microscopic examination of smears obtained from the second band and bacterial growth on blood agar/nutrient agar plates.

These sterile, purified oocysts were stored in 10% FCS in the presence of penicillin, streptomycin, fungizone (x 4) at 4°C and used for several immunological in vitro assays.

In order to use Cryptosporidium oocysts as an innoculum to feed animals, a less rigourous method was necessary. Fecal samples containing oocysts stored at 4°C in potassium dichromate solution, were washed with PBS (x 2) at 5000 rpm for 10 minutes. The pellet containing oocysts was counted using a hemocytometer. It was resuspended in PBS and used as an innoculum to feed mice.

2. Cryptosporidium Infection in Normal Neonatal BALB/c Mice vs Adult BALB/c Mice:

Cryptosporidiosis was elicited by the feeding of Cryptosporidium oocysts (fecal sample) to 8 day old BALB/c neonatal mice. The presence of disease and its severity was evaluated quantitatively by determining the number of oocysts present in stool samples over the course of infection. A high oocyst count reflected the severity of the disease. The relative susceptibility of neonatal and adult BALB/c mice to Cryptosporidium infection was examined. (Table 1).

Disease course. Neonatal (8 day old) BALB/c mice were fed oocysts varying in number from 5 x 10^3 to 1.5 x 10^5/mouse.(5 x 10^3, 2.5 x 10^4, 1.0 x 10^5, 1.5 x 10^5). The intensity and duration of infection in groups of mice given different doses of oocysts was monitored. These neonatal mice suffered severe intestinal cryptosporidial infection as demonstrated by the appearance of large numbers of excreted oocysts in semi-solid stools. Typically, the infected neonatal mice started shedding oocysts in their feces by 4-5 days p.i. The peak of infection was on day 7-8 p.i.; with the number of oocysts shed reduced to zero by 11-12 days p.i. In all groups of normal neonatal BALB/c mice, Cryptosporidium infection was self-resolving.

Adult (8-9 weeks old) BALB/c mice were fed with higher numbers of oocysts ranging from 4.0 x 10^4-3.0 x 10^6 oocysts/mouse (4.0 x 10^4, 1.0 x 10^5, 1.5 x 10^5, 1.0 x 10^6, 3.0 x 10^6). Adult mice stools were checked daily for a period of up to 60 days, using Kinyoun's acid fast staining techinque. It was observed that even with a large dose of oocysts (3 x 10^6/mouse), about 600 times that required for intense infection of neonates, adult mice failed to develop symptoms of infection nor excrete oocysts. (limit of sensitivity, 1 oocyst/60 fields at 400 X magnification).

3.Cryptosporidium Infection in Normal Neonatal BALB/c Mice, Anti-CD4 mAb Treated Neonatal BALB/c Mice vs Adult Anti-CD4 Treated BALB/c Mice:

We carried out experiments to study the role of T cell mediated specific immunity in cryptosporidiosis and the importance of the CD4+T cell subset in resistance to and in recovery from Cryptosporidium infection. CD4+T cells comprised the specific T cell subset that was depleted in vivo from both peripheral circulation and the gut by the administration of specific monoclonal antibody (anti-CD4 mAb). The CD4+T cell deficient mice served as an immunologically compromised model to study cryptosporidiosis.

Disease course. To determine the impact of CD4+T cell depletion on cryptosporidiosis, neonatal mice were treated in vivo with anti-CD4 mAb (in vivo administration of purified anti-CD4 mAb, YTS 191.1.2), one day prior to feeding of Cryptosporidium oocysts (1.5×10^5 oocysts/mouse). This mAb was selected in preference to GK 1.5 due to the higher yield of mAb obtained from the YTS hybridoma. Flow cytometry was used to compare the presence of CD4 positive T cells in the spleen of individual untreated (normal) mice, mice treated with anti-CD4 mAb and control mice treated with normal rat immunoglobulin (NR-Ig). Spleen cells were stained with affinity purified, FITC conjugated goat anti-rat Ig and the percentage of CD4+T cells present was quantitated. Anti-CD4 mAb treatment was begun 7 days after birth and continued 3 times a week. This intervention was shown to deplete >97 % of CD4+T cells. Treatment with NR-Ig had no detectable effect on numbers of CD4 and CD8 cells nor their function.

Untreated neonatal (8 days old) BALB/c mice, age matched NRIg-treated and anti-CD4 mAb treated neonates were fed 1.5 x 10^5oocysts/mouse. These neonatal mice developed severe, symptomatic intestinal Cryptosporidium infection as demonstrated by the appearance of large numbers of excreted oocysts in the semi-solid stools (Figure 1). Neonatal mice stools were examined daily for 21 days using Kinyoun's acid fast staining technique. Although the time of onset was simillar in all groups, anti-CD4 treated mice exhibited more severe diarrhea and a protracted disease course. The infection in anti-CD4 treated mice was charachterized by multiple cycles of oocysts shedding. The NR-Ig treated or untreated mice showed a single acute infection. However, despite the increased severity and duration of cryptosporidiosis observed in CD4 deficient neonates, the Cryptosporidium infection ultimately was self resolving in all three groups despite continued mAb treatment, without mortality in any of the mice. These mice were monitored for two additional weeks, but failed to demonstrate any recurrence of oocysts shedding.

Adult (8 weeks old) anti-CD4 mAb treated mice were innoculated with 1.5 x 10^5 oocysts/mouse. In contrast to the results obtained in neonates, CD4 depletion of adult mice failed to measurably increase their susceptibility to Cryptosporidium infection (Fig.1, Panel D). Ten fold increases in the dose of oocysts administered (not shown), failed to alter the resistance of these mice to cryptosporidiosis,

despite the fact that these mice exhibited <3% CD4 + lymphocytes in the spleen or MLN. The above results suggest that T cells play an important but not essential role in resolution of cryptosporidiosis.

4. Characterization of T Cell Dependent Immune Responses Elicited by Cryptosporidium Infection.

Proliferative responses of spleen and MLN cells from Cryptosporidium infected BALB/c neonates to $CsCl_2$ purified Cryptosporidium oocyst antigens were examined. Previous studies carried out by Dr.Kilani demonstrated the inability of splenic cells and T-enriched spleen cells from Cryptosporidium infected BALB/c neonates to proliferate upon reexposure to Cryptosporidium oocysts. This was confirmed by our experiments.

In order to directly examine gut derived cells, proliferative responses of MLN cells to Cryptosporidium oocysts restimulation in vitro was determined. MLN cells were selected as they provide a ready source of Peyer's patch derived T cells. Neonatal normal infected BALB/c mice (fed 1.5 x 10^5 oocysts/mouse) and uninfected control BALB/c mice were sacrificed on day 8 and 15 p.i. (i.e. at the peak of and following infection). MLN cells obtained failed to exhibit T cell proliferative responses to Cryptosporidium oocysts in vitro (data not shown), despite being examined under a number of different culture conditions (Kilani R.T). In

contrast, T enriched spleen cells or unseparated lymph node populations obtained from Cryptosporidium (CFA) immunized mice (data not shown) yielded strong proliferative responses following in vitro restimulation with intact or sonicated oocysts, thus indicating the integrity of the oocyst population used for in vitro restimulation and the presence of Cryptosporidium reactive T cells in the neonatal T cell repertoire.

5. Cytokine Production in Response to Cryptosporidium Oocysts Antigen (In vitro).

An alternative approach was then used to characterize T cell activation upon Cryptosporidium infection. Direct examination of cytokine production following antigen specific in vitro restimulation was carried out. The rationale for selecting IL-2, IL-4 and IFN-gamma as the three cytokines for initial examination is that IL-2 and IL-4 play central roles in T cell proliferation while IFN-gamma is of interest because : a) IFN-gamma appears to be the principal lymphokine responsible for both intracellular and extracellular killing of parasites (130). b) Several researchers have concluded that IFN-gamma is an important macrophage activator (121, 122, 123). c) Laskay et al (131) observed that when fractionated antigen preperations of Leishmania parasites were used to stimulate the peripheral blood lymphocytes of patients with cutaneous leishmaniasis, there was production of IFN-gamma and

TNF-alpha. d) Ungar et al (110) demonstrated an independent role for IFN-gamma in protective immunity in an adult mouse model of cryptosporidiosis.

Spleen cells and MLN cells from normal uninfected and Cryptosporidium infected BALB/c neonates at the peak of infection (day 8) and after recovery (day 15), were cultured to evaluate production of IFN-gamma, IL-2 and IL-4 in response to purified cryptosporidial oocyst antigens. No detectable IL-2 and IL-4 production was observed in these mice when spleen and MLN cells were cultured with increasing concentrations of Cryptosporidium oocysts (<1 U/ml). As a positive control, spleen and MLN cells were cultured with anti-CD3 mAb (1452C11). IL-2 and IL-4 responses elicited by polyclonal T cell activation were similar in uninfected and infected groups and were not affected at the peak (day 8 post-feeding) and following infection (day 15 post-feeding). (Table 2).

In marked contrast, spleen and MLN cells from Cryptosporidium infected BALB/c mice but not normal uninfected BALB/c mice, exhibited strong production of IFN-gamma when exposed to increasing concentrations of Cryptosporidium oocysts (Figure 2). IFN-gamma production by Cryptosporidium infected BALB/c mice was maximum at day 8 post-feeding of oocysts. Examination of kinetics of IFN-gamma production revealed that it was rarely detectable three days p.i. (data not shown) and approximately 50-70% of maximal at day 5 post feeding (prior to shedding of oocysts) or day 15 (recovery of

infection). In all cases comparison of infected with noninfected normal neonates at a given oocyst concentration yields $p < 0.001$. In terms of the intensity of IFN-gamma production which results following addition of exogenous antigen in vitro, comparison of cultures to which no oocysts were added to those with 10^2, 10^3 or 10^4 oocysts yielded $p < 0.05$ where indicated (*).

This induction of IFN-gamma synthesis by neonates, very early following feeding of oocysts may suggest that IFN-gamma is of importance in resistance to and resolution of Cryptosporidium infection invivo.

6. Phenotype of Cells Responsible for IFN-gamma Production in Cryptosporidium Infected BALB/c Mice.

Spleen and MLN cells from Cryptosporidium infected BALB/c mice were cultured 8 days p.i. with increasing concentrations of purified oocysts (0, 10^3, 10^4 /well) in the presence and absence of anti-CD4, anti-CD8 or anti-CD4 + anti-CD8 monoclonal Abs (Table 3). Supernatants were harvested at 20 hours and IFN-gamma production was determined by ELISA. Anti-CD4 mAb was found to abolish IFN-gamma production; anti-CD8 somewhat decreased it (although to a much lesser extent), while anti-CD4 + anti-CD8 abolished the IFN-gamma production completely.

An alternative approach to determine the phenotype of cells producing IFN-gamma in Cryptosporidium infected neonates

was carried out using the immunocompromised animal models (data not shown, results of a single experiment discussed). Spleen and MLN cells from untreated, Cryptosporidium infected; anti-CD4 mAb, anti-CD8 mAb, or anti-CD4 + anti-CD8 mAb treated Cryptosporidium infected neonates were cultured 8 days p.i. with increasing concentrations of oocysts (0, 10^3, 10^4/well). Supernatants were harvested at 20 hrs and IFN-gamma production was determined by ELISA. In vivo anti-CD4 mAb treatment was found to abolish IFN-gamma production, anti-CD8 mAb somewhat decreased it, while anti-CD4 + anti-CD8 mAb treatment abolished the IFN-gamma production completely.

The data obtained from the above experiments (Table 3) suggests that IFN-gamma production is a CD4+ T cell dependent and possibly to a lesser extent a CD8+ T cell dependent mechanism. The potential role of CD8+ T cells in IFN-gamma production needs to be investigated further.

7. Cryptosporidium Infection in Irradiated Mice.

In order to futher characterize the role of CD4 and CD8 T cells and cytokine production in the resolution of cryptosporidial infection, experiments on irradiated mice were performed.

A) B and T cell Responses in Irradiated Mice.

Neonatal mice (7 day old) were irradiated (550 rads), a dose found appropriate to effectively abolish T and B cell mediated responses while having minimum impact on the

reticuloendothelial system (132). Antigen and mitogen driven proliferation assays were carried out on spleen cell populations of irradiated and age matched normal non-irradiated mice. This was done to confirm the absence of functional B and T cell responses in irradiated, unreconstituted mice.

To do this, spleen cells were cultured, exposed to mitogens (B cell mitogen-LPS, T cell mitogen-Con- A) over a wide concentration range (as in methods), pulsed with ^{3}H-thymidine and thymidine uptake was determined by liquid scintillation counting. Spleen cells from irradiated mice failed to respond to LPS at day 4, 8 and 10 post irradiation (p.ir.); in contrast to age matched control mice, but regained this capacity by day 14 p.ir. (Fig.3). Similarly, spleen cells from irradiated mice did not respond to Con-A at day 4, 8 and 10 p.ir., in contrast to age matched control mice, but regained this capacity by day 14 p.ir. (Fig.4). These data are indicative of the absence of specific T or B cell responses in neonatal mice following the dose of radiation used (550 rads).

B) Infection Profile.

Neonatal, irradiated BALB/c mice infected with Cryptosporidium were reconstituted with CD4+ T cells or CD8+ T cells or left unreconstituted and their disease profile was evaluated (Fig.5). Suprisingly, no significant differences were found in the onset of infection, peak, clearance and

intensity of infection in unirradiated, unreconstituted; irradiated, CD4 T cell reconstituted; irradiated, CD8 T cell reconstituted and irradiated, unconstituted BALB/c mice. This suggests that specific immune responses may not be essential for resolution of cryptosporidiosis i.e. under the conditions tested, Cryptosporidium specific immune responses may not be essential for resolution of cryptosporidiosis.

In later experiments, the effect of irradiation on neonatal resistance to Cryptosporidium infection was monitored by comparing infection intensity and duration in irradiated and normal mice. Stool samples were collected from neonatal irradiated and age matched non-irradiated controls infected with Cryptosporidium oocysts (1.5×10^5/mouse). As can be seen from the data in Table.4, the time of onset, peak of infection and clearance of oocysts excretion and intensity of infection were not found to be different in these two groups of mice. Despite the absence of detectable B and T cell responses in irradiated mice, the capacity of these animals to resolve cryptosporidial infection was not impaired. No mortality was observed in any of these mice.

When adult BALB/c mice were irradiated and innoculated with Cryptosporidium oocysts, they remained resistant to infection. No oocysts were excreted in their stools over a period of three weeks (< 1/120 fields of view at 450X magnification). Irradiated adult mice reconstituted with either B or T cells did not exhibit any infection after they

were fed with oocysts.

C) IFN-gamma Production by Irradiated Mice.

The above results demonstrate the absence of specific B and T cell responses in neonatal mice that have been exposed to 550 rads of irradiation. The resolution of oocysts shedding in the absence of detectable B or T cell function suggests that in this particular experimental model resolution of cryptosporidiosis may be independent of T cell function and IFN-gamma production. However, previously primed lymphocytes are generally considered to be less radiosensitive than naive cells. Moreover, there may be a possibility that environmental antigens exist which are cross-reactive with cryptosporidial antigens. Also, T cells in the 8 day old neonates that are specific for such environmental antigens may not be detectable by polyclonal induced responses, yet yield IFN-gamma production in vivo following challenge with oocysts, despite irradiation.

To address the possibility that resolution of cryptosporidiosis in irradiated mice may in fact be dependent on the production of IFN-gamma by mature, environmental antigen primed, radioresistant T cell populations, we directly examined cytokine production by cells obtained from normal and irradiated mice. Both Cryptosporidium specific and polyclonal stimuli were used.

IFN-gamma production by irradiated (550 rad) neonatal mice and age matched non-irradiated controls, infected with

Cryptosporidium (1.5 x 10^5/mouse) was determined (Table.5). MLN cells obtained from Cryptosporidium infected, irradiated and non-irradiated neonatal BALB/c mice were cultured with increasing concentrations of purified oocysts (0, 10^3, 10^4) and IFN-gamma production was determined by ELISA (as in methods). Irradiated neonates failed to exhibit IFN-gamma production or responsiveness to Con-A (day 8 and day 15 p.i.) as compared to the age matched non-irradiated infected controls. This again suggests that in this experimental model resolution of cryptosporidiosis may not depend on IFN-gamma production.

8. Cryptosporidium Infection in BALB/c scid/scid Mice.

As an alternative approach to evaluating the role of T cell mediated specific immunity in Cryptosporidium infection, SCID mice were used. These mice are genetically deficient in their capacity to produce B or T lymphocytes and consequently humoral or cellular immunity; as a result of a selective defect in hematopoeitic stem cells. SCID mice have normal numbers of macrophages, phagocytes and granulocytes. As a genetically deficient model, BALB/scid mice play an important role in verifying the findings obtained with mice selectively depleted of specific lymphocyte subsets by immunotherapy.

Neonatal (8 day old) BALB scid/scid mice were fed with varied number of Cryptosporidium oocysts (5 x 10^3-1.5 x 10^5 /mouse) and the disease course in these infected mice vs age matched normal infected controls was determined (Table 6).

Onset of infection in these groups of mice was 4-7 days; peak of infection was 5-9 days and resolution of infection was 9-15 days p.i. The pattern of disease progression in both infected BALB scid/scid neonates and normal neonates was not significantly different. No mortality was observed in any of these mice. One difference that was observed was the stool consistency in the above two groups, scid/scid infected mice had watery stools while normal infected mice had semi-solid to solid stools. A major symptomatic expression of cryptosporidiosis is watery diarrhoea (29); which is indicative of an extensive increase in the lumenal water content. Various factors may be involved in the relationship between the main clinical symptoms and pathophysiology of cryptosporidiosis. Of significance are the distribution of Cryptosporidia within the intestine and the immunological status of the host (29). The relevance of stool consistency in cryptosporidial infection remains to be resolved.

Similarly, adult BALB scid/scid and age matched normal BALB/c mice were fed varying numbers of Cryptosporidium oocysts (4.0×10^4, 1.5×10^5, 1×10^6 and 3×10^6/mouse) and stools collected from the above two groups were examined for presence of oocysts. Disease was monitored for a period of 30 days. No infection was detected in any of these mice. Adult mice, both scid and normal were not susceptible to Cryptosporidium infection even at a high dose of 3×10^6oocysts/mouse.

9. Role of IFN-gamma in Cryptosporidium Immunity.

Susceptibility to Cryptosporidium infection in BALB/c mice is age dependent. Typically, neonatal (7-8 day old) and adult, normal BALB/c mice and BALB scid/scid mice fed Cryptosporidium oocysts varying in number from 1.5 x 10^5 to 3 x 10^6oocysts/mouse, showed no significant differences in the pattern of disease progression. The time of onset, peak and clearance of oocysts excretion and intensity of infection were not significantly different in the neonatal normal BALB/c and BALB scid/scid mice. Adult, normal BALB/c, anti-CD4 treated and BALB scid/scid mice failed to develop manifestations of infection even at a very high dose of 3.0 x 10^6 oocysts/mouse.

As shown in Table 2, no detectable IL-2 and IL-4 production was observed in normal uninfected and Cryptosporidium infected BALB/c neonates (<1 U/ml). In contrast, as seen in Fig.2, spleen and MLN cells from Cryptosporidium infected BALB/c mice (day 8 p.i.) but not uninfected BALB/c mice, produced IFN-gamma when exposed to increasing concentrations of purified Cryptosporidium oocysts antigen. These neonatal mice show a decrease in IFN-gamma production after the infection is resolved (day 15 p.i.). The finding of antigen specific induction of IFN-gamma production following infection and the corelation between disease stage and the kinetics of cytokine synthesis raises the possibility that IFN-gamma may be involved in resolution of cryptosporidial infection in neonates.

The role of IFN-gamma in adult BALB/c mice found to be resistant to cryptosporidiosis was then examined. Adult BALB/c mice (4 week old) were fed 1.0 x 10^6 oocysts/mouse. On day 3, 5 and day 8 p.i., spleen and MLN cells from Cryptosporidium fed adult mice and age matched unfed adult controls were cultured with increasing concentrations of purified Cryptosporidium oocysts (0, 10^3 and 10^4/well) and anti-CD3 mAb precoated wells (Table 7). The disease course monitored revealed that at a high dose of 1.0 x 10^6 oocysts/mouse, no oocysts excretion was detected in the Cryptosporidium fed adult mice suggesting that the adult mice failed to manifest the disease. However, on in vitro restimulation with purified Cryptosporidium oocysts, the Cryptosporidium fed adult mice produced IFN-gamma as measured by ELISA. A similar pattern of IFN-gamma production with respect to kinetics, dose dependency and intensity was observed on day 3, 5 and day 8 p.i. Also, cytokine production evaluated on day 3 and day 5 p.i. when harvested at 18 hours and 36 hours post in vitro culture, did not alter IFN-gamma production. In one of three experiments, normal unfed adult BALB/c mice also generated an IFN-gamma response following restimulation with Cryptosporidium oocysts; albeit at lower levels. The induction of IFN-gamma synthesis by adults (and neonates, as shown before) very early following feeding of oocysts suggests the importance of IFN-gamma synthesis in host immunity to Cryptosporidium. We futher investigated the role of IFN-gamma in resistance to

Cryptosporidium infection in adults by determining if there was any correlation between age, IFN-gamma production and lack of susceptibility to infection. To accomplish this we examined the capacities of unfed, Cryptosporidium resistant adult BALB/c mice to produce the cytokines IFN-gamma, IL-2 and IL-4, following in vitro polyclonal stimulation.

Evaluation of IFN-gamma, IL-2 and IL-4 Production by BALB/c mice of Different Age Groups Following Polyclonal Invitro Stimulation : The relative capacities of 4 day old, 1 week, 2, 3, 4, 6, 9 and 12 week old BALB/c mice (2/group, in duplicate) to produce cytokines IL-2, IL-4 and IFN-gamma, following polyclonal in vitro stimulation (with immobilized anti-CD3 (145 2C11) were determined (Fig.6). Cytokine production was optimal in all groups of mice using anti-CD3 mediated stimulation at 6 x 10^6 spleen cells/ml. An age dependent increase in IFN-gamma production following polyclonal stimulation of increasingly older mice was observed. Minimal IFN-gamma production was observed prior to 2 wks with large increases occuring in the capacity of mice to generate IFN-gamma between 2-12 weeks of age (2, 3, 4, 6, 9, 12 weeks). In contrast, IL-4 and IL-2 production reach adult levels by 2 weeks and 1-4 weeks of age respectively. Detailed intra-group comparisons of cytokine production will require more experiments and direct analysis of one vs. two week old neonates.

Figure 1. Shedding of Cryptosporidium oocysts in infected normal neonatal, NRIg-treated neonatal, anti-CD4 mAb treated neonatal and adult anti-CD4 mAb treated BALB/c mice. A minimum of 60 fields/data points were counted. The daily mean of the number of fecal oocysts seen in 10 microscopic fields ($\pm$ SEM) is shown (450X); comparing infection in infected normal neonatal (A), NRIg-treated neonatal (B), anti-CD4 mAb treated neonatal (C) and anti-CD4 mAb treated adult (D) BALB/c mice. No significant differences were seen in the time of onset and resolution in groups (A), (B) and (C); but day of peak oocyst shedding and the magnitude of oocyst shedding was much higher in group (C). Adult anti-CD4 mAb treated BALB/c mice failed to be infected at a high dose of 1.5×10^6 oocysts/mouse. "Cryptosporidiosis" in groups (A), (B) and (C) was a self resolving infection with no mortality in any of the mice. Data represent one of six similar experiments carried out.

Figure 1A

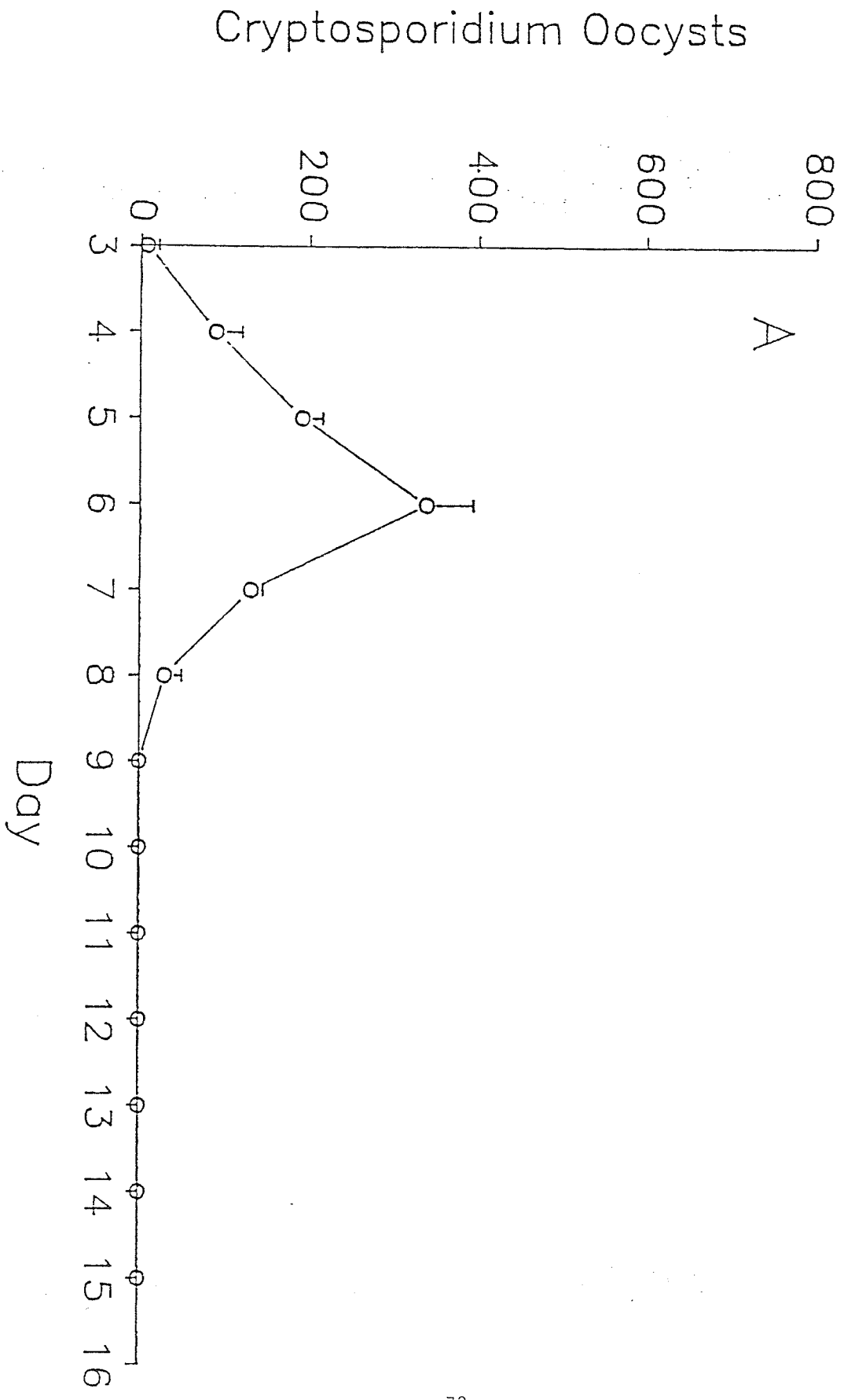
Cryptosporidium Oocysts
0
200
400
600
800
A
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Day

Figure 1B

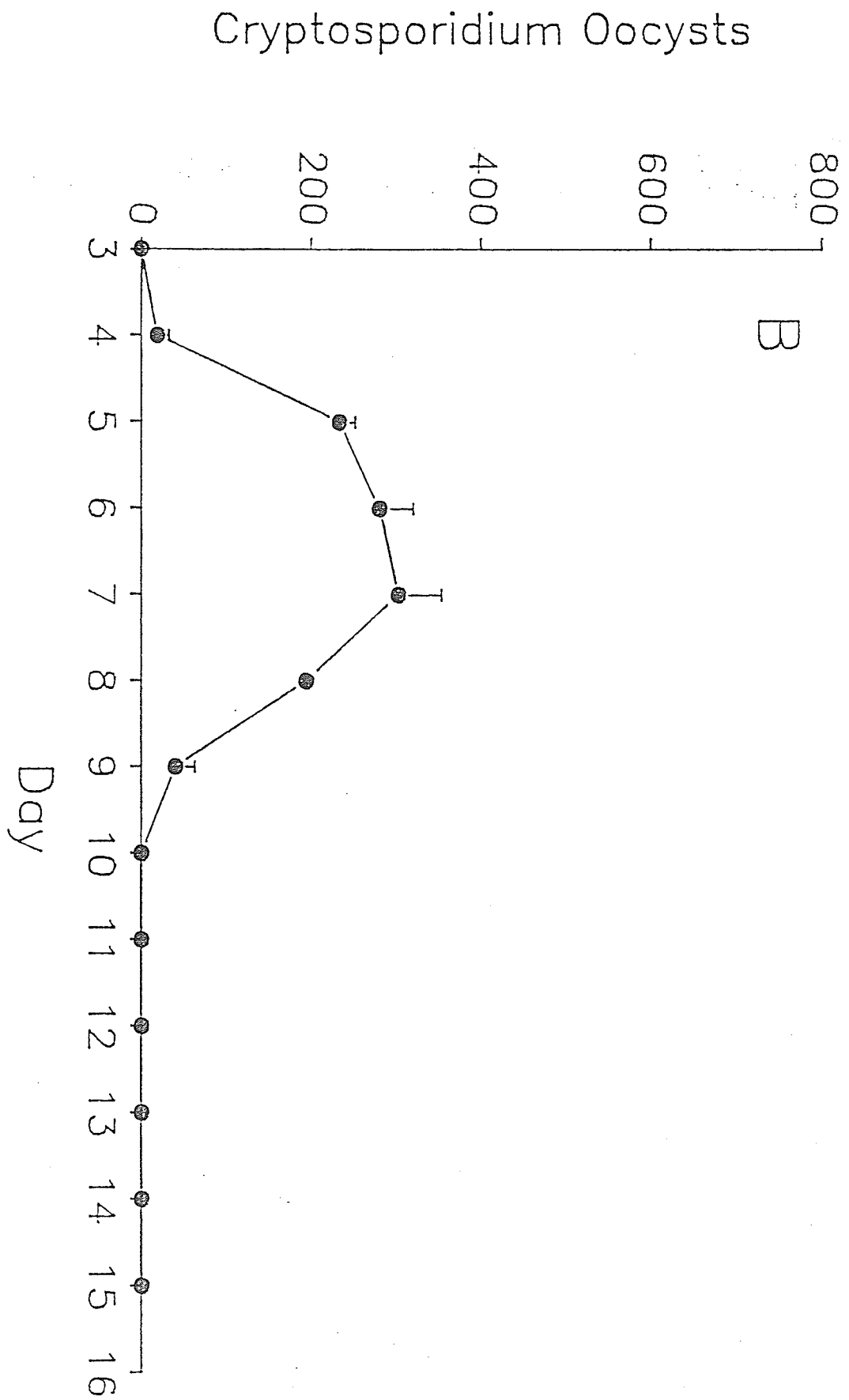
Cryptosporidium Oocysts
0
200
400
600
800
B
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Day

Figure 1C

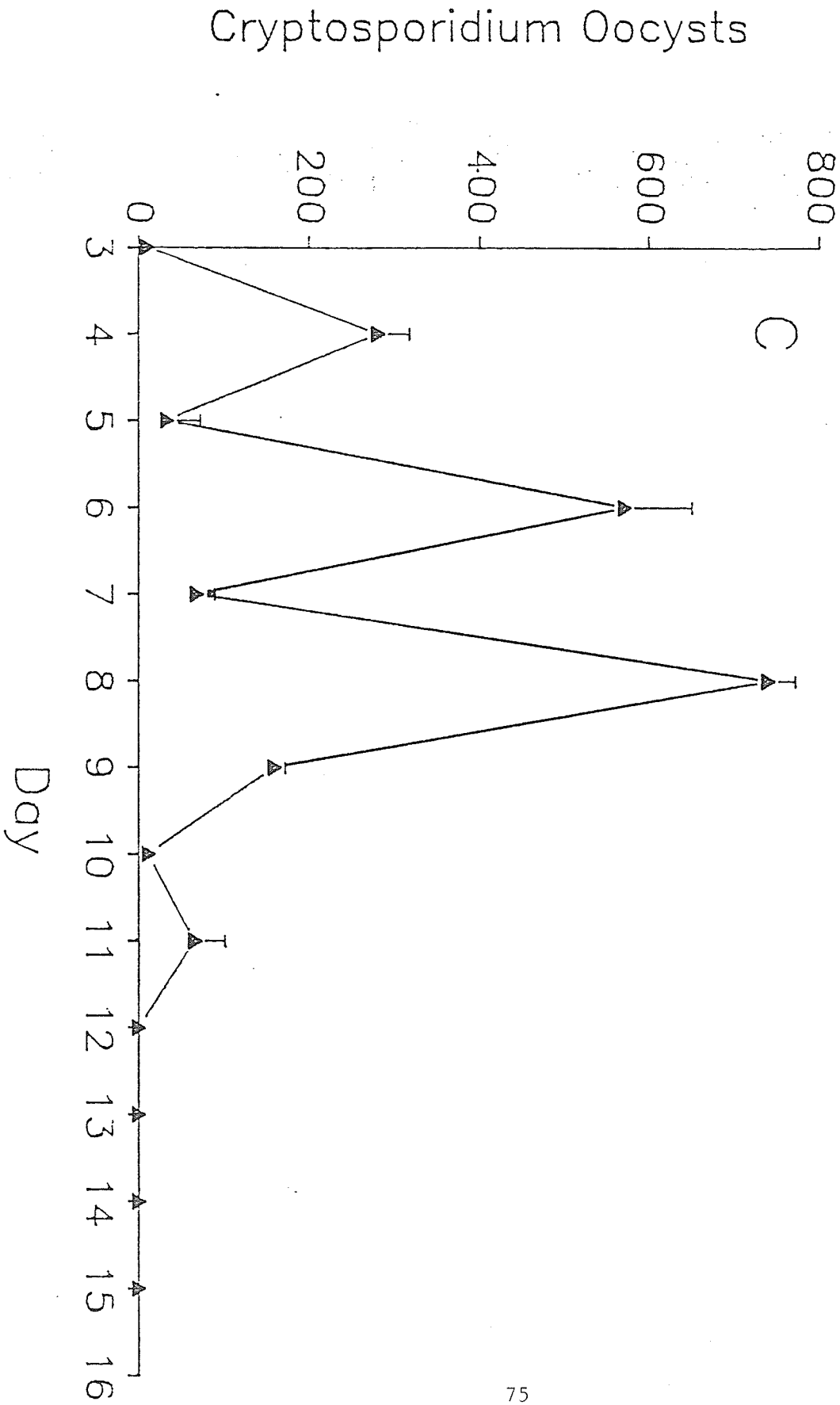
Cryptosporidium Oocysts
0
200
400
600
800
C
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Day

Figure 1D

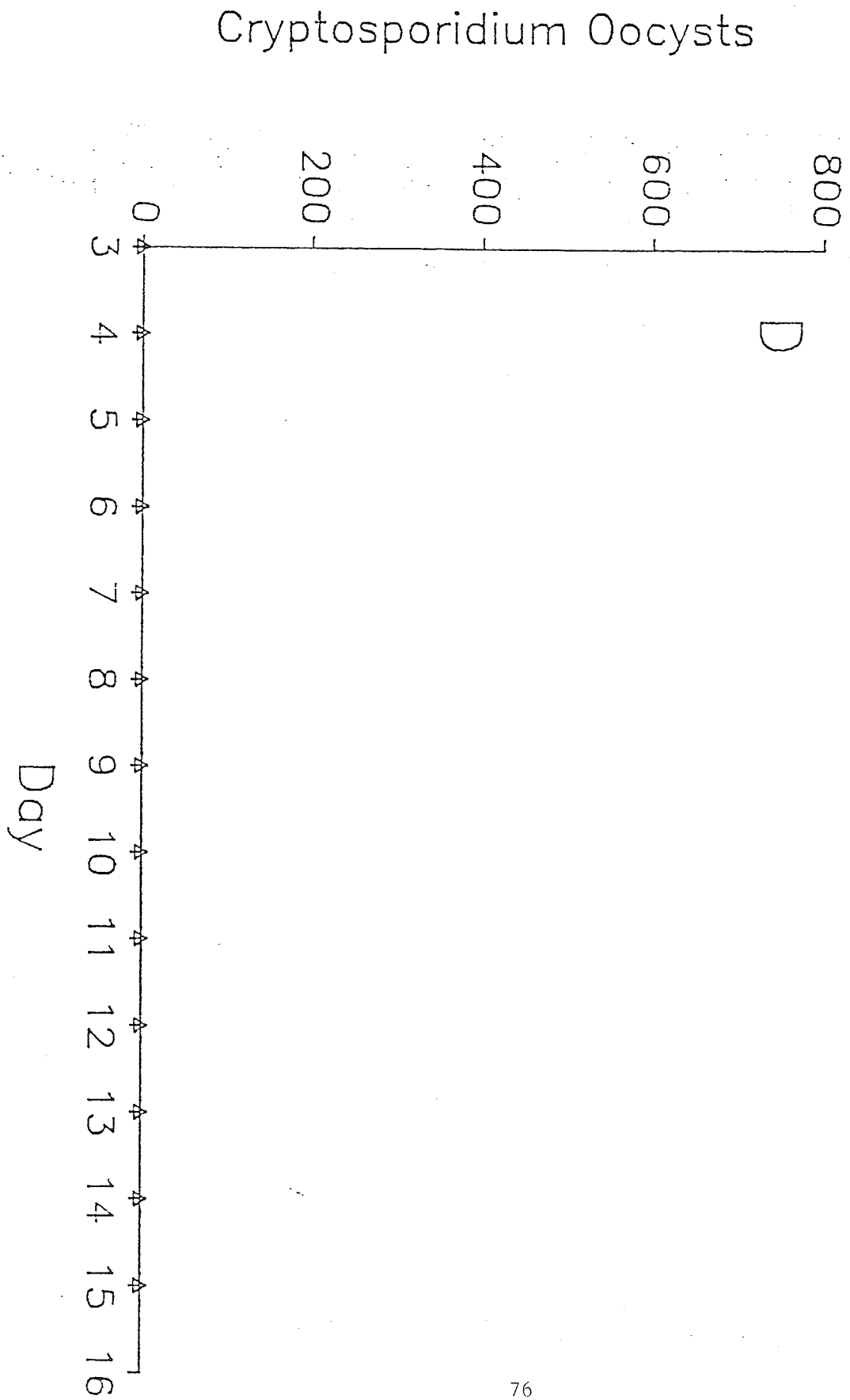
Cryptosporidium Oocysts
0
200
400
600
800
D
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Day

Figure 2. In vitro IFN-gamma production by splenic and MLN cells from Cryptosporidium oocysts infected and uninfected BALB/c neonates exposed to cryptosporidial oocysts antigens (in vitro). MLN cells (A) and spleen cells (B) from Cryptosporidium infected neonates (■) and non-infected neonates (□) were cultured on day 8 p.i. with increasing concentrations of purified Cryptosporidium oocysts. Supernatants were collected after 24 hours and assayed for IFN-gamma production as in materials and methods. Spleen and MLN cells from Cryptosporidium infected BALB/c neonates but not uninfected BALB/c neonates produce the lymphokine IFN-gamma when exposed to the indicated number of Cryptosporidium oocysts. Data represent one of four similar experiments. In all cases comparison of infected with noninfected neonates at a given oocyst concentration yields $p < 0.001$. An asterisk represents $p < 0.05$. (Student's t test).

Figure 2A

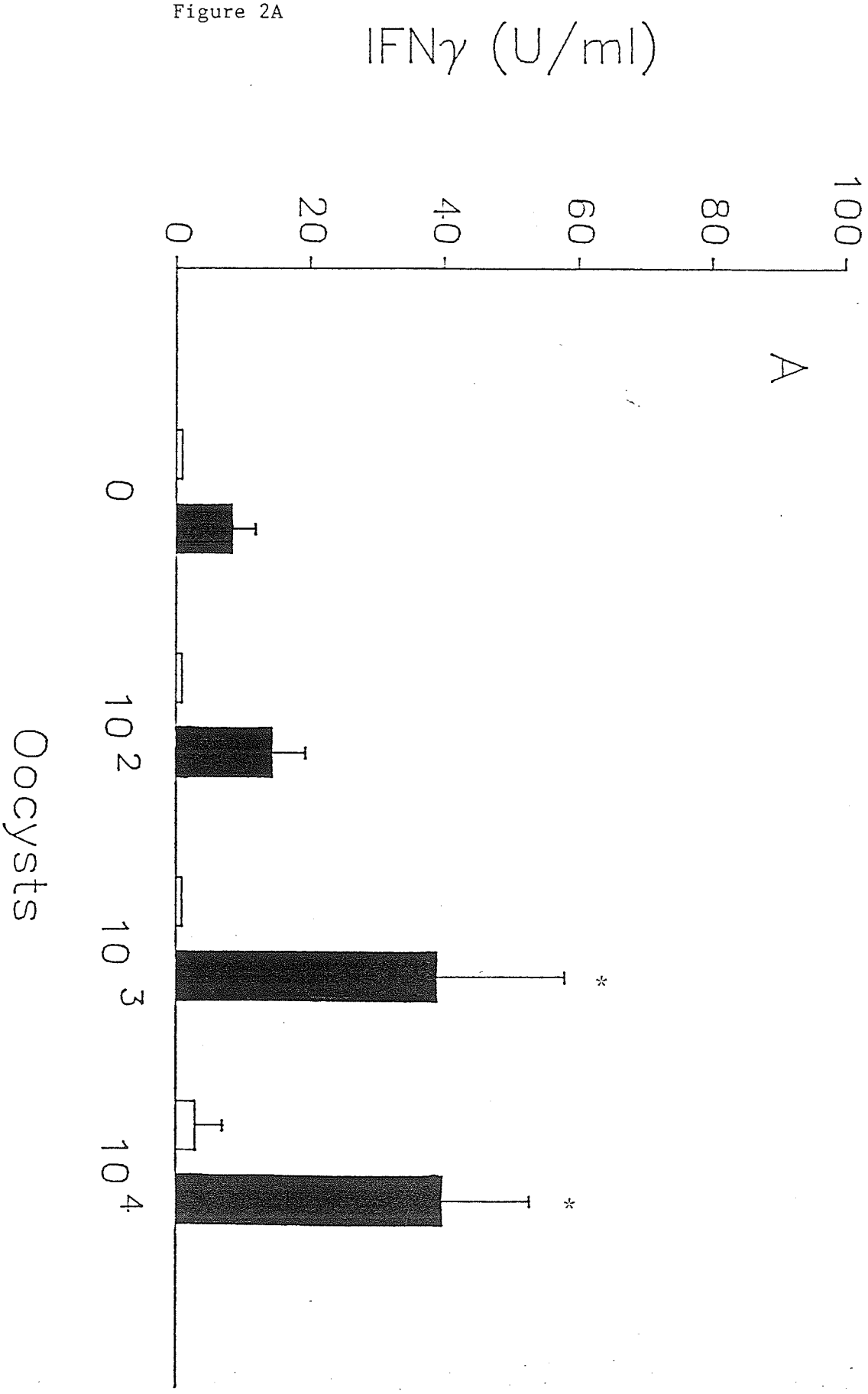

Figure 2B

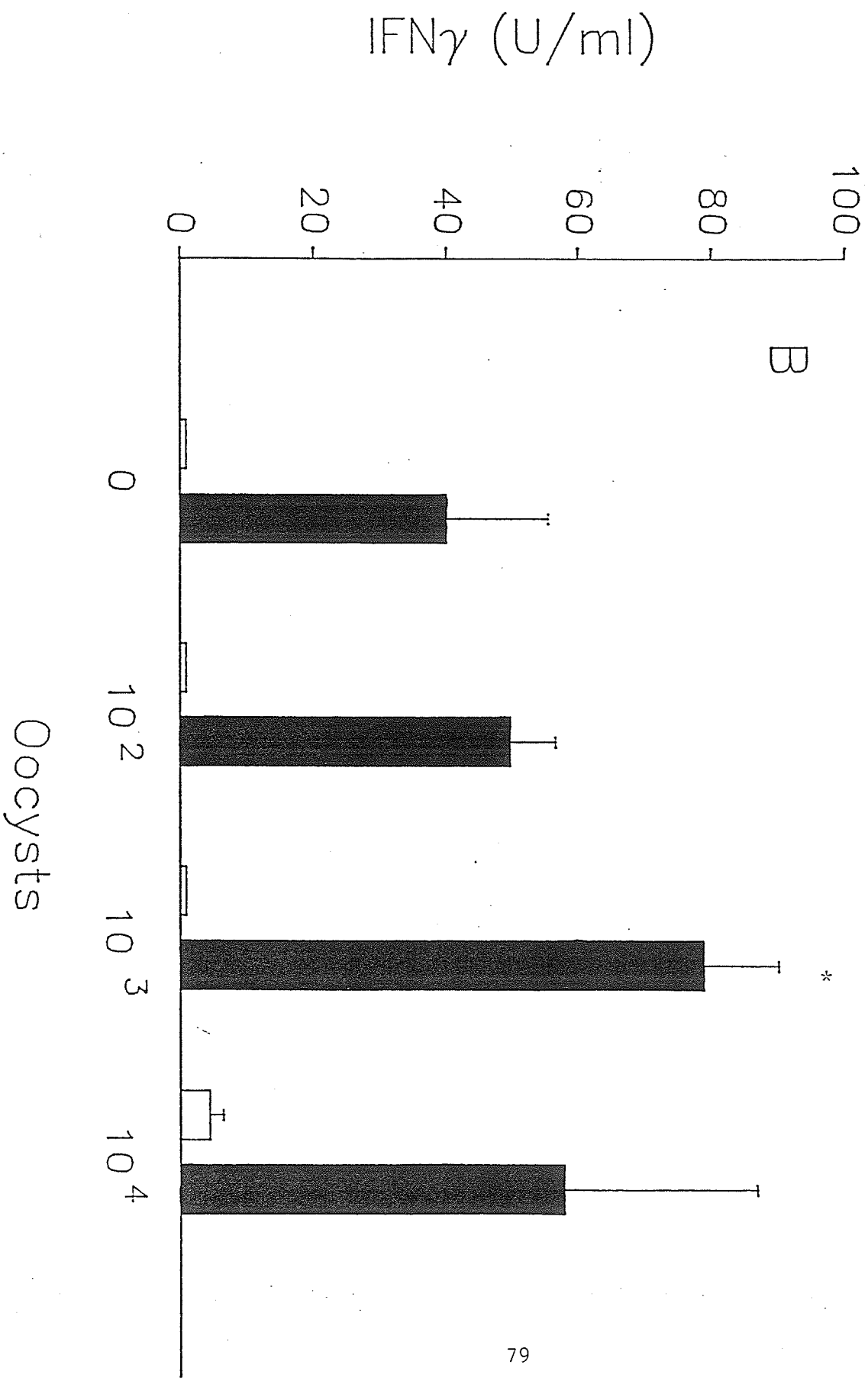

Figure 3. Effect of irradiation on proliferative responses of spleen cells from neonatal BALB/c mice to the B cell mitogen-LPS. Proliferation assays were carried out on spleen cell populations as described in materials and methods. Spleen cells were cultured, exposed to varied concentrations of B cell mitogen-LPS; pulsed with ^{3}H-thymidine and thymidine uptake was determined by liquid scintillation counting. Spleen cells from irradiated mice (■) failed to respond to LPS at day 4, day 8, and day 10 (in the comparison of normal vs irradiated neonates, $p < 0.001$) p.ir.; in contrast to age matched non-irradiated controls (□), but regained this capacity by day 14 p.ir.(asterisk represents $p < 0.05$, others $p > 0.05$). Responses are presented as mean dpm from duplicate cultures in one of four similar experiments. Student's t test.

Figure 3

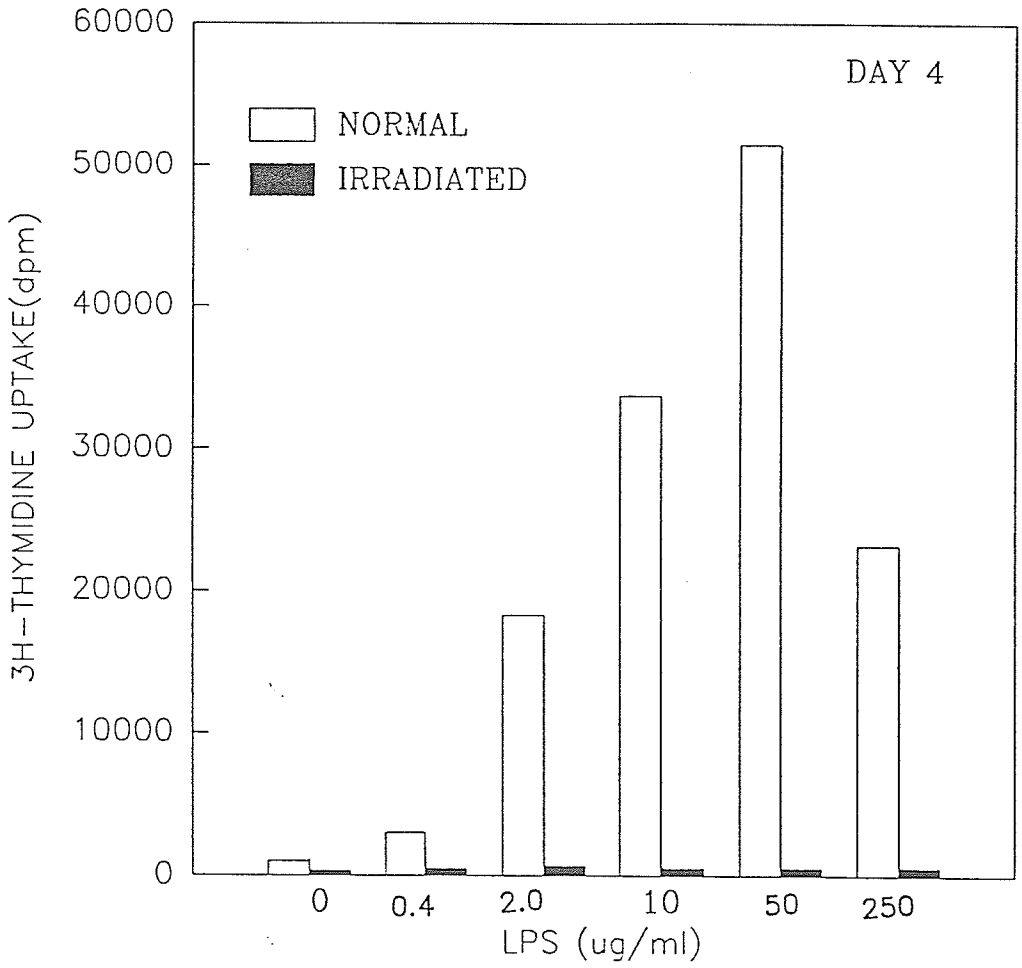
60000
50000
40000
30000
20000
10000
0
3H-THYMIDINE UPTAKE(dpm)
DAY 4
NORMAL
IRRADIATED
0
0.4
2.0
10
50
250
LPS (ug/ml)

Figure 3

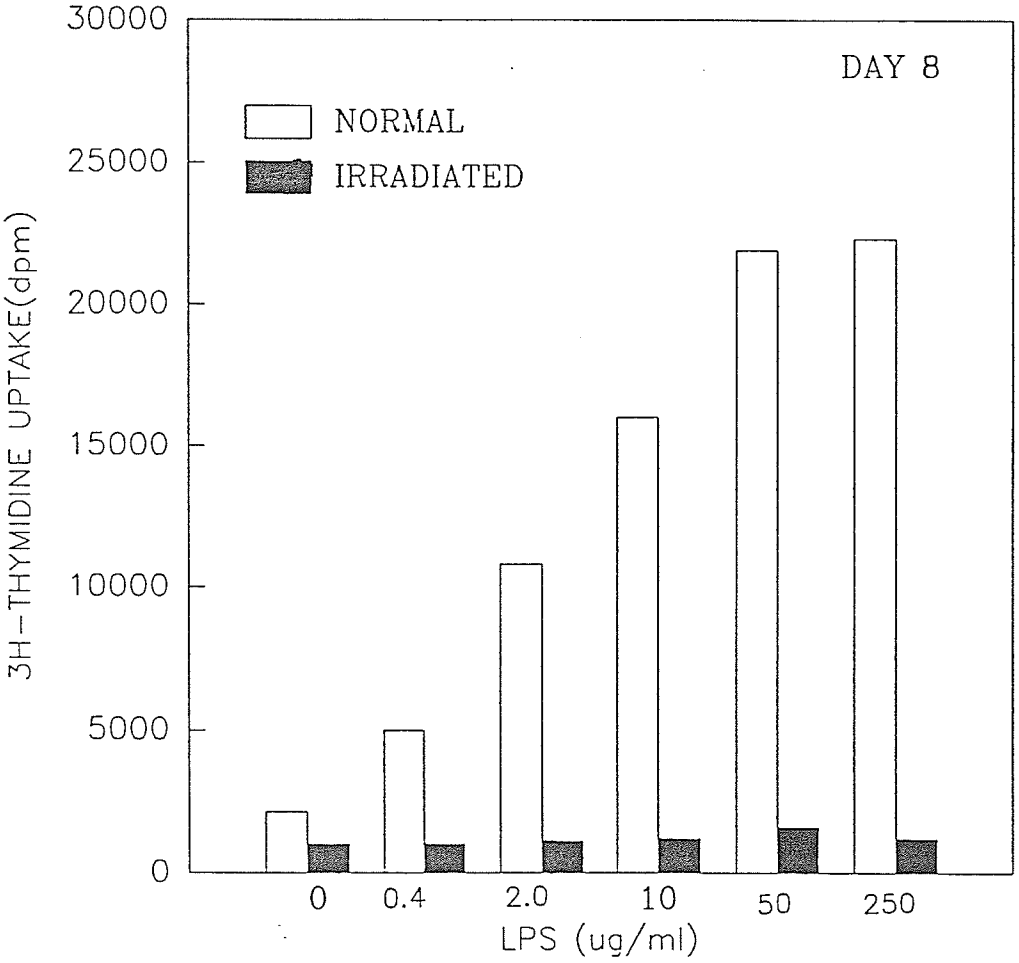
30000
25000
20000
15000
10000
5000
0
3H-THYMIDINE UPTAKE(dpm)
DAY 8
NORMAL
IRRADIATED
0
0.4
2.0
10
50
250
LPS (ug/ml)

Figure 3

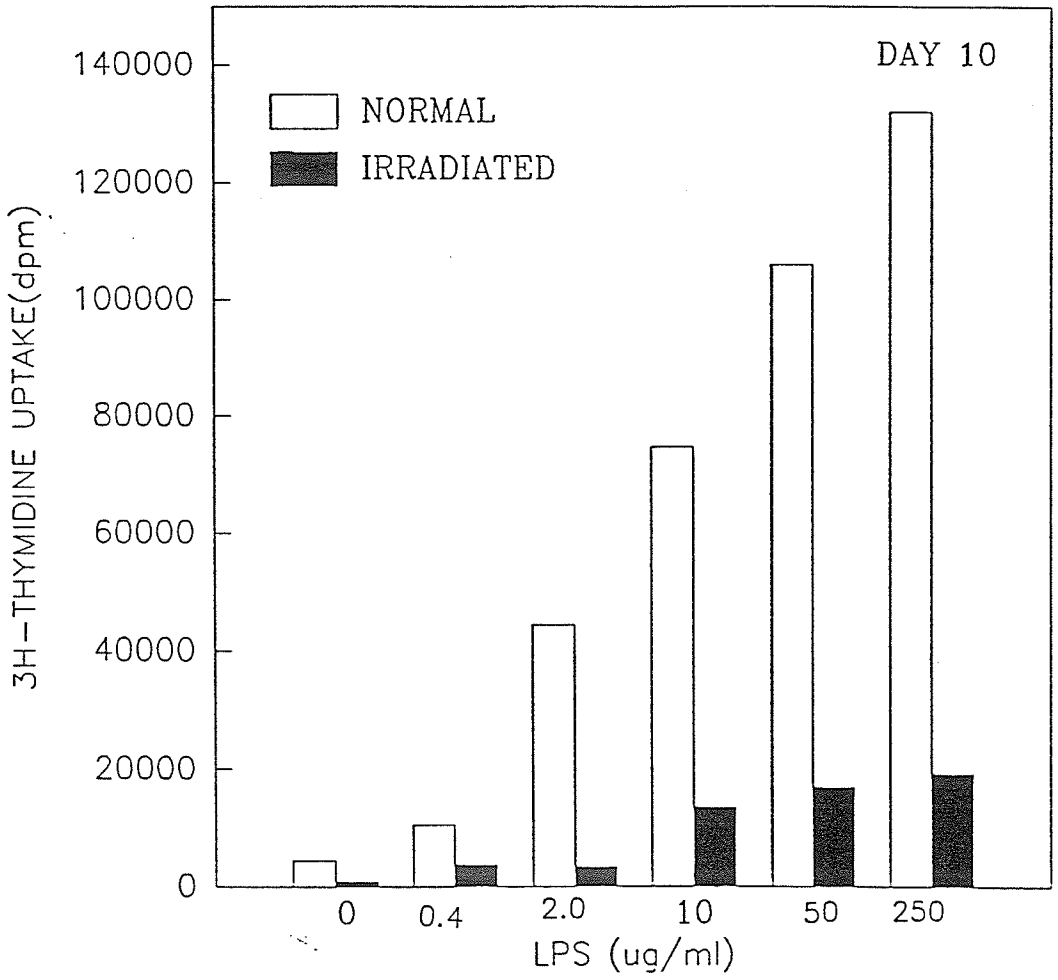
DAY 10
NORMAL
IRRADIATED
3H-THYMIDINE UPTAKE(dpm)
140000
120000
100000
80000
60000
40000
20000
0
0
0.4
2.0
10
50
250
LPS (ug/ml)

Figure 3

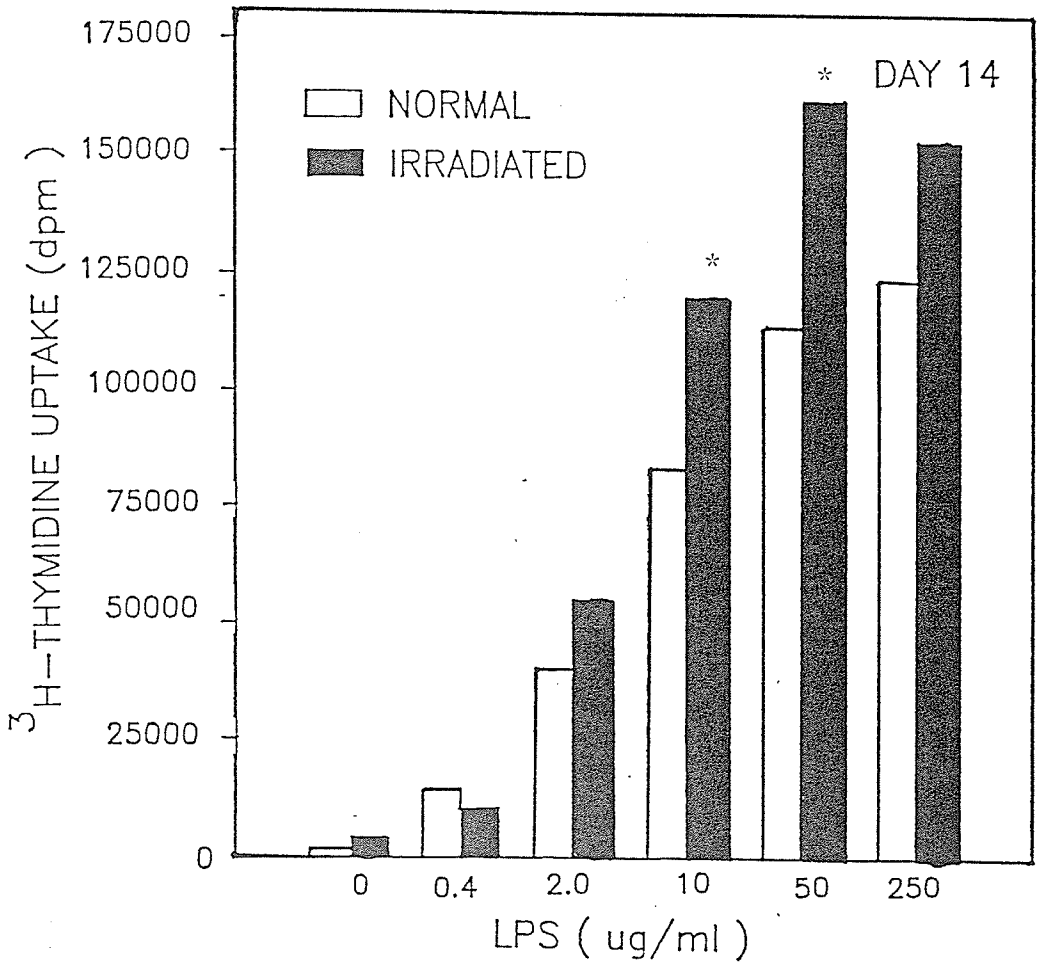
175000
150000
125000
100000
75000
50000
25000
0
^{3}H-THYMIDINE UPTAKE (dpm)
NORMAL
IRRADIATED
DAY 14
*
*
0
0.4
2.0
10
50
250
LPS (ug/ml)

Figure 4. Effect of irradiation on proliferative responses of spleen cells from neonatal BALB/c mice to the T cell mitogen-Con A. Proliferation assays were carried out on spleen cell populations as described in materials and methods. Spleen cells were cultured, exposed to varied concentrations of T cell mitogen-Con A; pulsed with ^{3}H-thymidine and thymidine uptake was determined by liquid scintillation counting. Spleen cells from irradiated mice (■) failed to respond to Con A at day 4, 8 and 10 p. ir.(in comparison of normal vs irradiated neonates, all cases $p < 0.001$); in contrast to age matched non-irradiated controls (□), but regained this capacity by day 14 p.ir.(asterisk represents $p < 0.05$, others $p > 0.05$). Responses are presented as mean dpm from duplicate cultures in one of four similar experiments.

Figure 4

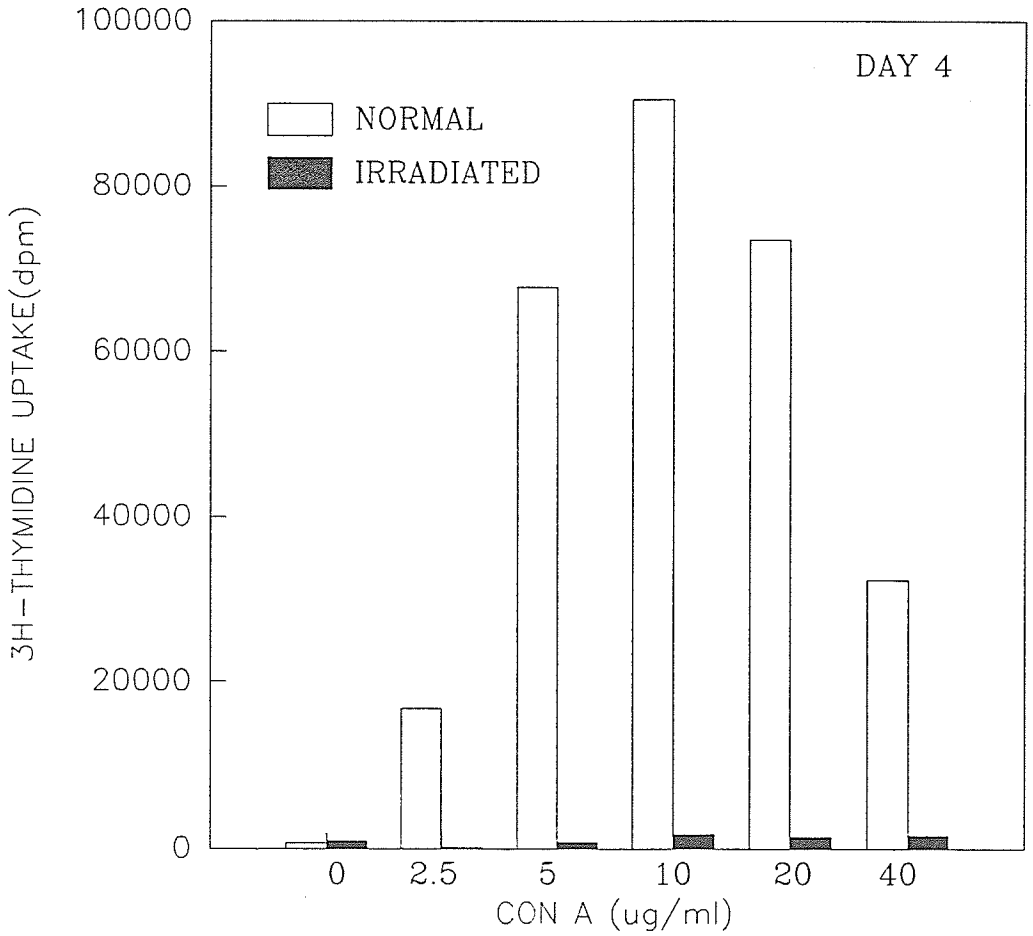
100000
80000
60000
40000
20000
0
3H-THYMIDINE UPTAKE(dpm)
DAY 4
NORMAL
IRRADIATED
0
2.5
5
10
20
40
CON A (ug/ml)

Figure 4

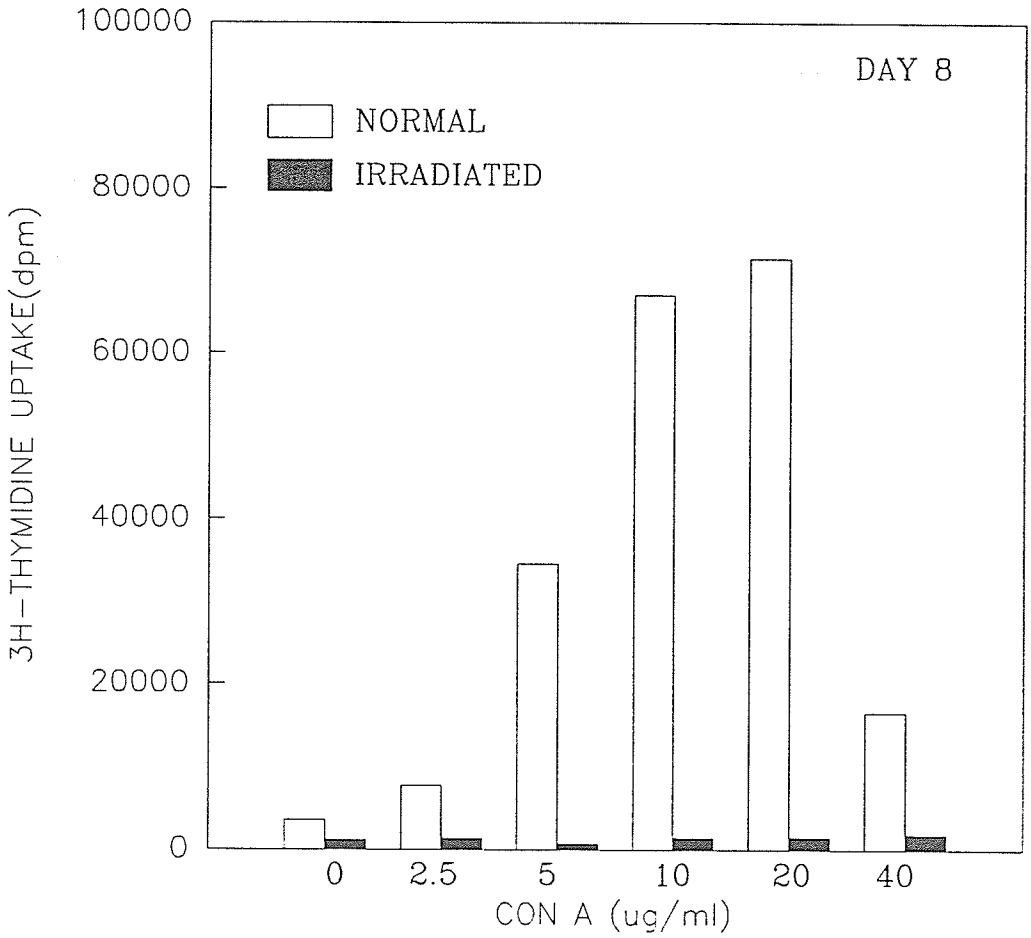
100000
80000
60000
40000
20000
0
3H-THYMIDINE UPTAKE(dpm)
DAY 8
NORMAL
IRRADIATED
0
2.5
5
10
20
40
CON A (ug/ml)

Figure 4

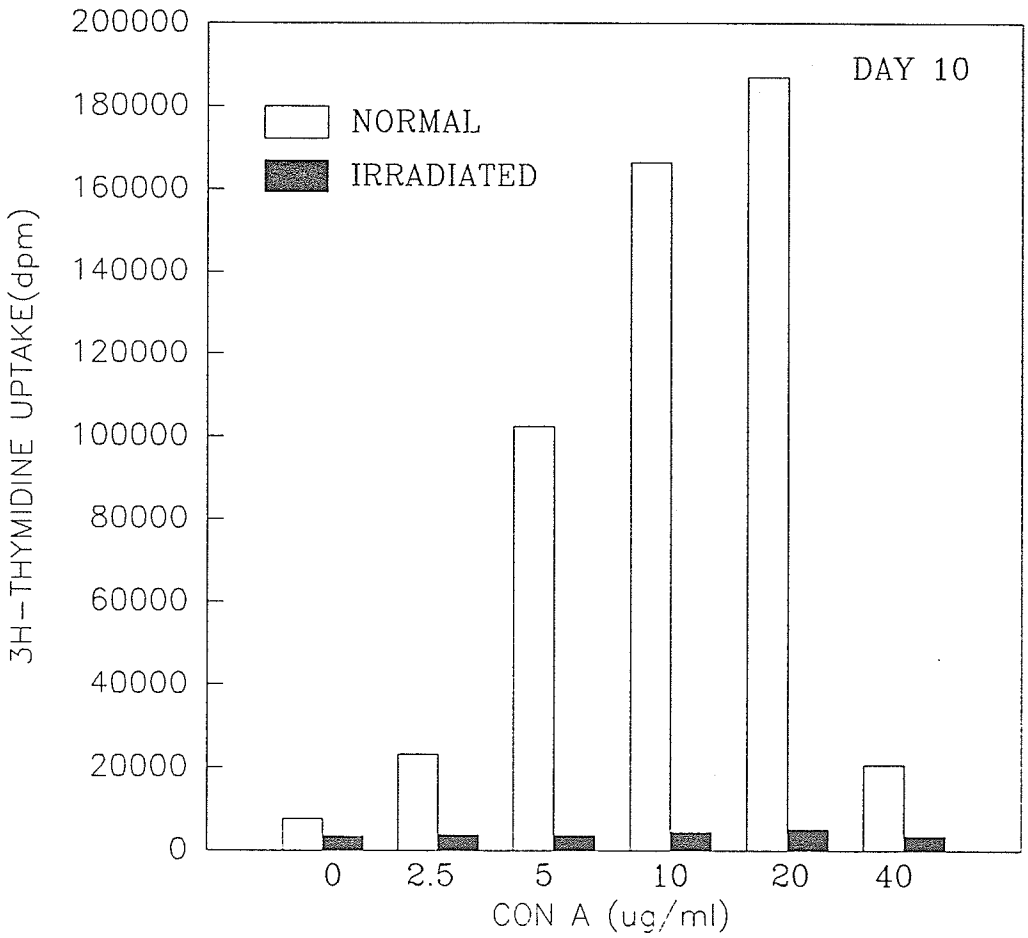
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
3H-THYMIDINE UPTAKE(dpm)
DAY 10
NORMAL
IRRADIATED
0
2.5
5
10
20
40
CON A (ug/ml)

Figure 4

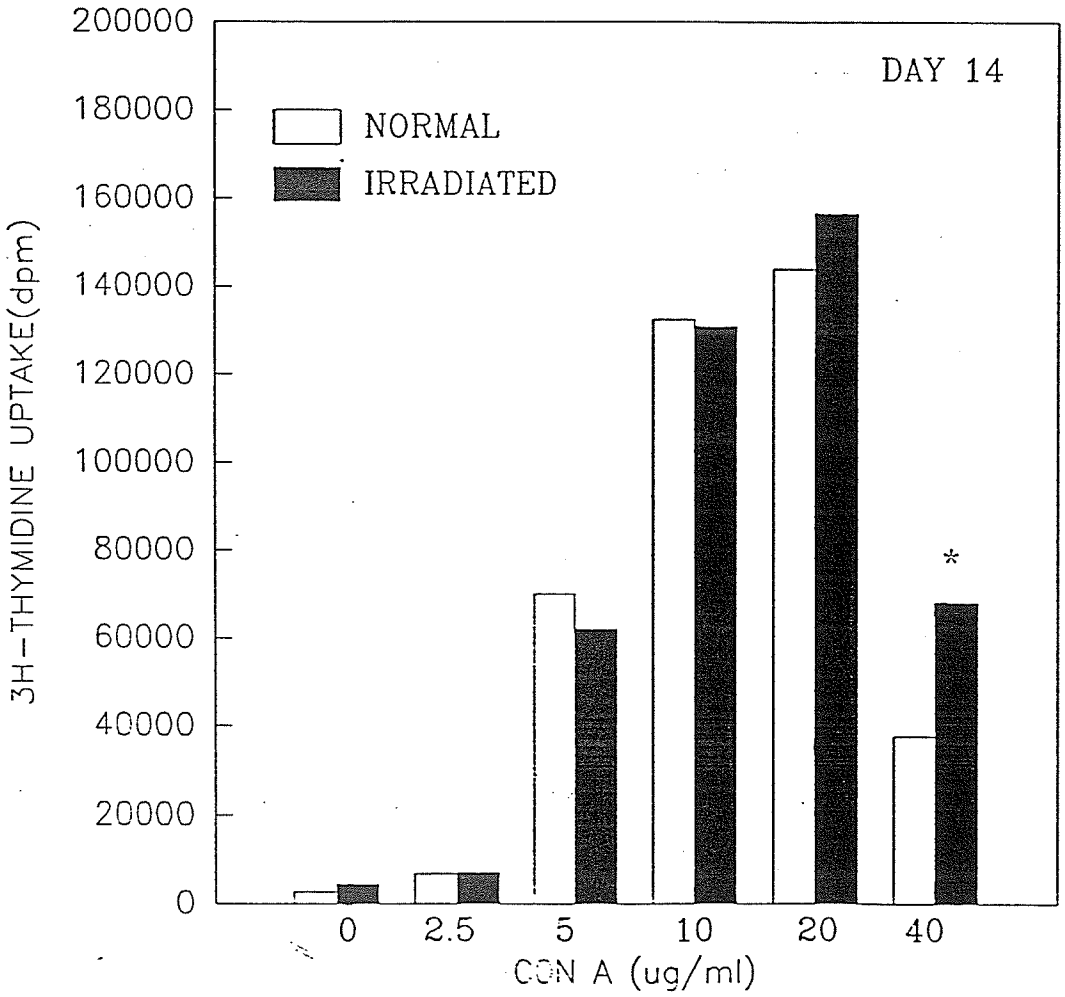
DAY 14
NORMAL
IRRADIATED
3H-THYMIDINE UPTAKE(dpm)
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
*
0
2.5
5
10
20
40
CON A (ug/ml)

Figure 5. Cryptosporidium oocysts shedding in unirradiated, unreconstituted; 550r CD4 + T cell reconstituted; 550r CD8 + T cell reconstituted and 550r unreconstituted neonatal BALB/c mice. The daily mean of the number of fecal oocysts (± SE) seen in 10 microscopic fields is shown. A minimum of 60 fields/data point were counted. No significant differences were found in the onset, peak, clearance and intensity of infection (as examined by oocysts shedding) in the above groups of mice. ($p > 0.05$). One of two similar experiments is shown.

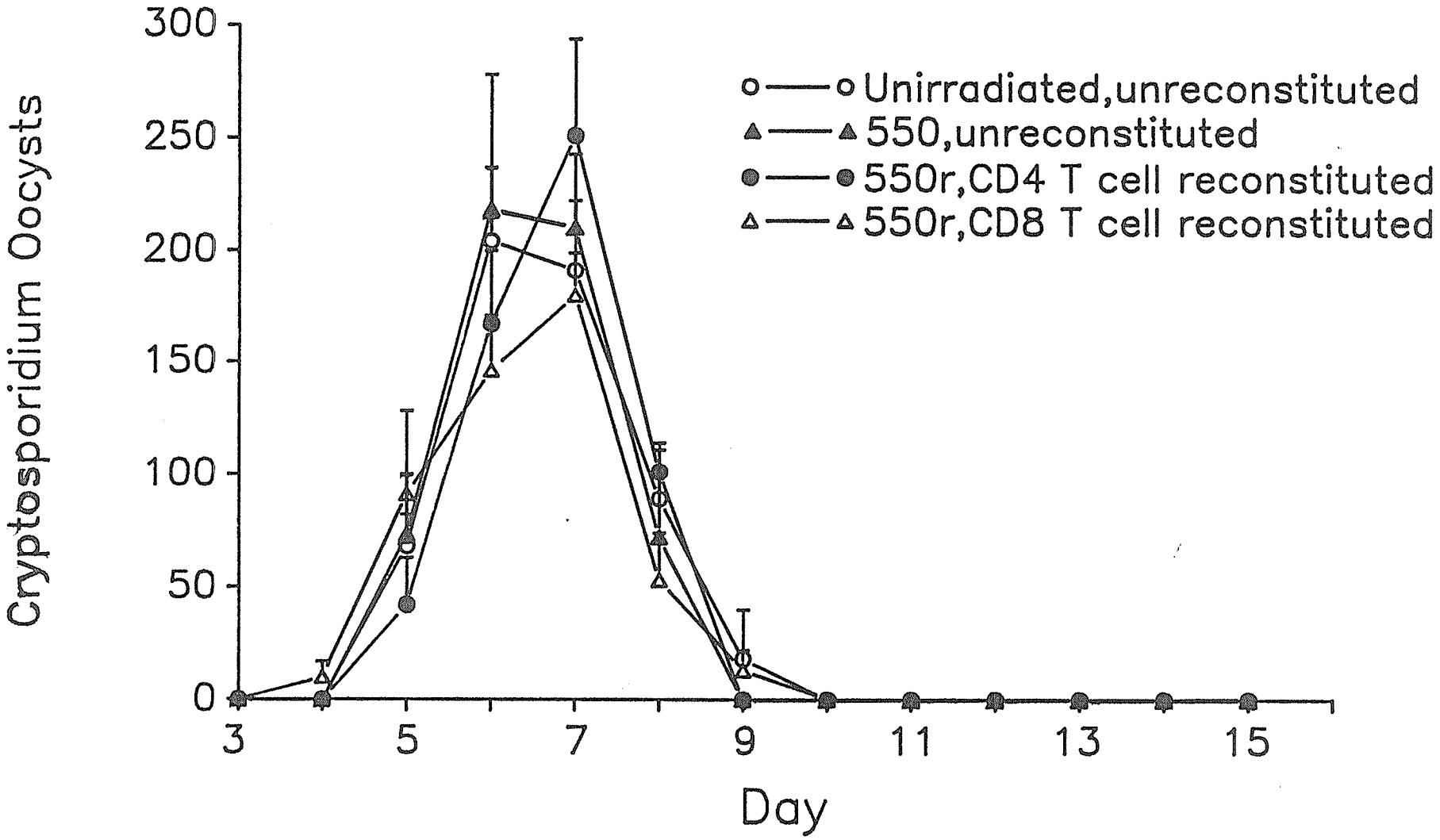
Cryptosporidium Oocysts
300
250
200
150
100
50
0
3
5
7
9
11
13
15
Day
Unirradiated,unreconstituted
550,unreconstituted
550r,CD4 T cell reconstituted
550r,CD8 T cell reconstituted

Figure 5

Figure 6. Cytokine production in response to polyclonal in vitro stimulation in BALB/c mice. The relative capacities of 4 day old, 1 week, 2, 3, 4, 6, 9 and 12 weeks old BALB/c mice to produce cytokines IFN-gamma, IL-2 and IL-4 following polyclonal in vitro stimulation of spleen cells with anti-CD3 mAb (145 2C11) was determined. Spleen cells were also cultured in the absence of anti-CD3 mAb. Supernatants were collected and assayed for IFN-gamma, IL-2 and IL-4 as described in materials and methods. Minimal IFN-gamma production is observed prior to 2 weeks with large increases occuring in the capacity of mice to generate IFN-gamma between 2-12 weeks of age. An age dependent increase in IFN-gamma production by increasingly older mice is observed. In contrast, IL-4 production looks "mature" by 2 weeks of age while IL-2 production by 1-4 weeks of age in BALB/c mice.

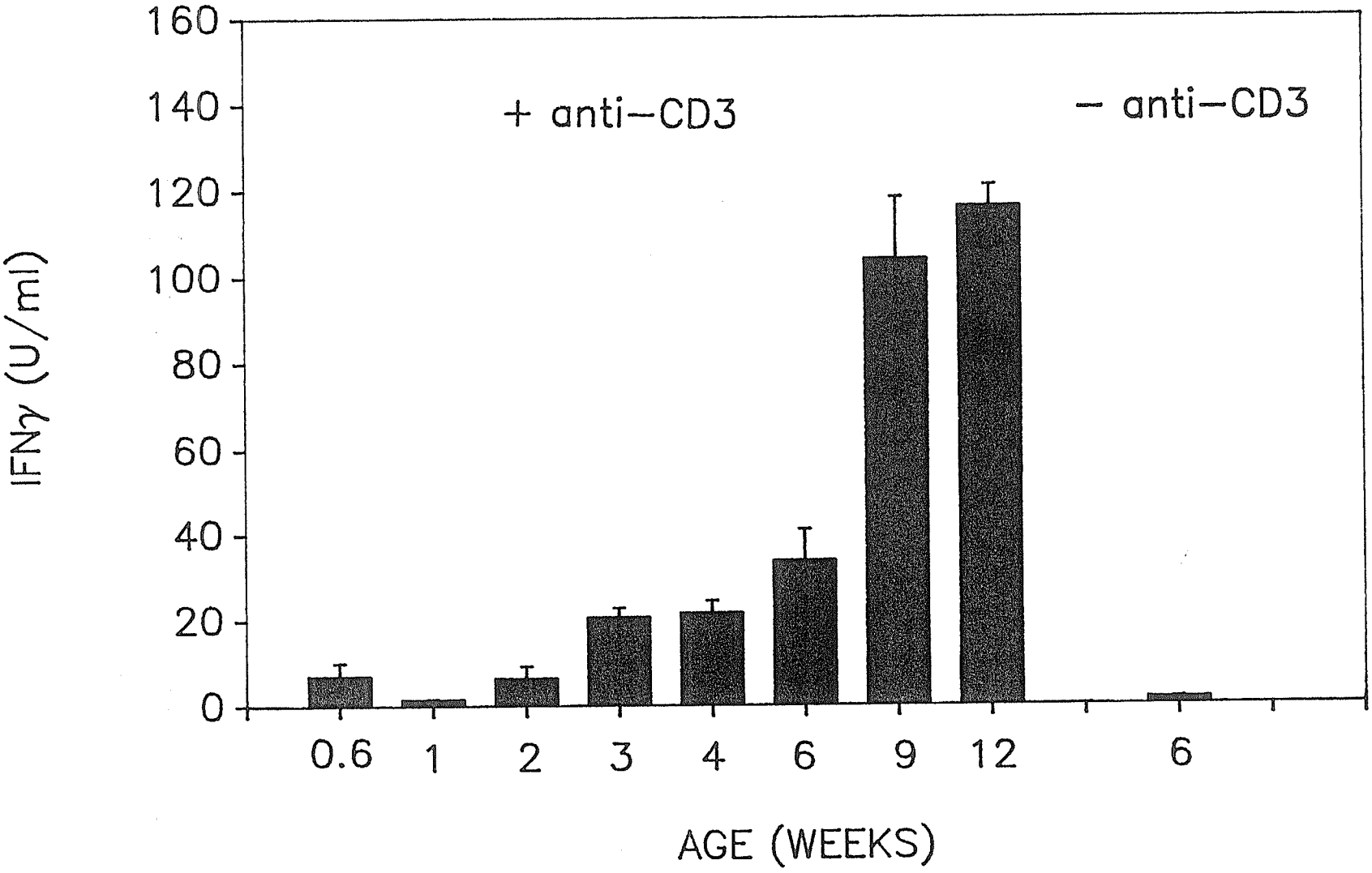
160
140
120
100
80
60
40
20
0
+ anti-CD3
- anti-CD3
IFNγ (U/ml)
0.6
1
2
3
4
6
9
12
6
AGE (WEEKS)

Figure 6

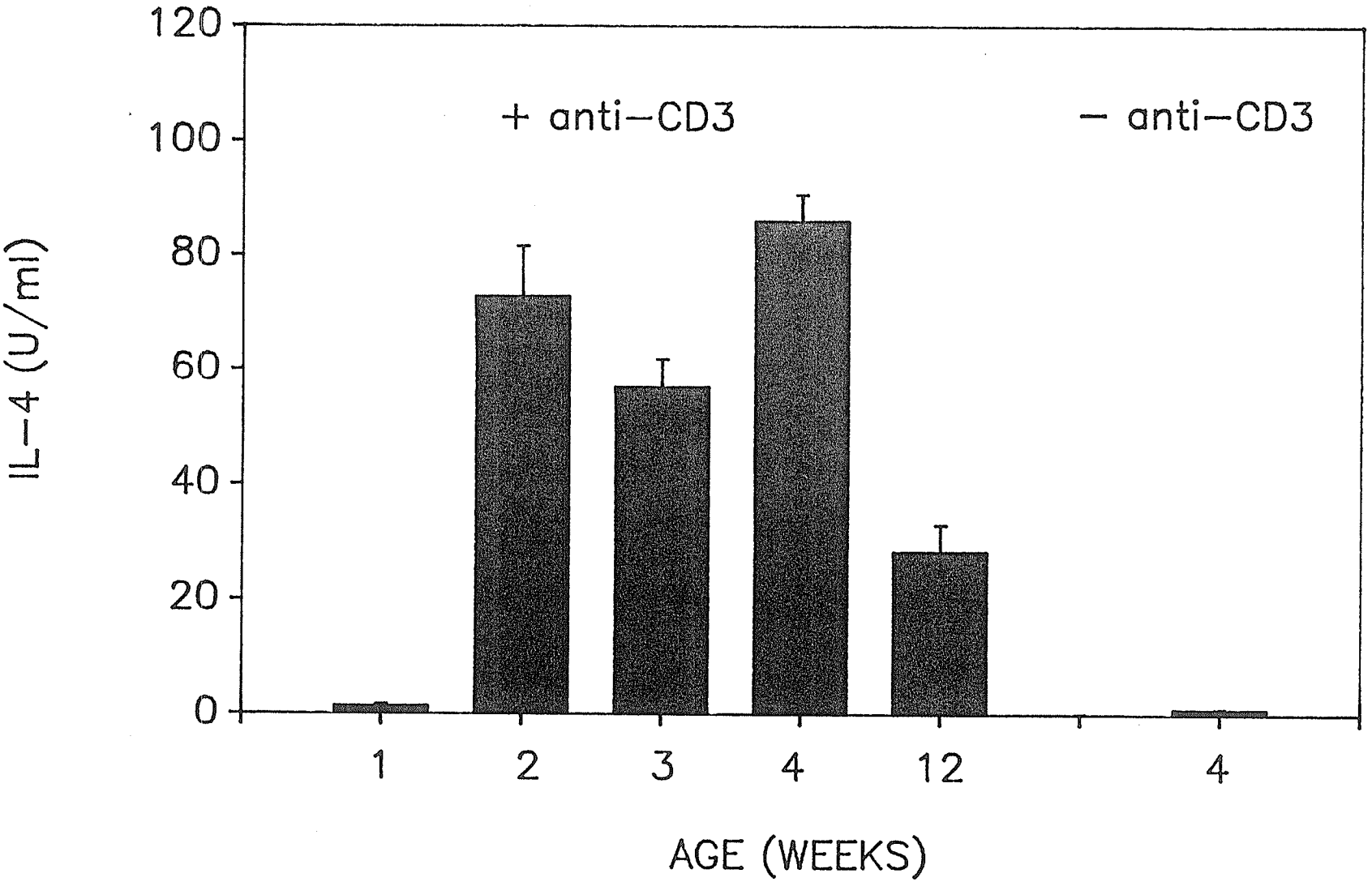
120
100
80
60
40
20
0
IL-4 (U/ml)
+ anti-CD3
- anti-CD3
1
2
3
4
12
4
AGE (WEEKS)

Figure 6

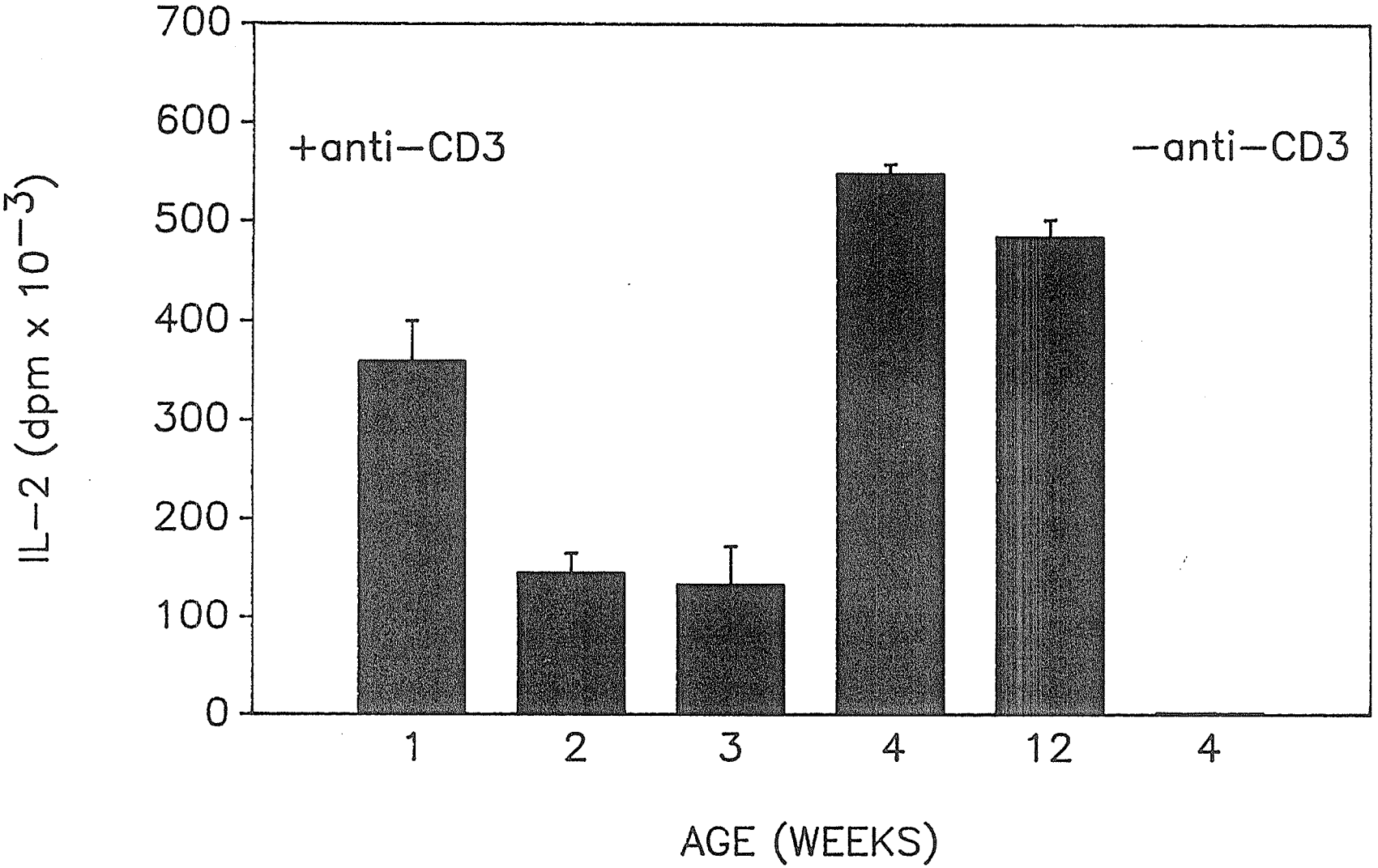
700
600
500
400
300
200
100
0
+anti-CD3
-anti-CD3
IL-2 (dpm x 10^{-3})
1
2
3
4
12
4
AGE (WEEKS)

Figure 6

Table 1. Cryptosporidium infection in normal neonatal BALB/c mice vs adult BALB/c mice.

Experimental Group	Cryptosporidium oocysts/ mouse	Excretion of oocysts (day) Onset	Peak	Recovery	Intensity* of infection
Neonates (8 day old)	5.0×10^3	5	7	12	997 ± 10
	2.5×10^4	6	7	12	877 ± 72
	1.0×10^5	5	7	12	1325 ± 187
	1.5×10^5	4	8	12	953 ± 39
Adults (8 weeks old)	4.0×10^4	-	-	-	<1
	1.0×10^5	-	-	-	<1
	1.5×10^5	-	-	-	<1
	1.0×10^6	-	-	-	<1
	3.0×10^6	-	-	-	<1

Dose titration of susceptibility of normal neonatal BALB/c mice and normal adult BALB/c mice to different doses of Cryptosporidium oocysts. No significant differences were noted in time of onset, peak and resolution of oocysts shedding in neonatal mice fed different doses of Cryptosporidium oocysts. Adult mice fed a very high dose of Cryptosporidium oocysts, failed to manifest disease. Oocysts not detected are indicated by (-). At least 60 microscopic fields were counted and mean number of oocysts/10 fields at the peak of infection (*) is shown. (± SEM).

Table 2. Absence of detectable IL-2 and IL-4 production during and following murine cryptosporidiosis.

Donor (BALB/c)	IL-2 production (dpm x 10^{-3}) to:					IL-4 production (U/ML) to:				
	Oocysts/well				anti-CD3	Oocysts/well				anti-CD3
	0	10^2	10^3	10^4		0	10^2	10^3	10^4	
Naive	<1	<1	<1	<1	87	<0.5	<0.5	<0.5	<0.5	39
Infected (day 8)	<1	<1	<1	<1	104	<0.5	<0.5	<0.5	<0.5	47
Infected (day 15)	<1	<1	<1	<1	71	<0.5	<0.5	<0.5	<0.5	30

Spleen (Table 2) and MLN (not shown) cells were obtained 8 and 15 days post-feeding of 1.5 x 10^5 oocysts to 8 day old BALB/c neonates. Spleen cells cultured with anti-CD3 mAb (145 2C11) were used as a positive control. Cells were cultured with increasing concentrations of Cryptosporidium oocyst antigens for 20 hours and cytokine production was assessed, as described in materials and methods. One of four similar experiments is shown.

Table 3. Phenotype of cells responsible for IFN-gamma production in Cryptosporidium infected neonatal BALB/c mice.

Culture conditions	Stimulus (Oocysts/well)	IFN-gamma (U/ML)
Spleen	0	<1
	10^3	<1
	10^4	14.7
Spleen+anti-CD4	0	<1
	10^3	<1
	10^4	1.1
Spleen+anti-CD8	0	<1
	10^3	<1
	10^4	2.0
Spleen+anti-CD4+ anti-CD8	0	<1
	10^3	<1
	10^4	<1
MLN	0	12.6
	10^3	28.4
	10^4	64.4
MLN+anti-CD4	0	<1
	10^3	<1
	10^4	4.2
MLN+anti-CD8	0	6.0
	10^3	8.6
	10^4	39.5
MLN+anti-CD4 +anti-CD8	0	<1
	10^3	<1
	10^4	<1

Spleen and MLN cells were obtained 8 days post-feeding (peak of infection) of 1.5×10^5 oocysts to 8 day old BALB/c neonatal mice. Cells were cultured for 20 hours, with increasing concentrations of purified Cryptosporidium oocysts in the presence and absence of anti-CD4, anti-CD8 and anti-CD4+anti-CD8 mAbs. IFN-gamma production was assessed by ELISA as in materials and methods. One of two similar experiments is shown.

Table 4. Effects of 550r whole-body irradiation on neonatal resistance to Cryptosporidium infection.

		Intensity of fecal oocysts shedding (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	0.5	2.0	0.1
	3	-	-	0.5	2.8	32	279	1.5	0.5	0.5	0.3	0.2	-
Normal control mice	1	-	-	13	246	77	97	2.0	3.0	1.5	2.5	-	-
	2	-	0.6	109	1.6	8.0	2.1	0.5	-	1.0	-	-	-
	3	-	-	8.3	189	307	18	4.3	-	0.8	-	-	-

No significant differences were observed, when time of onset, peak and resolution, as well as peak intensity (underlined) of fecal oocysts shedding were compared between irradiated and normal control (infected,non-irradiated) mice. In all cases SEM was <20%. In some time points oocysts were not detected and are indicated by (-).

Table 5. Absence of demonstrable T cell responses in irradiated BALB/c neonates.

Cell donor	IFN-gamma production (U/ML)		Con A (S.I.)	
	day 8	day 15	day 8	day 15
Unirradiated BALB/c neonates	47	13	93	140
Irradiated (550 r) BALB/c neonates	<2	<2	0.9	1.2

Peak IFN-gamma production (day 8 p.i.) and IFN-gamma production after recovery (day 15 p.i.) was determined by ELISA following in vitro culture of MLN cells with 10^4 oocysts/well. Irradiated neonates failed to show any IFN-gamma production. (One of two similar experiments). T cell proliferation assay was carried out on spleen cell populations (as described in materials and methods). Spleen cells were cultured, exposed to the T cell mitogen Con-A, pulsed with ^{3}H-thymidine and thymidine uptake was determined by liquid scintillation counting. Stimulation index (S.I.) for spleen cell populations of irradiated neonates exposed to Con-A was less than 2.

Table 6. *Cryptosporidium* infection profile for neonatal normal BALB/c mice vs neonatal BALB scid/scid mice.

1 Experimental group	2 Innoculum (oocysts)	3 Excretion of oocysts (day) Onset	Peak	Recovery	4 Infection intensity
BALB scid/scid neonates	5.0×10^3	4	5	9	487 ± 46
	2.5×10^4	7	7	11	581 ± 43
	1.0×10^5	7	7	10	1037 ± 125
	1.5×10^5	6	9	15	662 ± 81
Normal BALB/c neonates	5.0×10^3	5	7	12	743 ± 92
	2.5×10^4	5	7	12	820 ± 74
	1.0×10^5	5	7	11	1325 ± 188
	1.5×10^5	6	7	14	489 ± 57

No significant differences were observed when time of onset, peak and resolution, as well as peak intensity of fecal oocysts shedding were compared in infected normal BALB/c and BALB scid/scid neonates. (1), Neonatal mice used (8 day old). (2), Number of *Cryptosporidium* oocysts fed/mouse. (3), Time of onset, peak and recovery in experimental groups of mice. (4), Average number of *Cryptosporidium* oocysts observed/10 fields (± SEM). In all cases S.E. was <15%.

Table 7. IFN-gamma production by adult BALB/c mice following restimulation with Cryptosporidium oocysts.

Donor cells	Cryptosporidium oocysts fed/mouse	In vitro restimulation (IFN-gamma production,U/ML)		
		Cryptosporidium oocysts		
		0	10^4	anti-CD3
		-	---	--------
Spleen	10^6	day 5	day 5	day 5
		<0.5	25.4	56.7
		day 8	day 8	day 8
		<0.5	13.7	91
MLN	10^6	day 5	day 5	day 5
		<0.5	<0.5	9
		day 8	day 8	day 8
		<0.5	<0.5	5
Spleen	0	<0.5	<0.5	day 8
				52.3
MLN	0	<0.5	<0.5	19

Spleen and MLN cells were obtained 3, 5 and 8 days post-feeding of 10^6 oocysts to adult (4 week old) BALB/c mice. Cells were cultured for 18 and 36 hours with 10^3, 10^4 purified Cryptosporidium oocysts and anti-CD3 mAbs. IFN-gamma production was assessed as in materials and methods. IFN-gamma responses peak 5 days post-innoculation of Cryptosporidium oocysts. Three days post-feeding yeilds similar pattern of IFN-gamma production. In vitro restimulation with 10^3 oocysts did not elicit IFN-gamma responses. Spleen and MLN cells from adult unfed BALB/c mice served as controls. One of 3 similar experiments.

DISCUSSION

DISCUSSION

Despite a number of experimental studies, little is resolved about the effector mechanisms involved in resolution of infection with the protozoan parasite Cryptosporidium. Laboratory models are usually described as "permissive" or "non-permissive" hosts according to their capacity to allow a parasite to reach sexual maturation. The mouse is an example of a permissive host for the coccidian parasite Cryptosporidium and is widely used as an animal model to study mammalian cryptosporidiosis.

Experimental Infection of Mice.

Cryptosporidiosis was elicited by feeding Cryptosporidium oocysts to 8 day old neonatal mice. The presence of disease and its severity was semi-quantitatively evaluated by determining the number of oocysts present in stool samples obtained over the course of infection. Other investigators have used methods like histopathology which have greater sensitivity than our selected method of oocysts enumeration. But these methods are not as quantitative as the one we and several other investigators have used (56, 58, 61, 129).

Calf feces containing Cryptosporidium oocysts were obtained from the Provincial Veterinary Laboratory. These oocysts were used for experimental infection of neonates. Our

observations made on the experimental infection of neonatal mice are:

a) The neonatal mice developed intestinal cryptosporidiosis as demonstrated by the large number of oocysts that were detected in their feces.

b) For all infected mice; onset of oocysts excretion was 4-5 days post-feeding, peak of oocysts excretion was 7-8 days post-feeding and the number of oocysts shed declined to zero by 11-12 days post-infection.

c) All the experimentally infected mice survived the infection. No mortality was observed in any of the neonatal mice.

Purification of Oocysts.

Several methods for purification of oocysts have been suggested (66, 67, 133). Flotation in Sheather's sugar solution or in 33% zinc sulphate and sedimentation with Formalin-ether or Formalin-ethyl acetate are effective concentration techniques for clinical diagnosis of cryptosporidiosis (127). Fecal oocysts obtained by flotation techniques were found to be heavily contaminated with fecal material. The usefulness of concentrates for immunological studies is limited by non-specific binding caused by yeasts, fecal bacteria and vegetable matter. Toxoplasma oocysts recovered from cat feces have been similarly contaminated using Sheather's flotations leading investigators to develop

alternative recovery methods (134).

A modification of the $CsCl_2$ density gradient centrifugation (133), was sucessfully carried out. Large numbers of viable, contaminant free Cryptosporidium oocysts were obtained from the $CsCl_2$ gradients. About 70% recovery was obtained. These oocysts were suitable for immunological studies. Purification of Cryptosporidium oocysts by this labor intensive process is of particular value for in vitro applications.

Role of Humoral Immunuty in Cryptosporidium Infection.

Many of the early case reports of human cryptosporidiosis were from patients exhibiting Ig deficiencies such as congenital hypogammaglobulinemia (96, 99). Cryptosporidium specific serum antibody production is commonly noted in normal humans and animals (60, 100) over the course of self-resolving infections. Thus, stimulation of humoral immunity by the use of vaccines could be a potentially promising approach for prevention of cryptosporidiosis. The potential therapeutic role of passively administered anti-Cryptosporidium antibodies from hyperimmune xenogeneic colostrum (102), serum (101) or monoclonal antibodies (103) further supported this strategy.

Our laboratory recently investigated the role of humoral immunity in Cryptosporidium infection (129). Using an experimental animal model of cryptosporidiosis, the protective role of antibody responses in protection from and resolution

of the disease was examined. Purified oocysts (1 x 10^5/mouse) were fed to 7-8 day old neonates (BALB/c mice) thus eliciting cryptosporidiosis. The presence of disease and its severity was quantitatively evaluated by determining the number of Cryptosporidium oocysts present in stool samples obtained over the course of infection (14-21 days). The disease pattern observed in these mice was found to be independent of the intensity of the specific antibody response (predominantly IgM Abs) produced in the mice. The relative susceptibility of normal and B-cell deficient BALB/c mice to Cryptosporidium infection was examined. Chronic treatment of mice beginning at birth, with rabbit anti-mouse IgM Abs (anti-u) yielded mice that were virtually B cell deficient (104). These mice displayed very few (<5%) sIg expressing B cells in spleen or lymph nodes and had extremely reduced levels of serum and gut immunoglobulins of all isotypes. The majority of T cell and macrophage functions were found to be intact (104, 105). It was found that although normal neonatal BALB/c mice exhibited good IgM and IgG antibody responses to Cryptosporidium; no correlation could be drawn between the intensity of these responses and duration or severity of infection. No significant differences in the frequency, intensity or duration of cryptosporidiosis were found in B cell deficient, NR-Ig treated and untreated neonatal BALB/c mice, inspite of the absence of anti-cryptosporidial IgG, IgM, IgA and IgE responses in B cell deficient mice. Adult BALB/c mice were

treated with doses of Cryptosporidium oocysts ten times the dose required to infect 100% of normal Ig producing neonates. The adult mice were not susceptible to infection. These results suggest that the role of specific in vivo antibody responses in the resolution of murine cryptosporidiosis is minor. Whether these results observed in the murine system can be readily extrapolated to humans remains to be seen. Thus, it would be essential to evaluate the likelihood of success of strategies used to develop cryptosporidial vaccines that are aimed to stimulate humoral immunity.

Role of Cellular Immunity (T Cell Mediated Immunity) in Cryptosporidiosis.

Investigators have used congenitally athymic (nude, Nu/Nu) mice to study T cell immunity in the resolution of cryptosporidiosis. These nude mice were shown to be infected with the parasite Cryptosporidium for more prolonged periods as compared to their age matched controls with functional T cell repertoires (Nu/+) (106, 118). These T cell deficient mice exhibited persistent infections which had several characteristics in common with those observed in immunocompromised humans. But it is difficult to attribute the increased severity of the disease in nude mice to one specific immunological mechanism (defect), because such mice exhibit very low antibody levels (due to T-helper cell depletion) and poor macrophage function (107).

The present studies were undertaken to examine the role of specific T cell immunity in cryptosporidiosis. The role of this arm of the immune response in protection from and resolution of Cryptosporidium infection was studied in an experimental animal model of human infection with Cryptosporidium.

A) Cryptosporidiosis in CD4 T Cell Deficient Neonatal and Adult Mice.

The prevelance of severe cryptosporidial diarrhea in AIDS patients correlates well with the stage of the disease and is inversely related to levels of CD4 + T cells. It is not known whether this reflects a direct or secondary effect of CD4 T cell depletion/dysfunction. Previous studies carried out in animal models have used systemically derived T-lymphocytes, CD4 enriched T-lymphocytes or serum IgS instead of gut derived lymphocytes and secretory antibody, making the results of such studies difficult to interpret. We used an alternate approach by utilizing "immunologic surgery" in which specific lymphocyte subsets are depleted from the peripheral circulation and gut by administration of specific monoclonal antibodies.

Utilization of neonatal BALB/c mice deficient in CD4 +T cells produced by in vivo administration of purified anti-CD4 mAb provides an animal model (immunocompromised model) for studying the role of CD4 +T cells in Cryptosporidium infection

of mice. Recently, Ungar et al (106) observed that depletion of CD4 +T cells leads to an increase in the intensity and duration of Cryptosporidium infection in neonatal mice. The authors argue that a lack of T cells is crucial to the establishment of persistent Cryptosporidium infection, and reconstitution with a T cell population that includes CD4 + T cells was necessary for successful recovery from cryptosporidiosis, a finding that differs from our observations.

In contrast to our data, Ungar et al recently reported that low levels of cryptosporidial infection could be estsblished in CD4 depleted adult BALB/c mice (110). This was obtained following the administration of 200 fold larger Cryptosporidium innocula (than that used in our experiments for neonatal infection). These mice exhibited extremely low levels of Cryptosporidium infections; oocysts shedding was 0.07-0.31 oocysts/10 microscopic fields in contrast to the 300-800 oocysts/10 fields typically observed following infection of neonates. The conclusion from their studies is that CD4 T cell depletion of adults leads to extremely low level Cryptosporidium infection. In our experiments CD4 depleted neonatal mice received 1.5 x 10^5oocysts/mouse (2 logs less than 1.0 x 10^7oocysts/mouse given by Ungar et al), and cleared their infection in about 2 weeks. From our experiments it was concluded that in vivo CD4 T cell depletion in neonates markedly enhances the intensity of infection and causes a

protracted disease course, but even in the absence of CD4 T cells, the infection is spontaneously resolving without mortality in any of the groups examined. The multiple cycles of oocysts shedding observed in CD4 T cell deficient mice may suggest that the phenomenon of autoinfection (infectious sporozoites released from the thin-walled oocysts penetrate into the microvillous border of enterocytes) is prevelant in these mice. Although in vivo CD4 T cell depletion results in more intense and protracted infection in neonates, it fails to alter the resistance of adults (normal or anti-CD4 mAb treated) to infection. Adult anti-CD4 mAb treated mice exhibited <3% CD4 T cells but were not found to be susceptible to cryptosporidiosis even at 10 fold increases in the dose of oocysts administered. The above observations thus suggest that CD4 T cells may play an important but ancillary role in resolution of Cryptosporidium infection in neonatal mice.

B) In Vitro T Cell Proliferation.

Our studies examined Cryptosporidium specific proliferative responses by spleen cells and MLN populations. There was failure to elicit significant T cell proliferation in response to in vitro restimulation with purified intact oocysts while immobilized anti-CD3 mAb or Con-A elicited strong proliferative responses in neonatal infected and uninfected BALB/c mice. In the current experiments, sacrifice of Cryptosporidium fed neonates at induction, peak, and

following recovery of infection gave similar results. In contrast, it was observed previously in our laboratory (Kilani.R.T.) that T enriched spleen cells or unseparated lymph node cells obtained from Cryptosporidium (CFA) immunized mice yielded strong proliferative responses following in vitro restimulation with oocysts (intact, sonicated). This implied that oocysts are good immunogens in vitro and that Cryptosporidium-reactive T cells are present in the neonatal T cell repertoire.

C) Cytokine Production in Vitro in Response to Cryptosporidium.

As an alternative approach to characterize T cell activation upon Cryptosporidium infection, we examined cytokine production following antigen specific in vitro restimulation with purified Cryptosporidium oocysts or immobilized anti-CD3 mAb as a positive control. Short term in vitro restimulation of MLN and spleen lymphocytes obtained prior to onset, peak and following recovery of infection failed to elicit any detectable IL-2 or IL-4 production (<1 U/ml). In marked contrast, strong IFN-gamma responses were elicited following in vitro culture of spleen and MLN cells obtained from Cryptosporidium infected BALB/c neonates, but not from normal uninfected mice. These were coincident with peak neonatal infection. Kinetics of IFN-gamma production revealed that it was rarely detectable 3 days p.i. and

approximately 50-70% of maximal at day 5 post-feeding (prior to oocyst shedding) or day 15 p.i. (recovery). Maximum IFN-gamma production was observed 8 days post-innoculation of oocysts.

Adult mice fed 1.5 x 10^6 oocysts/mouse (300 times the minimal infective dose of oocysts for neonates) failed to become infected (on the basis of fecal oocysts shedding) yet generated strong IFN-gamma production in response to in vitro stimulation with Cryptosporidium oocysts. IFN-gamma responses peaked 5 days post-innoculation of oocysts. Moreover, an age dependent increase in IFN-gamma production following polyclonal stimulation of increasingly older mice (oocysts not administered) was observed.

Thus, the induction of IFN-gamma production by normal neonates and adults, early, following the feeding of oocysts, suggests a significant role for IFN-gamma synthesis in resistance to and resolution of Cryptosporidium infection in vivo.

Further experiments (in vitro and in vivo) were carried out to determine the phenotype of cells responsible for IFN-gamma production in Cryptosporidium infected BALB/c mice.
In vitro:- We cultured spleen (representing the systemic response) and MLN cells (selected as they provide a ready source of Peyer's patch derived T cells) from Cryptosporidium infected BALB/c mice with purified Cryptosporidium oocysts and anti-CD4, anti-CD8 and anti-CD4+anti-CD8 mAbs. When IFN-gamma

production was assayed, we found that anti-CD4 mAb abolishes IFN-gamma production, anti-CD8 mAb somewhat decreases it while anti-CD4+anti-CD8 mAb abolish IFN-gamma production completely. In vivo:- Using the immunocompromised animal models (by in vivo treatment of anti-CD4, anti-CD8 and anti-CD4 + anti-CD8 mAbs in BALB/c mice), an alternate approach was used to determine the phenotype of cell responsible for IFN-gamma production in Cryptosporidium infected neonates. When IFN-gamma production was assayed, similar results were observed as in the in vitro experiments (data not shown). From the results obtained, we suggest that IFN-gamma production by Cryptosporidium infected BALB/c neonates is a CD4+T cell dependent and possibly to a lesser extent a CD8+T cell dependent mechanism. These results need to be confirmed, the next approach being : Determining the cytokine production by CD4 and CD8 enriched T cell populations from Cryptosporidium infected mice (prepared in vitro by using anti-CD4 mAb, anti-CD8 mAb and complement or using positively selected CD4 and CD8 T cell populations obtained by flow cytometry). Stimuli that could be used are Con-A, Cryptosporidium oocysts, recombinant protein to detect antigen specific responses and an unrelated antigen (ovalbumin) as a control. Alternatively, cytokine responses by gut derived cells i.e.IEL and identification of cytokine secretion patterns of IEL derived alpha-beta and gamma-delta T cells can be looked at. Although our studies demonstrate that the source of IFN-gamma may be of

T cell origin (CD4 T cell), studies carried by Ungar et al (110) on nu/nu mice suggest that at least some of this cytokine is not of T cell origin. The authors suggest that NK cells (natural killer cells) which have been shown to secrete IFN-gamma in some systems are not the only non-T cell source of IFN-gamma in Cryptosporidium infected mice, because rabbit anti-asilo-GM1 Ab, which kills splenic NK cells failed to increase oocysts shedding in Cryptosporidium innoculated nu/nu mice. By using NK 1.1 mAb a NK cell deficient mouse model can be obtained which can then be used to monitor disease profile and IFN-gamma production by NK cells.

D) Cryptosporidium Infection in Irradiated Mice.

To futher investigate the role of T cells in clearance of Cryptosporidium infection; irradiated (550 rad) BALB/c neonates were used. To better characterize the role played by CD4, CD8 T cells and cytokine production in resolution of cryptosporidiosis; neonatal mice were irradiated, reconstituted with CD4 enriched T cells, CD8 enriched T cells or left unreconstituted and then fed Cryptosporidium oocysts. Surprisingly, irradiated, CD4 reconstituted, CD8 reconstituted and unreconstituted neonates all exhibited infections that were simillar to the infection seen in normal controls (non-irradiated, unreconstituted). This suggests that under the experimental conditions tested, specific immune responses may not be required for resolution of cryptosporidiosis. Our

hypothesis is that in healthy normal neonates the intestinal immunity to Cryptosporidium infection may be primarily mediated by phagocytic cells. Cryptosporidium infection normally leads to the induction of CD4 T cell dependent IFN-gamma production. IFN-gamma activates the phagocytic cell function in the small intestine (the primary site of murine cryptosporidial infection), resulting in enhanced phagocytic capacity and eventual elimination of Cryptosporidium and resolution of the infection. Consistent with this hypothesis is our observation that absence of detectable specific immune responsiveness (as seen by the absence of IFN-gamma, IL-2 and IL-4 production following antigen-specific (oocysts) or polyclonal (Con-A) stimulation) does not interfere with the capacity of irradiated neonates to resolve cryptosporidial infection. We observed that despite the absence of detectable T cell responses in irradiated mice, the capacity of these neonatal mice to resolve the infection was not impaired. Moreover, irradiated adult mice fed Cryptosporidium oocysts failed to excrete oocysts. The above results leads to our hypothesis that in this irradiation model resolution of cryptosporidiosis is associated with, but not dependent on IFN-gamma production. Also, most lymphocytes are sensitive to as low as 50rads of radiation whereas phagocyte (macrophage) activity is markedly enhanced by irradiation (135). This suggests that though the resolution of neonatal murine cryptosporidiosis may be aided by IFN-gamma production, it is

primarily mediated by cells which are radioresistant.

Thus, we were presented with a paradoxical situation in which there was an intense antigen driven IFN-gamma response to Cryptosporidium oocysts, while irradiated neonates who have no demonstrable T cell mediated immunity (as demonstrated by the absence of antigen specific or polyclonally driven proliferation or IFN-gamma synthesis) have the ability to recover from cryptosporidiosis, exhibiting a disease pattern similar to that of normal controls. O'Garra.A. et al established one of the important activities of IFN-gamma as a macrophage activating factor. Recently, studies have been carried out on lethally irradiated bone marrow chimeras, demonstrating that moderate doses of gamma radiation have a similar macrophage activating effect (135).

IFN-gamma contributes to protective immunity against several intracellular microorganisms by inducing macrophage enzymes that allow these cells to kill the ingested parasite (136). A similar mechanism may have a role in the IFN-gamma contribution to protective immunity against Cryptosporidium. Alternative possibilities may include a direct toxic effect of IFN-gamma on Cryptosporidium, induction of the production of other cytokines that may be toxic to the parasite (i.e.TNF-alpha) and induction of changes in gut epithelium that make it less favourable for the proliferation and survival of the parasite.

We hypothesize that the speed and efficiency with which

irradiated mice resolve Cryptosporidium infection in the absence of specific immunity results from irradiation dependent (rather than IFN-gamma dependent) phagocyte activation. Whole body irradiation (550 rad) results in elimination of specific immune responses but results in non-specific activation of macrophages (phagocyte activity), which resolves the infection in absence of detectable IFN-gamma production. Thus, we can speculate that the role normally played by T cell dependent IFN-gamma production in activating the macrophages, is replaced by the non-specific effects of this dose of radiation in the irradiated mice. The mechanism and effects of radiation on non-specific macrophage activation (in vivo and in vitro) has been studied by several investigators using animal models. Elzer et al (137) observed that sublethal irradiation of mice infected with Brucella abortus caused significant reductions in the bacterial numbers as compared to the unirradiated controls. Rats immunized with irradiated Schistosoma japonicum cercariae (138) were examined for macrophage activation in terms of spreading, adhesion and ingestion of sheep erythrocytes and pinocytosis of horse-radish peroxidase. Macrophage from the above rats was found to be more active than from the normal rats. Spontaneous IL-1 secretion was found to be increased in patients undergoing postoperative radiation treatment (139) which was an indication of macrophage activation. Yui.S et al (140) observed that ultraviolet radiation caused the peroxidation of

phosphatidylserine and this resulted in the augmentaion of its macrophage growth-stimulating activity. In vivo radiation of mice was found to increase Prostaglandin E2 and leukotriene C4 synthesis (141) and elevation of the activities of acetyltransferase and acetylhydrolase in the adherent peritoneal cells. The use of microwave radiation (142) was found to increase the adherence, opsonization, phagocytosis, nitroblue tetrazolium reduction and antibody dependent cell cytotoxicity of peritoneal macrophages from BALB/c mice. Irradiated mice showed a spontaneous release of Prostaglandin E and tumor cytostatic activity by liver macrophages of Listeria monocytogenes infected mice (143). The authors suggest that these radioresistant Kupffer cells can be activated to express enhanced effector function during in vivo infection. The precise mechanism involved in the non-specific activation of macrophages by whole-body irradiation in Cryptosporidium infected mice needs to be investigated.

Collectively our data indicate that under normal conditions (i.e.in the absence of experimental irradiation), two immunological processes would be responsible for resolution of cryptosporidiosis : a) Activation of Cryptosporidium-specific T lymphocytes to produce IFN-gamma and b) Clearance of Cryptosporidium infection by IFN-gamma activated phagocytes. One piece of evidence in support of this hypothesis comes from studies of murine Listeria monocytogenes infection (76) in which, this parasite elicits IFN-gamma

production which is secondary to the phagocyte dependent protection necessary for disease resolution. The above hypothesis leads to two main predictions :

A) Even in the presence of CD4 T cell dependent IFN-gamma production, if macrophage function in vivo were depleted, then there would be a significant increase in the disease intensity in Cryptosporidium infected neonates. Preliminary experiments (not shown) demonstrate that silica treatment results in Cryptosporidium infection 400-600% more intense than that observed in normal controls. Silica has been reported as an agent which selectively inactivates macrophages (94). To test the above prediction, two approaches can be examined : 1) Cohorts of normal and Cryptosporidium infected neonates can be treated with silica to deplete phagocytic cell function prior to infection (oocyst innoculation) i.e.day 0; in order to eliminate T cell activation and therefore induction of IFN-gamma production and phagocytic cell function. 2) An alternative approach would be to deplete phagocytic cell function at the time of peak Cryptosporidium infection (day 8) resulting in abrogation of macrophage activity but having no effect on the ability of specific T cells (Cryptosporidium primed) to produce IFN-gamma. Our speculation is that abrogation of the phagocytic cell function, in the presence or absence of IFN-gamma production will have a severely negative effect on disease intensity in Cryptosporidium infection.

B) We predict that blocking of IFN-gamma function in

infected, unirradiated mice via in vivo administration of anti-IFN-gamma mAb (rat anti-mouse IFN-gamma hybridoma XMG 1.2) would enhance the severity of Cryptosporidium infection, thus providing indirect evidence of antigen specific induction of IFN-gamma production in protection from or resolution of cryptosporidiosis.

E) Cryptosporidium Infection in Adult Mice.

A number of previous studies have shown that adult mice are resistant to Cryptosporidium infection. Sherwood et al (144) reported that CBA nude mice innoculated at 28 days of age were not susceptible to Cryptosporidium infection. Also, nude mice deficient in certain T cell subsets, are relatively more resistant to cryptosporidiosis at 42 days of age than at 6 days of age (118). Even Ungar et al using gastric gavage and an extremely high dose of 1.0×10^7oocysts/mouse reported that CD4-deficient adult mice (by anti-CD4 mAb treatment) were much more resistant to infection than neonates (106). They did manage to infect adult nude mice using a large dose of oocysts (1.0×10^7oocysts/mouse) and gastric gavage to feed the mice; the infection was fatal within 4 months in most adult athymic mice while a few developed stable infections.

Recently Perryman et al (145) innoculated orally four week old female C.B-17/Icr/Imd-scid mice with 1.0×10^7 Cryptosporidium parvum oocysts. Examination of hematoxylin and eosin or giemsa-stained horizontal sections of

gastrointestinal tracts showed numerous organisms in the distal 2-3 mm of the pyrolic stomach and in the cecum, as early as 2 weeks and persisting for greater than 8 weeks p.i.

The mechanism of resistance of adult mice to cryptosporidiosis is not very well established. Harp et al (146), tested the role of a mature intestinal flora in the resistance of adult mice to Cryptosporidium parvum. Germfree CD1 and BALB/c mice were colonized at day 7 following innoculation with Cryptosporidium parvum oocysts, but age-matched conventional mice of the same strains 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. They suggest that activation of the immune system (eg:non-specific macrophage activation), by previous association with the intestinal flora, may contribute to the resistance of adult mice.

Some authors suggest that in mice, resistance to Cryptosporidium is related to age; neonates being more susceptible to infection while adults are resistant to the infection. The majority of human studies are consistent with the interpretation that age related differences are the result of acquired immunity i.e. likely to be exposure dependent. An interesting finding by Current.W.L. (1) and Crawford et al (4) was that in humans, children usually had a significantly

higher prevalence of diarrheal illness (caused by Cryptosporidium) than did adults. An increased rate of exposure to enteropathogens (Cryptosporodium, Giardia, Salmonella, Shigella, and Campylobacter), as a result of fecal-oral contamination and age-specific differences in host defence mechanisms was the suggestion made by Cohen (147) to account for the increased susceptibility to and severity of certain enteric infections in infants as compared to older children and adults.

Previous studies in our laboratory have shown that irradiated, B cell deficient adult BALB/c mice fed 1.5 x 10^6 oocysts/mouse could not be infected (Kilani.R.T). In our studies, adult mice 4 wks-9 wks old, fed increasing ammounts of Cryptosporidium oocysts (upto 3 x 10^6 oocysts/mouse) failed to be infected. Our experiments also showed that adult, anti-CD4 treated and irradiated BALB/c mice remained resistant to Cryptosporidium infection even when attempts were made to feed them with 300 fold higher doses of Cryptosporidium oocysts than those used to cause infection in neonates, an expected result based on previous data. Quantitative enumeration of oocysts in the fecal samples from adult mice did indicate the absence of manifestation of disease but does not exclude the possibility that histopathological examination of intestinal sections might reveal the parasite. CD4 deficiencies in adult mice, unlike in neonates does not cause diarrhea or heavy oocyst shedding which is observed in CD4 deficient neonates.

The reason for this difference between CD4 deficient neonates and adults remains unsolved.

We suggest a central role for IFN-gamma synthesis in protection from cryptosporidiosis in adult mice. In our study, adult BALB/c mice (4 week old) failed to be infected when innoculated with 1.5 x 10^6 oocysts/mouse. But on in vitro stimulation with purified Cryptosporidium oocysts, the spleen cells from these Cryptosporidium fed adult mice but not unfed, age-matched controls produce IFN-gamma (5 days p.i.). Also, an age dependent increase in IFN-gamma production following polyclonal stimulation (anti-CD3 mAb) of increasingly older mice (unfed) was observed (Figure 6). Large increases occur in the capacity of these Cryptosporidium unfed mice to generate IFN-gamma (following polyclonal stimulation) between 2-12 wks of age. This correlate with the loss of susceptibility to infection. These preliminary data suggest that the association between the ontogeny of T cell derived, IFN-gamma capability and the loss of susceptibility to Cryptosporidium should be investigated further.

This induction of IFN-gamma synthesis by adults very early (5 days post-innoculation) following feeding with Cryptosporidium oocysts and the observed large increases in the capacity of Cryptosporidium unfed mice to generate IFN-gamma between 2-12 wks of age is consistent with our hypothesis that IFN-gamma plays a significant role in resistance to Cryptosporidium infection in adults.

CONCLUSION

CONCLUSION

A useful murine model of human cryptosporidiosis was used and disease profile was evaluated following experimental infection by the oral route. Cryptosporidiosis was elicited in 8 day old neonatal mice by feeding of Cryptosporidium oocysts found in calf feces. The calves in turn were experimentally infected by the oral route.

For immunological studies, Cryptosporidium oocysts were purified successfully by a modification of the $CsCl_2$ density gradient centrifugation method. These purified Cryptosporidium oocysts were used for in vitro cell culture assays.

The primary objective of the work presented in this thesis was to begin characterization of the role played by cellular immunity (T cells) in protection from and resolution of Cryptosporidium infection using an experimental animal model of human cryptosporidiosis. We examined the role of CD4 T cells in host resistance using an immunocompromised model (CD4 T cell deficient) of cryptospotidiosis. Neonatal, CD4+T cell deficient mice exhibited markedly more severe diarrhea, significantly more fecal oocyst shedding and protracted infection than age-matched normal mice. However, the observation that infection in these neonates is self-resolving (as determined by fecal oocysts shedding) in the continued absence of CD4+T cells, and the inability of in vivo CD4 T cell depletion to alter the resistance of adult BALB/c mice to infection, led to our interpretation that CD4 T cells play an

important but auxillary role in resolution of Cryptosporidium infection.

We then examined the role of Cryptosporidium driven cytokine production in host resistance. Our results suggest a significant role of IFN-gamma in resistance to and resolution of Cryptosporidium infection in vivo. We were able to demonstrate that spleen and MLN cells from Cryptosporidium infected BALB/c neonates, but not non-infected ones, produce IFN-gamma in response to in vitro restimulation with purified Cryptosporidium oocysts. Also, adult mice fed Cryptosporidium oocysts, and which did not exhibit detectable infection, generated strong IFN-gamma responses upon in vitro stimulation with purified Cryptosporidium oocysts. This induction of IFN-gamma synthesis by normal neonates and adults very early (peak at 8 days post-innoculation for neonates and 5 days post-innoculation for adults) following feeding with Cryptosporidium oocysts is consistent with a significant role of IFN-gamma in resistance to and resolution of Cryptosporidium infection in vivo. Our hypothesis is that neonates are highly susceptible to Cryptosporidium infection since their capacity to produce IFN-gamma is not yet mature (as seen by polyclonal stimulation using anti-CD3 mAb). Large increases occur in the capacity of mice to generate IFN-gamma between 2-12 wks of age. We thus conclude that the lack of susceptibility of adult mice to Cryptosporidium may reflect the fact that these mice have a mature immune system that is

capable of responding to Cryptosporidium challenge immediately. Neonates, lacking the capacity to generate IFN-gamma responses even to a very strong stimulus like polyclonal activator anti-CD3 mAb, may exhibit a window of accessibility to cryptosporidiosis due to this hole in their developing system.

Definite evidence of a critical role for IFN-gamma in Cryptosporidium infection could be sought by the administration of anti-IFN-gamma mAbs in Cryptosporidium infected mice (110). Our hypothesis is that, blocking of IFN-gamma function in infected, unirradiated mice via passive administration of anti-IFN-gamma mAb should enhance the severity of Cryptosporidium infection in these mice, thus providing a convincing evidence of antigen specific induction of IFN-gamma production in resistance to or resolution of cryptosporidiosis.

Two immunological processes may be responsible for recovery from and resolution of cryptosporidiosis in the model studied:

A) Activation of Cryptosporidium specific CD4 + T lymphocytes to synthesize IFN-gamma.

B) Activation of phagocytic cells in the small intestine (the primary site of replication of murine Cryptosporidium) by IFN-gamma. IFN-gamma activates the gut macrophages, resulting in enhanced phagocytic capacity, elimination of Cryptosporidium and resolution of infection.

The absence of IFN-gamma needed for activation of macrophages, may explain the severe cryptosporidiosis seen in CD4+T cell deficient neonates.

Experiments comparing infection intensity and duration in irradiated vs normal neonatal BALB/c mice showed that the absence of specific immune responsiveness did not interfere with the ability of these irradiated neonates to resolve cryptosporidiosis. Suprisingly, irradiated, unresistant neonates resolve cryptosporidiosis with the same kinetics as do CD4 or CD8 T cell reconstituted irradiated recepients or normal non-irradiated controls. Collectively, these results suggest that CD4 T cell dependent IFN-gamma production is an important but ancillary event in the resolution of cryptosporidiosis, secondary to activation of phagocytic cells present in the gut. Whole body irradiation (550 rad) results in elimination of specific immunity but may be accompanied by non-specific activation of macrophages and resolution of cryptosporidial infection in the absence of IFN-gamma.

To futher explain the mechanism(s) of clearance of cryptosporidial infection, the role of gamma-delta T cells, the predominant population of IEL (intestinal intraepithelial lymphocytes) needs to be investigated. It has been proposed that TCR gamma/delta positive cytotoxic IEL may play a role in surveillance of epithelia and may be involved against bacterial and viral invasion via specific interaction with epithelium associated antigen. The physiological role for

gamma-delta T cells in cryptosporidiosis can be investigated by using gamma-delta T cell deficient mice (148) which are chronically treated with mAbs GL-3. The role played by CD8 T cells in resistance to and resolution of Cryptosporidium infection needs to be investigated. It is essential to explore the role of mucosal associated lymphoid tissue (MALT) because a notable feature of MALT is the close association of the lymphoid tissue with the mucosal epithelium, a characteristic we believe is of particular relevance to Cryptosporidium. Due to the relevance of TNF-alpha in many protozoan infections, it is also important to focus on the TNF-alpha production by cells of the intestinal epithelia and its role in cryptosporidiosis. Finally, the role of phagocytic cell function in cryptosporidiosis needs to be extensively researched. The involvement of T cell derived IFN-gamma and macrophages in host-defence mechanisms against Cryptosporidium may be of potential importance in immunodeficient hosts eg:in AIDS patients in which, together with CD4 + T cell loss, abnormality of macrophage function allows the developement of invasive disease. New approaches towards the clinical management of Cryptosporidium infection (and related pathogens) in AIDS patients could involve activation of phagocytic cells in the intestinal mucosa by different methods.

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