

## Basic Science In Medicine

### THE ROLE OF B-CELLS IN THE DEVELOPMENT OF IMMUNITY TO *CRYPTOSPORIDIUM* INFECTION

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#### ABSTRACT

Studies have shown that symptomatic improvement of cryptosporidiosis in immunocompromised patients is possible following treatment with "hyperimmune" colostrum containing anti-sporozoite antibodies from cattle immunized with *Cryptosporidium parvum* antigens. The contribution to immunity of antibodies developed during *Cryptosporidium* infection is unclear, however. Previously, we showed that SCID (severe combined immunodeficiency) mice acquired immunity to *C. muris* infection if injected with immunocompetent splenocytes. To investigate the role of the splenic donor B-cells in this immunity, groups of CB-17 SCID mice received BALB/c splenocytes, or splenocytes depleted of B-cells by immunomagnetic separation before infection. Both groups recovered from infection at similar times and displayed little difference in patterns of oocyst excretion. Subsequently, B-cells and serum anti-parasite antibodies were found only in mice reconstituted with unfractionated spleen cells. These results suggested that B-cells may not normally be required in immunity against *Cryptosporidium*.

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#### INTRODUCTION

Cryptosporidiosis of man and domestic animals is a diarrheal disease caused by *Cryptosporidium parvum*, a coccidian parasite which develops in gastrointestinal epithelial cells and is transmitted from host to host in a fecal-oral manner through the oocyst stage. The illness normally lasts a few days but immunocompromised hosts—including those with AIDS—often develop serious chronic diarrhea which may contribute to the cause of death.<sup>1</sup> Antimicrobial chemotherapy usually has only limited or no beneficial effect but certain immunotherapies have proven to be at least partially effective in relieving symptoms.<sup>2</sup>

One of the most effective approaches in immunotherapy has been the use of hyperimmune colostrum from cattle immunized mucosally with oocyst antigens containing a high titer of anti-parasite antibody capable of neutralizing parasite invasive stages.<sup>3</sup> Cryptosporidial infection stimulates the production of antibodies in both serum and mucosal secretions,<sup>4</sup> but the contribution of these antibodies to protection is not clear. Recent investigation with murine infection models have emphasized the importance of cell-mediated immune mechanisms involving interferon (IFN)- $\gamma$  activity in the clearance of infection.<sup>5,6</sup> Some epidemiological studies, however, have suggested that breast-fed infants are less likely to develop

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cryptosporidiosis,<sup>7</sup> although experimentally no passive transfer of immunity to offspring was obtained with colostrum from previously infected mothers.<sup>8</sup> Few studies, however, have directly examined the role in immunity of B-cells or parasite-specific antibody induced by infection. The results of one study suggested that antibody played a minor or no part in immunity since neonatal mice depleted of B-cells by anti- $\mu$ -chain antibody treatment were no more susceptible to *C. parvum* infection than age-matched controls.<sup>9</sup> In contrast, results from another study, also involving rodent models of *C. parvum* infection, indicated an important role for humoral immunity as there was a significant reduction in the level of infection and pathology in immunocompromised neonatal nude mice following treatment with bile containing parasite-specific IgA isolated from rats infected with *C. parvum*.<sup>10</sup>

In a new attempt to clarify the contribution of B-cells to immunity to cryptosporidial infection we employed a different approach, using a *C. muris* murine infection model. We have previously shown that immunity to this parasite involves CD4+ T-cell and IFN- $\gamma$  activity.<sup>6</sup> It was found that severe combined immunodeficiency (SCID) mice given oocysts of *C. muris* developed chronic infection and failed to recover, but injection of these animals with histocompatible spleen or mesenteric lymph node cells from immunocompetent donor animals allowed the animals to recover. In the present study the role of B-cells in immunity to *C. muris* was investigated in SCID mice which received immunocompetent spleen cells either depleted of B-cells or with B-cell population intact.

The results suggested that B-cell activity may not be an essential component of the protective immune response against *C. muris*.

### MATERIALS AND METHODS

#### Animals

Eight-to-twelve weeks old female CB-17 SCID mice (with a BALB/c background) in groups of 6 were used. These mice were bred under aseptic conditions in isolators at the London School of Hygiene and Tropical Medicine. During the experiments they were housed individually in wire-floored cages with filter top lids in a clean room and provided with UV-sterilized food and autoclaved drinking water. BALB/c mice were purchased from Charles River U.K. Ltd. and used at 12-16 weeks of age.

#### Parasite

The RN 66 strain of *C. muris* was passaged in SCID mice and oocysts were isolated and purified from fecal pellets as described previously.<sup>11</sup> Mice were infected following oral inoculation with  $2 \times 10^5$  oocysts and infections monitored by measurement of oocyst excretion from total 24 h fecal outputs.<sup>11</sup>

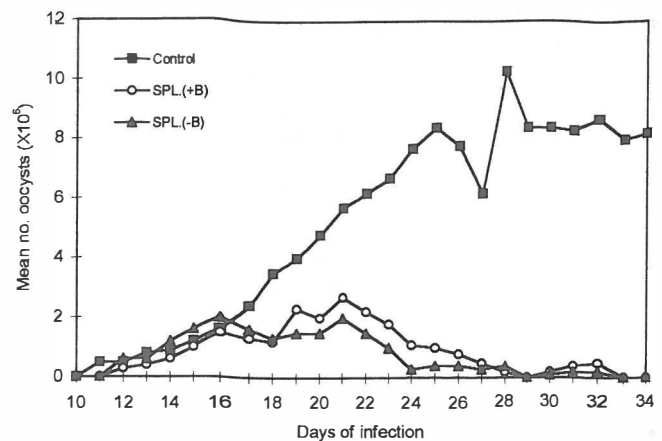


Fig. 1. Measurement of oocyst production in *C. muris*-infected CB-17 SCID mice which received either no lymphoid cells (square), BALB/c spleen cells with B-cells (open circles) or BALB/c spleen cells depleted of B-cells prior to oocyst inoculation (triangles). Each point represents the mean total number of oocysts during a 24 h period.

#### Spleen cell preparations

Single cell suspensions were prepared aseptically by passing spleens, and also MLNs, of three to four mice through a sterile stainless steel mesh, into RPMI 1640 medium supplemented with 200 U/mL penicillin, 200  $\mu$ g/mL streptomycin and 10% heat-inactivated fetal calf serum (FCS) (GIBCO).<sup>6</sup> After leaving on ice for 10 minutes, the cells in suspension were removed from the settled clumps and washed three times in supplemented medium (250 g for 7 min). Red blood cells were lysed by resuspending cell pellets in 2-3 mL of ammonium chloride solution (0.83% weight/volume) and leaving for 3-5 min at room temperature. After 3 washes in supplemented medium, cells were ready to be used in this study. SCID mice were injected iv with  $1.2 \times 10^6$  cells prior to infection with *C. muris*.

#### B-cell depletion from spleen cells and detection

B-cells were depleted from spleen cells using a magnetic activated cell sorter (MACS).<sup>12</sup>  $2 \times 10^7$  spleen cells were incubated with 2  $\mu$ L rat-antimouse (B 220 B-cell antigen) monoclonal antibodies conjugated with superparamagnetic biotinylated microparticles (100 nm diameter) at 4°C for 15 minutes before being passed through a size A2 column in a MACS separator unit (Eurogenetics U.K. Ltd.). In several experiments B-cell numbers were determined in the original spleen cell population and in the MACS-eluted spleen cells by quantification of cells with surface immunoglobulin (Ig) using immunofluorescence or flow cytometry.<sup>6</sup> The original unfractionated spleen cells contained 35-40% Ig-bearing cells whereas after B-cell separation by MACS less than 1% (0.3-0.5% by immunofluorescence) of the eluted cells were Ig-bearing.

**Table I.** Measurements of oocyst production, splenic B-cell numbers and parasite-specific antibodies in the serum of SCID mice five weeks after reconstitution with BALB/c spleen cells and infection with *C. muris*.

Treatment of SCID mice <sup>a</sup>	Total mean no. of oocyst production ( $\times 10^6$ ) <sup>b</sup>	B-cells in spleen <sup>c</sup> (%)	Parasite-specific antibody (O.D.)
control	133 $\pm$ 24	1.4 $\pm$ 1.5	0
spleen (+B)	20 $\pm$ 7	8.9 $\pm$ 1.7	0.22 $\pm$ 0.04
spleen (-B)	18 $\pm$ 12	0.3 $\pm$ 0.4	0

a. The SCID mice received either no cells (control), BALB/c spleen cells with an intact B-cell population ( $\pm$ B) or BALB/c spleen cells depleted of B-cells (-B).

b. The total numbers of oocysts produced by each group up to day 34 post-infection.

c. Numbers of Ig-bearing cells in 1000 cells were determined from four mice by immunofluorescence. These cells could not be detected below 1% in flowcytometry.

### ELISA for detection of parasite-specific antibodies

Antibodies against *C. muris* in serum were detected by ELISA using 96-well plastic Immulon II plates (Dynatech). Oocyst antigen for coating wells was prepared by homogenizing purified oocysts by vortex mixing with No.8 glass beads (Jencons) in PBS pH 7.6.<sup>13</sup> The equivalent of  $3310^4$  oocysts in a volume of 50  $\mu$ L was placed in each

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## RESULTS

### Oocyst production in SCID mice

Figure 1 shows the patterns of oocyst production in

groups of SCID mice which received spleen cells prior to infection and a control group which received no cells. Infection in the control mice had a prepatent period of about 10 days following which oocyst levels in feces increased sharply, reaching a peak between days 25 and 28. These mice continued to shed large numbers of oocysts for the remainder of the experiment. In contrast, the levels of oocyst production in mice which had been given spleen cells were lower, reaching a maximum around day 21 and subsequently decreasing until all mice showed subpatent infections by day 33. There were no significant differences in either the patterns of oocyst production or in the mean total numbers of oocysts produced in mice given unfractionated spleen cells and those receiving spleen cells depleted of B-cells (Table I).

### B-cell numbers and antibody detection in recovered SCID mice

Following recovery of the spleen cell-reconstituted SCID mice from infection, comparative measurements were made of both their splenic Ig-bearing cell numbers and serum parasite-specific antibody levels. Table I shows that nearly 9% Ig-bearing cells were present in the spleens from recipients of unfractionated spleen cells. (In earlier experiments in which we studied the role of T-cells in immunity,<sup>6</sup> the corresponding percentage of CD3 + T-cells in recovered mice was a similar value of approximately 10% (unpublished data)). In contrast, Ig-bearing cells were virtually undetectable in spleens of both SCID control mice or SCID mice which received B-cell-depleted splenocytes.

Parasite-specific antibodies were present in the sera of all SCID mouse recipients of unfractionated cells following recovery from infection. A mean O.D. value of  $0.22 \pm 0.04$  was obtained for positive control sera from BALB/c mice following *C. muris* infection. However, there was no evidence for the presence of parasite-specific antibody in the sera of

SCID mice which had received spleen cells depleted of B-cells.

### DISCUSSION

The main finding of this study was that the ability of SCID mice to resolve *C. muris* infection following injection with functional splenic lymphocytes was not diminished by depletion of B-cells from the donor population. This contrasted with our previous observation, using this experimental system, that depletion of CD4+T-cells from donor splenocytes abrogated the adoptive transfer of immunity and depletion of CD8+ cells moderately reduced the protective effect of spleen cells.<sup>6</sup> The present results suggested, therefore, that B-cells were not necessary for the development of the protective immune response against cryptosporidial infection.

B-cells were depleted from a normal BALB/c population by magnetic separation on a MACS separator, reducing the percentage of B-cells from >35% to <1% (see *Materials and Methods*). It cannot be discounted that following transfer of the B-cell depleted splenocytes to SCID mice any small numbers of B-memory cells present could have proliferated and contributed to the immune response against *C. muris*. However, after recovery of these mice from infection (and five weeks after cell transfer) we failed to detect any Ig-bearing cells in the spleens above the background level found in control SCID mice whereas recipients of unfractionated spleen cells at that stage had nearly 9% Ig-bearing cells. This latter value was substantially lower than that found in the BALB/c donor mice, but similar to values numbers ( $1.2 \times 10^6$  in the present study) these animals received.

Parasite-specific antibody was not detected in sera of mice given B-cell-depleted splenocytes whereas specific antibodies were present in recipients of unfractionated splenocytes. The levels of antibody in the latter mice were low compared with that found in immune BALB/c mice, but since the BALB/c mice would have had significantly more Ig-bearing cells (see above) this difference should not have been surprising. The possibility remained that SCID mice reconstituted with spleen cells depleted of B-cells had significant B-cell numbers in the gut, but this seems unlikely.

Our results, therefore, strongly support the conclusions of an earlier study by Taghi-Kilani et al.<sup>9</sup> who observed that infection of neonatal mice with *C. parvum* was not exacerbated following depletion of B-cells by anti- $\mu$  treatment. Indeed, immunity to other intestinal coccidia which infect epithelial cells has also appeared not to require B-cell involvement.<sup>14</sup>

It remains to be established whether during cryptosporidial infection secretory antibody has a secondary or redundant role in elimination of the parasite. Clearly, parasite-specific antibodies developed by immunization with

parasite antigen have been able to inhibit parasite reproduction,<sup>3</sup> but there is little evidence that secretory antibody induced by infection is protective. The demonstration that bile from *C. parvum*-infected rats containing parasite-specific IgA provided nude mice with partial protection against infection with this parasite suggested that there might be a role in immunity for secretory antibody,<sup>10</sup> although antibody was not clearly identified as the protective component of "immune" bile. High titers of parasite-specific secretory IgA have been observed in AIDS patients with chronic cryptosporidiosis, perhaps suggesting an insignificant protective role for antibody,<sup>15</sup> but it is possible that in such cases of depressed cellular immunity secretory antibody is partially effective in limiting parasite reproduction. The antibody response may be more effective when complementing a cell-mediated response or as part of an antibody-dependent cellular mechanism of immunity.

In conclusion, the results of this study indicated that the development of immunity against cryptosporidial infection in SCID mice following injection of immunocompetent splenic lymphocytes was not dependent on a significant contribution by B-cells.

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### REFERENCES

1. Garcia LS, Current WL: Cryptosporidiosis. *Clin Microb Rev* 4: 325-8, 1991.
2. Tzipori S, et al: Remission of diarrhoea due to cryptosporidiosis in an immunodeficient child treated with hyperimmune bovine colostrum. *Br Med J* 239: 1276-7, 1986.
3. Riggs MW, et al: Bovine antibody against *Cryptosporidium parvum* elicits a circumsporozoite precipitate-like reaction and has immunotherapeutic effect against persistent cryptosporidiosis in SCID mice. *Infect Immun* 62: 1927-9, 1994.
4. Peeters JH, et al: *Cryptosporidium parvum* in calves: kinetics and immunoblot analysis of specific serum and local antibody response (Immunoglobulin A {IgA}, IgG, and IgM) after natural and experimental infections. *Infect Immun* 60: 2309-16, 1992.
5. Ungar BLP, et al: Cryptosporidium in mice: a murine model: independent roles for IFN- $\gamma$  and CD4+ T lymphocytes in protective immunity. *J Immunol* 147: 1014-22, 1991.
6. McDonald V, et al: *Cryptosporidium muris* in adult mice: adoptive transfer of immunity and protective roles of CD4+ versus CD8+ cells. *Infect Immun* 62: 2289-94, 1994.
7. Mata L: *Cryptosporidium* and other protozoa in diarrheal disease in less developed countries. *Paed Infect Dis J* 5: S117-30, 1986.

8. Moon HW, et al: Lacteal immunity to enteric cryptosporidiosis in mice: *Infect Immun* 56: 649-53, 1988.
9. Taghi-Kilani R, et al: The role of humoral immunity in *Cryptosporidium spp.* infection: studies with B cell-depleted mice. *J Immunol* 145: 60: 3325-31, 1992.
10. anticryptosporidial immune rat bile. *Am J Trop Med Hyg* 50: 112-9, 1994.
11. McDonald V, et al: Immune response to *Cryptosporidium muris* and *Cryptosporidium parvum* in adult immunocompetent or immunocompromised (nude or SCID) mice. *Parasit Immunol* 13: 251-9, 1991.
12. Miltenyi S, et al: High gradient magnetic separation with MACS immune dams do not. *Cytometry* 11: 231-8, 1990.
13. McDonald V, et al : Characteristics and specificity of hybridoma antibodies against oocyst antigens of *Cryptosporidium parvum* from man. *Parasit Immunol* 13: 251-9, 1991.
14. Albert MM, et al: Treatment of n intestinal immune responses to *Eimeria* parasites. *Clin Microb Rev* 9: 349-60, 1996.
15. Cozon G, et al: Secretory IgA antibodies to *Cryptosporidium parvum* in AIDS patients with chronic cryptosporidiosis. *Infect Dis* 169: 696-9, 1994.

