

NONSPECIFIC IMMUNOMODULATION INFLUENCES THE COURSE AND LOCATION OF *CRYPTOSPORIDIUM PARVUM* INFECTION IN NEONATAL BALB/c MICE

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SUMMARY

The influence of nonspecific immunomodulation with Thymomodulin (a calf thymic extract with immunomodulatory activity) and hydrocortisone on the course and location of *Cryptosporidium parvum* infection in neonatal BALB/c mice (infected with 10^6 or 10^5 oocysts on day 7 of life) was studied using scanning electron microscopy of the inner surface of different parts of intestine. Daily peroral treatment of suckling mice with 20 mg/kg/day of Thymomodulin for 5 days before inoculation resulted in an earlier peak and earlier termination of cryptosporidial infection when compared with control infected mice. On the other hand,

peroral administration of 25 mg/kg of hydrocortisone every second day led to the persistence of cryptosporidial infection in the ileum of immunosuppressed mice until the end of observation (day 15 post infection), whereas only transient infection was observed in the intestine of control infected mice. The location of infection was also altered in hydrocortisone-treated mice—the severe infection was observed in more proximal parts of the intestine (anterior and middle jejunum), whereas no cryptosporidia were found in these parts of the intestine in nontreated infected mice.

RÉSUMÉ : Influence d'une immunomodulation non spécifique sur le cours de l'infection et la localisation de *Cryptosporidium parvum* chez les souriceaux nouveau-nés BALB/c.

Une immunomodulation non spécifique par la Thymomoduline (extrait de thymus de veau à activité immunomodulatrice) et l'hydrocortisone est réalisée chez des souriceaux nouveau-nés BALBN/c, afin d'étudier le cours de l'infection et la localisation de *Cryptosporidium parvum*. (Les souris âgées de 7 jours sont infectées avec 10^5 ou 10^6 oocystes). L'étude est fondée sur l'examen au MEB de la surface interne des différentes parties de l'intestin. Une dose de 20 mg/kg par voie orale chaque jour, pendant les 5 jours qui précèdent l'inoculation des souriceaux, détermine un pic d'infection plus précoce et une guérison plus rapide que chez les témoins. Par ailleurs l'administration perorale, un jour sur deux, de 25 mg/kg d'hydrocortisone entraîne la persistance de l'infection dans l'iléon des souriceaux immunodéprimés jusqu'à la fin de l'observation (15e jour de l'infection), alors que l'infection est transitoire chez les témoins. La localisation de l'infection est également modifiée chez les souris traitées à l'hydrocortisone : l'infection sévère est plus proximale (jejunum antérieur et moyen), alors que ces localisations n'existent pas chez les témoins.

INTRODUCTION

Cryptosporidiosis (*Cryptosporidium parvum*) is a protozoan infection frequently associated with certain immunodeficient states (Lockwood and Weber, 1989; Mead *et al.*, 1986). Both cell-mediated and humoral immune responses are elicited by *Cryptosporidium*, but functional cell-mediated immunity is necessary for the recovery from infection (Current and Garcia, 1991). The strong thymic-dependence of anti-cryptosporidial resistance was demonstrated in experiments on athymic animals, persistent infection being observed in adult athymic nude mice (Heine *et al.*, 1984; Ungar *et al.*, 1990; Mead *et al.*, 1991b) and

rats (Gardner *et al.*, 1991), whereas immunocompetent animals developed only transient asymptomatic infection (Enriquez and Sterling, 1991). Persistent infection similar to that in athymic mice was reported in neonatal suckling BALB/c mice treated with monoclonal antibody that eliminated CD4⁺ T-lymphocytes (Ungar *et al.*, 1990, 1991). On the other hand, the course of infection was not altered in B-cell-depleted mice (treated with rabbit anti-mouse IgM antibodies, Taghi-Kilani *et al.*, 1990), nor in mast-cell-deficient W/W^v mice (Harp and Moon, 1991).

The most widely used laboratory animal model for cryptosporidiosis involves infection of neonatal suckling mice within first the 2 weeks of life, which leads to the development of a self-limited infection (Current and Garcia, 1991; Novak and Sterling, 1991). Due to its relative immunoincompetence the neonatal mouse (Murgita and Wigzell, 1981) represents a suitable model for a study of

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the impact of immunomodulation on resistance to cryptosporidial infection. In this study we examined the possibility of influencing the course and location of cryptosporidial infection in neonatal BALB/c mice by administration of Thymomodulin (a partially purified calf thymic extract, Cazzola *et al.*, 1987) or hydrocortisone-nonspecific immunomodulators acting preferentially on T-cell immunity (Renoux and Renoux, 1980; Bach, 1987; Oates *et al.*, 1989).

MATERIALS AND METHODS

MICE

The neonatal BALB/c mice originated from pregnant SPF mice (Velaz, Prague). Each litter of neonatal mice was kept individually and remained with their dam during the whole experiment.

PARASITE

Cryptosporidium parvum oocysts were isolated from the feces of a naturally infected calf by filtration through a metal sieve and centrifugation on a sucrose density gradient, sterilised by incubation with commercial bleach, washed 5 times with phosphate-buffered-saline (PBS) and stored (for less than 2 months) in 2.5 % potassium dichromate at 4° C until used. Immediately before administration, the oocysts were washed several times with sterile PBS to remove potassium dichromate and counted using a hemocytometer. All experimental mice were inoculated with 0.1 ml inoculum by gastric gavage using a tuberculin syringe and a 22-gauge feeding needle.

IMMUNOMODULATORS AND EXPERIMENTAL DESIGN

Experiment 1: Neonatal mice (both sexes) of the same age (born within 24 hours) were divided into three groups. The TM-group (25 animals) were treated perorally with 20 mg/kg/day of Thymomodulin (« Leucotrofina 50 » kindly provided by Dr. Cazzola, ELLEM Industria farmaceutica Milano) starting from day 2 of life up to the day preceding inoculation. The HC-group (25 animals) were treated perorally with 25 mg/kg/dose of hydrocortisone (Solu-Cortef, UpJohn) every second day from day 2 of life until day 13 post infection (DPI 13). The nontreated infected mice (25 animals) served as control group. All mice were inoculated with 107 oocysts of *C. parvum* on day 7 of life (DPI 0).

Experiment 2: The experimental mice were divided into three groups and treated perorally with 25 mg/kg/dose of hydrocortisone (Solu-Cortef, UpJohn) every second day starting from day 2 of life up to the end of experiment (group HC-1, 20 animals) or up to DPI 5 (group HC-2, 20 animals) or up to the day preceding inoculation (group HC-3, 20 animals). The nontreated infected mice (20 animals) served as a control group. All the mice were inoculated on day 7 of life with 107 oocysts of *C. parvum*.

EVALUATION OF INTENSITY OF INFECTION

Fecal samples were collected directly from the rectum from 5 mice of each experimental group every day throughout experiments in order to evaluate the prepatent and patent periods of infection. The fecal pellets were smeared onto microscopic slides and stained with aniline-carbol-methyl-violet (Miláček and Vítová, 1985) to detect oocysts. Every second day (starting from DPI 1 in experiment 1 or DPI 3 in experiment 2) 3 mice from each group were

killed by cervical dislocation and 3 (ileum, cecum and colon, in experiment 1) or 5 (anterior and middle jejunum, ileum, cecum and colon, in experiment 2) 1.0-1.5 cm-segments of intestine were removed, opened longitudinally and fixed in phosphate-buffered 4 % paraformaldehyde (pH 7.4). The fixed intestinal segments were dehydrated using ascending ethanol gradients. Specimens were further desiccated by critical point drying and then coated with gold-palladium. After mounting on aluminium stubs, the intestinal mucosa was examined with the scanning electron microscope (SEM) TESLA BS-300, using an accelerating voltage of 19 kV. All specimens were routinely examined at intermediate (500x), and high (3,500x, 5,000x) magnifications. Other levels of magnification were used to highlight a particular finding.

On the basis of the SEM findings the degree of infection was classified as follow:

degree 0: no cryptosporidia found on the mucosal surface;

degree 1: moderate infection, sporadic cryptosporidia distributed on the surface of villi or in crypts, representing less than 100 cryptosporidia per jejunal or ileal villus (10 villi examined at random), and less than 100 cryptosporidia seen in any of 10 cecal or colonic SEM fields at magnification 500x;

degree 2: medium infection, regularly disseminated cryptosporidia on the surface of villi and in crypts representing from 100 to 300 cryptosporidia per jejunal or ileal villus (10 villi examined at random), or from 100 to 300 cryptosporidia seen in any of 10 cecal or colonic SEM field at magnification 500x;

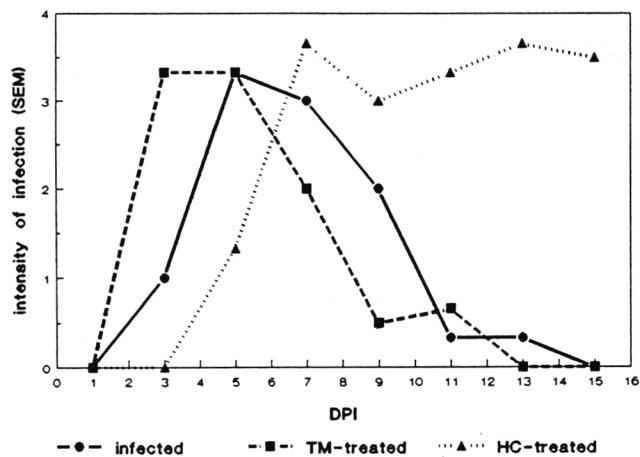
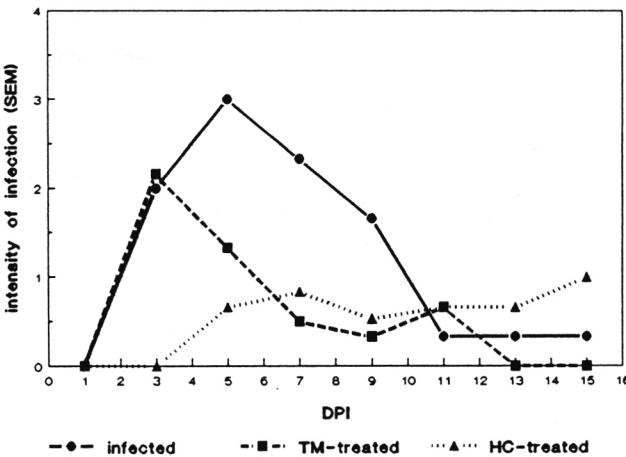
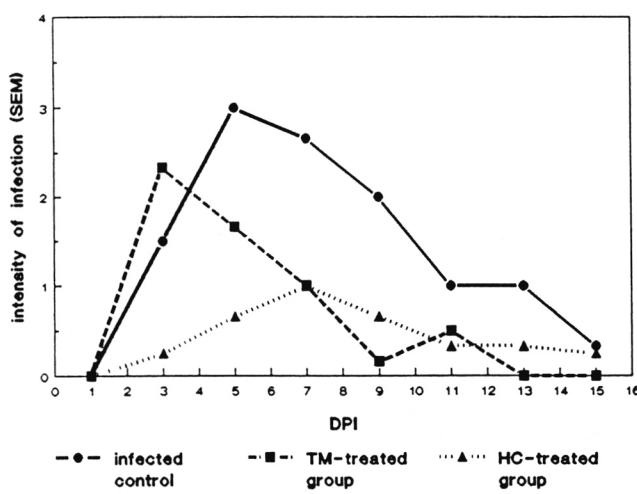
degree 3: severe infection, most of the epithelial surface covered by cryptosporidia representing more than 300 cryptosporidia per jejunal or ileal villus (10 villi examined at random), or more than 300 cryptosporidia seen in any of 10 cecal or colonic SEM field at magnification 500x, but less than 100 cryptosporidia per jejunal or ileal SEM field at magnification 5,000x, or less than 100 cryptosporidia per cecal or colonic field at magnification 3,500x;

degree 4: massive infection, almost the whole epithelium covered by cryptosporidia representing more than 100 cryptosporidia per jejunal or ileal SEM field at magnification 5,000x (10 SEM field examined), or more than 100 cryptosporidia seen in any of 10 cecal or colonic SEM field at magnification 3,500x.

Each point in the graphs represents the mean degree of infection observed in 3 mice. This methodological approach does not allow statistical analysis of the data, but has a great advantage in the complex evaluation of the intensity of cryptosporidial infection in that part of intestine examined.

RESULTS

In experiment 1, oocysts of *C. parvum* in the smears of rectal content from the control infected mice as well as from the Thymomodulin-treated mice were first detected on DPI 3 and the shedding of oocysts peaked between DPI 5 and 7. Cryptosporidial oocysts were observed until DPI 12 in smears from nontreated infected mice, indicating a patent period of 9 days. The patent period in the Thymomodulin-treated mice was shorter (7 days) and the shedding of cryptosporidial oocysts ceased on DPI 10. In both experiments, the hydrocortisone-treated mice began to shed oocysts on DPI 5 or DPI 6 and shedding persisted until the end of our observation (DPI 15 in experiment 1 and DPI 13 in experiment 2).

*a**b**c*

The influence of the administration of Thymomodulin and hydrocortisone on the course of cryptosporidial infection in neonatal BALB/c mice (experiment 1) is summarised on *Figure 1*. Treatment with Thymomodulin for 5 days before inoculation resulted in the earlier peak and earlier termination of infection (*Figs. 3A, 3C*). On the other hand, infection persisted in hydrocortisone-treated mice until the end of observation (DPI 15), whereas no cryptosporidia were observed in the ileum of untreated infected mice at this time. The small developmental stages of *C. parvum* together with the large mature stages were observed on the inner surface of ileal mucosa of hydrocortisone-treated mice until the end of experiment 1 (*Fig. 3E*). This influence of hydrocortisone on the course of infection was confirmed with a smaller inoculum of cryptosporidia in experiment 2 (*Fig. 2c*, group HC-1). Moreover, the location of cryptosporidial infection was altered in all of the treated mice. A medium to severe degree of infection was observed in proximal parts of the intestine (anterior and middle

FIG. 1. — The course of cryptosporidial infection in the ileum (*a*), cecum (*b*) and colon (*c*) of immunomodulated neonatal BALB/c mice (experiment 1). Two days old mice were treated *p. o.* with 20 mg/kg/day of Thymomodulin for 5 days (TM-group) or with 25 mg/kg/dose of hydrocortisone every 2nd day until the end of the experiment (HC-group). All experimental and control animals were inoculated at day 7 of life with 10^6 oocysts of *Cryptosporidium parvum*.

jejunum), whereas no parasites were detected in these parts of the intestine in nontreated infected mice (*Figs. 2a, 2b*). Infection persisted in the middle jejunum and ileum of mice treated with hydrocortisone throughout the whole experiment (HC-1 group), however no cryptosporidia or only moderate infection were observed on DPI 13 in mice treated until DPI 5 (HC-2 group) or until the day preceding inoculation (HC-3 group, *Figs. 2a, 2b, 2c*). The intensity of infection in the large intestine (cecum and colon) of hydrocortisone-treated mice was much lower than in nontreated control mice (*Figs. 2d, 2e*).

DISCUSSION

The present results clearly indicated that (*i*) the prophylactic administration of Thymomodulin resulted in the earlier recovery of neonatal BALB/c mice from *C. parvum* infection, compared with nontreated infected mice;

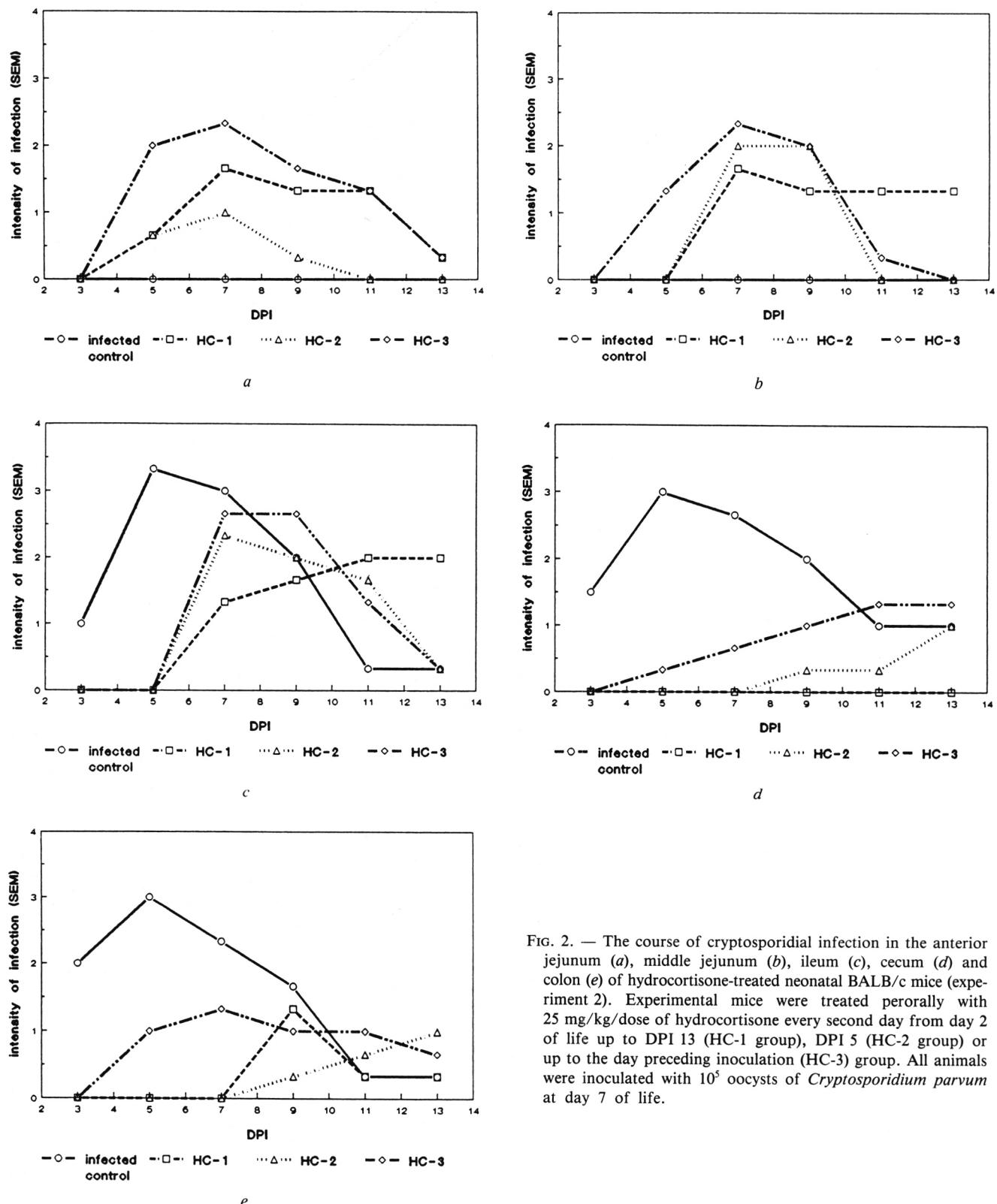


FIG. 2. — The course of cryptosporidial infection in the anterior jejunum (a), middle jejunum (b), ileum (c), cecum (d) and colon (e) of hydrocortisone-treated neonatal BALB/c mice (experiment 2). Experimental mice were treated perorally with 25 mg/kg/dose of hydrocortisone every second day from day 2 of life up to DPI 13 (HC-1 group), DPI 5 (HC-2 group) or up to the day preceding inoculation (HC-3 group). All animals were inoculated with 10^5 oocysts of *Cryptosporidium parvum* at day 7 of life.

(ii) immunosuppression by hydrocortisone resulted in the persistence of cryptosporidial infection in suckling mice; (iii) cryptosporidial infection was restricted to the anterior

and middle jejunum of hydrocortisone-treated mice, and shifted to the large intestine after treatment ceased. Considering that the immune status of the host appears to be

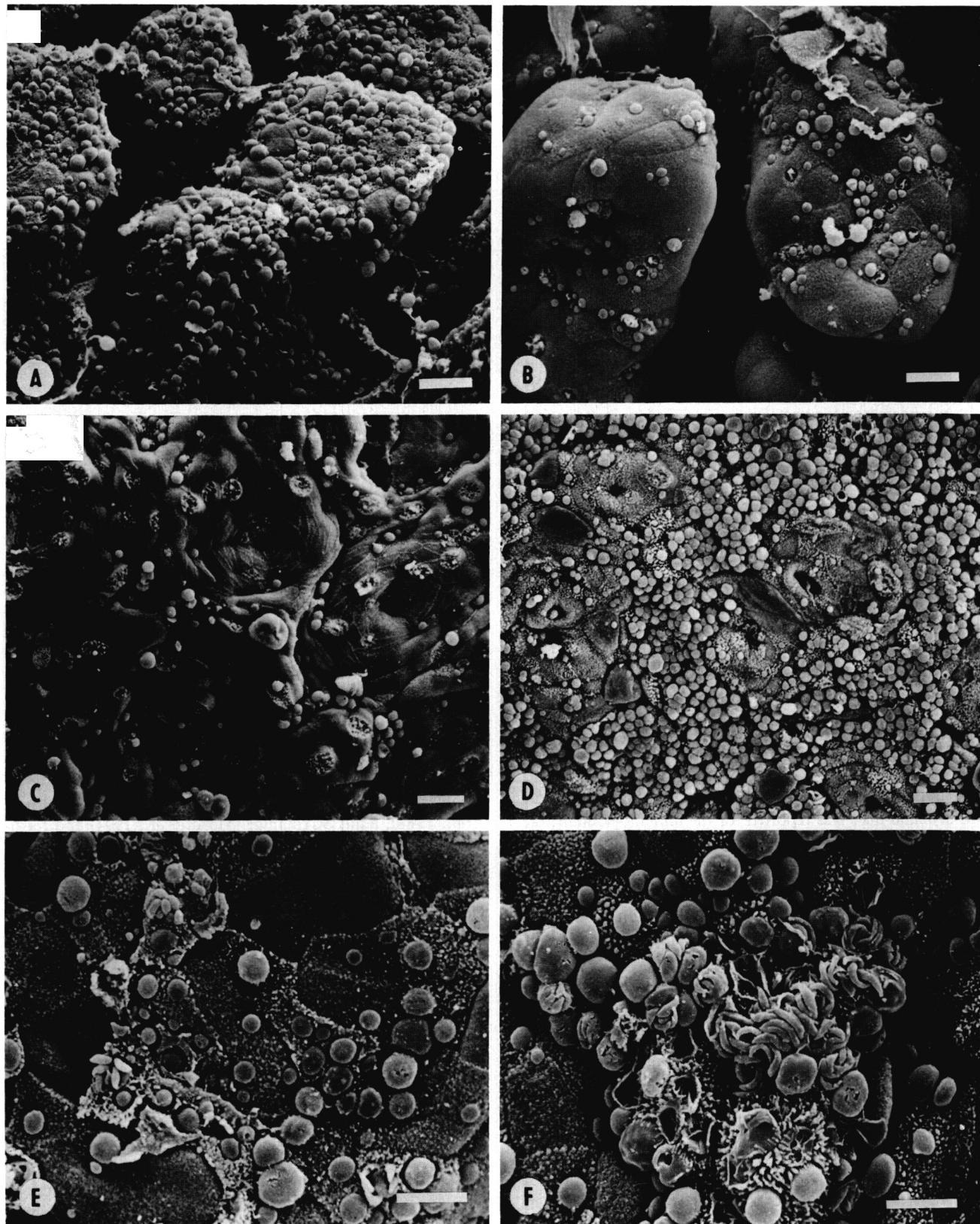


FIG. 3. — Scanning electron micrographs of the mucosal surface of neonatal BALB/c mice experimentally infected with *Cryptosporidium parvum*. (A, B) The ileal surface of Thymomodulin-treated (A, 500x) and nontreated control (B, 500x) mice on DPI 3. (C, D) The colonic mucosa of Thymomodulin-treated (C, 500x) and nontreated control mice (D, 500x) on DPI 5 (experiment 1). (E, F) The mucosal surface of the ileum of hydrocortisone-treated mice (E, 1 800x) on DPI 15 and of the ileum of nontreated control mice on DPI 5 (F, 1 800x).

the major factor determining the severity and duration of cryptosporidial infection and that effective chemotherapy of cryptosporidiosis is not yet available, immunological intervention seems to be a hopeful approach to control. Transfer of specific resistance against *C. parvum* infection has been already discussed in literature (especially the possible roles of humoral and cellular factors in such a transfer). Moon *et al.* (1988) showed that immune mice were unable to transfer protective immunity to their offspring, and Harp and Whitmire (1991) failed to transfer resistance to neonatal mice by the intraperitoneal injection of spleen or mesenteric lymph node cells from immune adult mice. On the other hand, Ungar *et al.* (1990) demonstrated that these cells were able to transfer resistance to infected athymic nude mice. Controversial results have also been reported on the protective efficacy of an immune (specific) bovine transfer factor (dialyzable leukocyte extract, DLE). Fayer *et al.* (1987) showed that such a preparation failed to protect neonatal calves against cryptosporidiosis, however McMeeking *et al.* (1990) described therapeutic effect of DLE in AIDS patients suffering from cryptosporidial diarrhoea. There have been many attempts to use different antibody-containing preparations, but the efficacy of these preparations remains questionable (Current and Garcia, 1991). Oral administration of colostrum from naturally infected cows did not alter the course of cryptosporidial infection in an AIDS patient (Saxon *et al.*, 1987), however hyperimmune colostrum showed a therapeutic effect in immunodeficient patients (Tzipori *et al.*, 1986) and protective effect in calves (Fayer *et al.*, 1989a) and neonatal mice (Arrowood *et al.*, 1989; Fayer *et al.*, 1989b). A similar immunotherapeutic effect on cryptosporidiosis in neonatal mice was reported for the immunoglobulin fraction of hyperimmune colostrum (Fayer *et al.*, 1990) or hyperimmune serum (Riggs and Perryman, 1987). Administration of a monoclonal anticryptosporidial antibody significantly reduced the intensity of infection in neonatal mice (Arrowood *et al.*, 1987) and athymic nude mice (Bjorneby, 1991), but not in SCID foals (Perryman and Bjorneby, 1991).

Considering these problems with development of specific immunotherapy then it is possible that non-specific immunomodulation could play an important role in the control of cryptosporidiosis, especially in immunocompromised hosts. The complete resolution of intestinal cryptosporidiosis after discontinuation of immunosuppressive chemotherapy, allowing restoration of immune function, was reported in several patients (Miller *et al.*, 1983; Stine *et al.*, 1985). Moreover, Mead *et al.* (1991) demonstrated complete eradication of cryptosporidia in SCID mice reconstituted with murine thymocytes, spleen or mesenteric lymph node cells from naive congenic donors. Indirect evidence of a possible role for non-specific immunomodulators in the therapy of cryptosporidiosis has been reported for recombinant interleukin-2 (Kern *et al.*, 1985). Given the

key role of T-cell mediated immunity in resistance to *C. parvum* infection, we presumed that thymic factors, which facilitate the differentiation of T-lymphocytes (Bach, 1987; Oates *et al.*, 1989), might be a promising group of immunomodulators for the control of cryptosporidiosis, especially in immunocompromised hosts. Protective effect of thymic preparations in experimental parasitic infection was firstly reported by ourselves (Heřmánek, 1991). This study is the first report of a beneficial influence of thymic extract on cryptosporidiosis in neonatal mice.

Peroral prophylactic administration of Thymomodulin resulted in the earlier recovery of neonatal BALB/c mice from cryptosporidial infection. The beneficial effect of Thymomodulin may reflect the faster maturation of immunocompetent cells in neonatal mice, because *in vitro* responsiveness of splenocytes to the T-cell mitogen ConA in the infected Thymomodulin-treated mice also increased more rapidly than in nontreated mice (data not shown). This suggestion is supported by the observation of Goldstein and Coworkers (1971), that the administration of another thymic preparation (thymosin) significantly accelerated the development of immunocompetence in neonatal mice. Our conclusions were also supported by the description of the enhancing activity of Thymostimulin in the chemotherapy of acute cryptosporidiosis in AIDS-patients (Barbaro *et al.*, 1990). Chronic cryptosporidiosis has been reported in immunosuppressed rats (Brasseur *et al.*, 1988; Regh *et al.*, 1987, 1988), hamsters (Rossi *et al.*, 1990), guinea pigs (Angus *et al.*, 1985; Chrisp *et al.*, 1990), immunosuppressed adult mice (Rasmussen and Healey, 1992), athymic and T-cell subset depleted mice (Heine *et al.*, 1984; Ungar *et al.*, 1990, 1991), germ-free adult mice (Harp *et al.*, 1988), adult SCID mice (Mead *et al.*, 1991) and retrovirally infected mice (Darban *et al.*, 1991). Hydrocortisone-immunosuppressed models for cryptosporidiosis have been characterized for rats and hamsters (Brasseur *et al.*, 1988; Rossi *et al.*, 1990), however our study is the first attempt to characterise the course of cryptosporidial infection in neonatal mice immunosuppressed with various regimens of hydrocortisone administration. SEM examination of the mucosal surface of treated mice showed that the infection persisted for at least for 15 days. Both small developmental stages of *C. parvum* as well as large mature stages were observed on the ileal mucosa of hydrocortisone-treated mice. We suppose that the presence of these small developmental stages in chronic infection reflects the autoinfective character of cryptosporidial infection (Current and Garcia, 1991).

The persistence of cryptosporidial infection in the ileum associated with lower intensity of infection in the cecum and colon of hydrocortisone-treated neonatal mice (experiment 1) suggest that the location of infection is immune-dependent and may therefore be influenced by non-specific immunosuppression. To confirm this hypothesis we analysed the course of infection in more proximal parts of the intes-

tine and in different regimes of hydrocortisone administration. The results (experiment 2) indicated that infection was found more proximally in hydrocortisone-treated mice, whereas no parasites were found in these parts of intestine in nontreated infected animals. In other immunocompromised animal models of chronic cryptosporidiosis only Ungar *et al.* (1990) and Rasmussen and Healey (1992) have demonstrated cryptosporidia in the anterior part of gastrointestinal tract. Ungar *et al.* (1990) observed cryptosporidia in the pyloric ring and duodenum of neonatally infected BALB/c mice treated with anti-CD4 monoclonal antibodies. Rasmussen and Healey (1992) found the greatest numbers of parasites in the jejunum, ileum, and colon, but cryptosporidia were also localised in the gastric glands of the stomach and in the duodenum of adult mice treated with dexamethasone. In our study, a gradual shift of the infection to the more distal parts of the intestine was observed after cessation of hydrocortisone-treatment. It is possible that this shift represents the expulsion of cryptosporidia following reconstitution of the impaired immune system. Similar clearance of cryptosporidia was described by Rehg *et al.* (1987, 1988) after withdrawal of cyclophosphamide or dexamethasone treatment in immunosuppressed rats. Moreover, a complete resolution of intestinal cryptosporidiosis after discontinuation of immunosuppressive chemotherapy, allowing restoration of immune function, has been reported in several patients (Miller *et al.*, 1983; Stine *et al.*, 1985).

Further investigations of the influence of nonspecific immunomodulation on mechanisms controlling the resistance to *Cryptosporidium* infection are in progress.

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