Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 149 (2007) 77–84
veterinary parasitology
www.elsevier.com/locate/vetpar

Methylprednisolone acetate immune suppression produces differing effects on *Cryptosporidium muris* oocyst production depending on when administered

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Received 18 August 2005; received in revised form 21 June 2007; accepted 2 July 2007

Abstract

At different times after inoculation with *Cryptosporidium muris*, infected CF-1 female mice were immunosuppressed with a single subcutaneous dose of methylprednisolone acetate (MPA; 600 mg/kg). MPA immunosuppression decreases circulating CD3, CD4 and CD8 T-lymphocytes and B-lymphocytes by greater than 90% for approximately 14 days with numbers not returning to pre-suppression levels until after 41 days post-suppression. Immunosuppression was initiated at selected times before, during, and after oocyst production. Immunosuppression initiated prior to oocyst production delayed the start of production by 4–5 days and extended oocyst shedding by 16 days. Initiation of immunosuppression during oocyst production both extended oocyst shedding and greatly increased the number of oocysts shed per day over most of the extended shedding period. Immunosuppression during the decline of oocyst production resulted in only a moderate extension of shedding and a moderate increase in oocyst numbers. Immunosuppression initiated soon after oocyst shedding had ceased resulted in the re-initiation of limited oocyst production for only a few days. Suppression initiated on days 40 and 46 post-infection, 11 and 17 days after oocysts could no longer be detected in the feces, did not result in a resumption of oocyst production. In all cases, where oocyst production was extended or reinitiated, the shedding of oocysts halted between days 45 and 53 post-oocyst inoculation. These studies demonstrate that the effect of MPA immunosuppression depends on the immunologic conditions existing in the host at the time immunosuppression was initiated. Immunosuppression initiated during oocyst production allows an overwhelming parasitism to exist, implying that T- and B-lymphocytes play an important role in moving the host immune process along during this period of the infection. Conversely, severe suppression of T- and B-lymphocytes initiated as oocyst production is decreasing does not result in a complete relapse of the disease suggesting that T- and B-lymphocytes are not critical to the continuation of the immune process after this point. These studies also show that the *C. muris* infection persists beyond the end of the detection of oocysts in the feces.

Keywords: *Cryptosporidium muris*; Immunosuppression; Glucocorticoids; Methylprednisolone acetate; Oocysts; Lymphocytes

1. Introduction

The course of a naturally occurring *C. muris* infection in mice has been well described (Yoshikawa and Iseki, 1992, 1991; Iseki et al., 1989). Clinical infection begins, when oocysts can be demonstrated in the feces and ends when oocyst production stops, indicating the elimination of the parasite by the host (O'Donoghue, 1995). Mice which have stopped shedding oocysts are considered recovered and are immune to reinfection. Individual CF-1 mice infected with *C. muris* begin shedding oocysts between days 7 and 10 post-exposure (Miller and Schaefer, 2007; Iseki

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doi:10.1016/j.vetpar.2007.07.005

et al., 1989). Oocyst shedding peaks between days 16 and 22 post-exposure, then gradually declines and finally stops around day 29.

A single subcutaneous dose of methylprednisolone acetate (MPA) at 600 mg MPA/kg decreases circulating T- and B-lymphocytes by greater than 90% by 24 h and lasts for up to 14 days. T- and B-lymphocytes do not return to pre-suppression levels until after day 41 post-suppression (Miller and Schaefer, 2006). Circulating mature segmented neutrophils rise over 160% through day 14 and gradually decrease through day 27. These immunosuppressed mice can be successfully infected with *C. parvum* but stop shedding oocysts by day 14 post-suppression. Mice which recovered from a *C. muris* infection can be reinfected with *C. muris* following MPA suppression.

Our hypothesis was that the effect of immunosuppression would be the same no matter when immunosuppression with MPA was initiated during the clinical infection. The expectation was that we would see a complete resumption of the infection provided the parasite was still present in the host. We found that this was not the case. To more clearly understand the immune process involved in eliminating the parasite and to establish the true duration of the infection, we selectively suppressed T- and B-lymphocytes by initiating MPA immunosuppression at different times during a *C. muris* infection and monitored its effects on oocyst production. To characterize the effect of immunosuppression the duration and intensity of oocyst shedding, the end of MPA effect, the days of peak intensity of oocyst shedding, and the days that 100% of a group were shedding oocysts were determined.

2. Methods and materials

2.1. Mice

CF-1 female mice which were 5–6 weeks of age were obtained from Charles River Laboratories (Portage, MI). All mice were initially housed in groups of 10 and were allowed food and water *ad libitum*. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the U.S. Environmental Protection Agency.

2.2. Cryptosporidium muris oocysts

C. muris oocysts, RN 66 strain, obtained from Iseki and Wehl (Osaka City University Medical School, Japan), were propagated in 5–7-week-old CF-1 female mice. Ten days after oral inoculation mice were placed in suspended cages and the feces were collected from day 13 through day 20. Oocysts were prepared as previously described (Miller and Schaefer, 2006). Briefly, after homogenization the feces were washed sequentially through sieves with 0.01% (v/v) Tween-20, purified on a 1.0 M sucrose gradient, followed by a 0.85 M sucrose gradient. Centrifugations and washes were done at 1000 × *g* (Ash and Orihel, 1987). *C. muris* oocysts were stored at 4 °C in an aqueous antibiotic–antimycotic solution (GibcoBRL, Grand Island, NY), containing penicillin (1000 units/ml), streptomycin (1000 μg/ml), and 2.5 μg amphotericin B/ml.

2.3. Methylprednisolone acetate preparation and dosing

MPA (DepoMedrol, Pharmacia-UpJohn, Kalamazoo, MI) was obtained as a 20 mg/ml aqueous suspension. The appropriate volume of MPA for 600 mg MPA/kg was aliquoted into a 1.5 ml microcentrifuge tube and, if less than 400 μl total volume, drawn into a 100 U insulin syringe and injected. For volumes greater than 400 μl, the microcentrifuge tube was centrifuged at 10,000 × *g* for 45 s. All but approximately 300 μl of the supernate were removed, the pelleted MPA was resuspended, and then drawn into a 100 U insulin syringe for injection. Each mouse was injected subcutaneously between the two scapulae along the dorsal midline (Miller and Schaefer, 2006).

2.4. Zinc sulfate processing of feces

Mice were transferred into individual clean cages without bedding for fecal collection. A minimum of four fecal pellets were collected per mouse. The feces were transferred to a 15 ml polyethylene tube containing 500 μl distilled water in which the sample was well macerated. Five milliliters of aqueous zinc sulfate (Sp. Gr. 1.18) was added, and the sample was thoroughly mixed. The mixture was centrifuged at 650 × *g* for 5 min without braking and then allowed to stand for a minimum of 15 min. One drop from the surface of each tube was transferred to a clean slide using a sterile inoculation loop (Ash and Orihel, 1987). To each drop 0.5 μl of Crypt-a-Glo™ (Waterborne Labs, New Orleans, LA) was added. After the coverslipped slide was incubated in the dark for 20 min at room temperature, it was examined at 400× using an epifluorescence microscope. The number of fluorescent oocysts counted in 10 high power fields (HPF) were averaged and recorded (Miller and Schaefer, 2006).