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Dynamic Monitor on Immunologic Protective Mechanism in Mice Induced by Recombinant *Echinococcus granulosus* p-29 Protein (Reg.P-29)

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Research Article

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ABSTRACT

Echinococcosis is a worldwide lethal zoonotic infection caused by cestodes species of the genus *Echinococcus. Echinococcus granulosus (Eg)* antigens have been proved to possess protective immunity against secondary infection. In the present study, a diagnostic antigen P-29 was recombined, expressed and purified. There combining *Eg*.P-29(r*Eg*. P-29) as a vaccine candidate was used to immunize mice, and the immune protection and mechanism of this protection was analyzed. Three groups of female mice, group A, B, and C were immunized intraperitoneally with r*Eg* P-29 with complete Freund adjuvant (CFA) in PBS, or phosphate buffered saline (PBS), respectively. Two weeks after additional two boosters, mice were challenged with 2000 *Eg* protoscoleces (PSCs) intraperitoneally. Serum samples and spleens were collected from mice at different time point by sacrificing 5 mice in each group. Antibodies in serum and cytokines in supernatant of lymphocytes cultures were measured. The results showed that compared to control groups, mice immunized with r*Eg* P-29 showed that the level of IgG, IgG1, IgG3 and IgG2b as well as IL-2, IL-4 and IFN-γ were significantly increased after one or more times of immunization, but neither IgG2a nor IgE did. Nevertheless, after mice inoculated with r*Eg*.P-29 was challenged, CD_4^+ and CD_8^+ subsets significantly enhanced which implied that CD_4^+ and CD_8^+ subsets could enlist the protective immune mechanism ($P<0.05$). Therefore, as a promising candidate for an effective vaccine to prevent secondary Echinococcosis, protection against *Eg* protoscoleces induced with r*Eg*.P-29 was associated with humoral and Th1 cellular responses.

INTRODUCTION

Echinococcosis, an ancient but still prevalence zoonotic infection with high incidences in most of the world including Eurasia, north and east Africa, Australia, south America and most provinces of China, is caused by adult or larval stages of cestodes belonging to the genus *Echinococcus* [1,2]. It occurs on the various organs (mainly liver and lungs) of both human and animals which can cause severe and even fatal disease, and the high rate of this infection also represents a considerable public health burden since the treatment is very costly and infection is lethal in most untreated patients [2], thus a series of treatments including improved surgical techniques, chemotherapy and interventional procedures have been developed [3]. It is believed that radical

surgery is the first-choice treatment may lead to complete cure; however, resection is often incomplete because of diffuse and undetected parasite infiltration into host tissues. Chemotherapy is recommended for limited or long-term periods after radical surgery or incomplete resection of lesions in inoperable cases, but chemotherapy itself cannot usually kill the parasite and followed with a series of side effects and drug resistance.

Recently, great effects have been made to develop vaccines, a highly effective and convenient approach, to reduce the incidence and transmission of hydatid disease [4]. It was proved that the intermediate hosts of *E. granulosus* are long-lived and infection by eggs provokes a high degree of protective immunity. Therefore, it has been used for the development of highly effective vaccines. The vaccine has been shown to confer a high degree of protection against challenge with different geographical isolates of *E. granulosus* implied that it could have wide applicability as a new method for use in hydrated control campaigns [2]. The vaccine provides a valuable way to aid in control of transmission of this important human pathogen, and it also has the potential to prevent hydatid disease directly through vaccination of humans, and most of the grazing animals are already vaccinated successfully against viral or bacterial diseases, and it indicated that vaccine against parasitic diseases can greatly helpful to normal farm practice [5]. Vaccination to intermediate host is a burgeoning area that has moved forward considerably in recent years following the development of a recombinant vaccine against Taeniaovis infection in sheep [6]. Any success in recognizing the immunologic reactions of intermediate hosts and finally in producing an effective vaccine could lead to a decline in human infections and also in economic loss of animals. A number of recombinant proteins are available now as candidate antigens for the immune-detection or vaccine candidate selection of *Eg* [7-10].

P-29, a 29-kDa antigen from *Eg*, is a metacestode-specific component which might be another useful diagnostic antigen of *Eg* by the evaluation of the immunologic cross-reactivity between P-29 and a major diagnostic antigen 5 of *Eg.* (Ag5) [11]. In our previous studies, we successfully constructed plasmid P-29/pET28a, transformed it into *Eg coli* BL21 (DE3) pLysS and purified for antigen preparation. Mice vaccinated with r*Eg*.P-29 and challenged intraperitoneally with *Eg* protoscoleces showed significant protective immunity of 96.6% compared with the control group [12]. In this study, we further evaluate the dynamics of immunologic protective mechanisms of purified r*Eg*.P-29 in ICR mice to reveal the related immunologic protective action of this antigen.

METHODS

Materials

Preparation of antigens

Plasmids *Eg*.P-29/pET28a was transformed into *E. coli* BL21 (DE3) pLysS and recombinant *E.coli* was constructed in our previous studies. Protein expression was induced at 37°C overnight in the presence of isopropyl-β-D-thiogalactoside (IPTG, Promega) at a final concentration of 1 mM. The r *Eg*.P-29 was purified from the extract of transformed *E. coli* BL21 (DE3) by nickel chelate affinity chromatography (Novagen) according to the manufacturer's instructions. The purified His6-tagged protein was analyzed on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Protein concentration was determined by Bradford method [13].

Animals and parasite

All animal experiments were strictly carried out according to the guide for the care and use of laboratory animals and were approved by the Bioethics Committee of the Ningxia Medical University. 132 female ICR mice, aged 6 weeks old, were obtained (Experimental Animal Centre of Ningxia Medical University, Yinchuan, China) and randomly divided into 3 groups (group A, B and C, n=44 in each group). *Eg.* protoscoleces (PSCs) was collected aseptically from fertile *Eg* and cysts from livers and lungs of infected sheep. The collected PSCs were washed in phosphate buffered saline (PBS-1%) and Hanks' balanced salt solution (Sigma, St. Louis, USA) containing 100 U/ml of penicillin G and 100 mg/ml of streptomycin sulfate. The viability of PSCs was determined by trypan blue exclusion assay. Only those batches containing more than 90% viable PSCs were used for mice infection.

Vaccination and Challenge infection

Mice in the control group (group C) were vaccinated subcutaneously in the back with only 100 µl PBS, group B with 50 µl CFA in 50 µl PBS, and group A vaccinated with 10 µg of r*Eg*.P-29 in 50 µl PBS emulsified in Freund adjuvant for three times (first immunization in CFA at week 0 and followed by two booster immunizations in incomplete Freund adjuvant (IFA☐ at weeks 2 and 4). Two weeks after the third vaccination, mice were challenged with 2000 viable PSCs intraperitoneally.

Investigation of specific antibodies

Serum antibody responses after immunization with r*Eg*P-29 or PBS were quantified by ELISA according to the published reference [14]. Compared with them, serum antibody responses were quantified by ELISA at 0, 2, 4, 6, 7, and 10 and 31 week from the commencement of experiments which covered the stages of immunization and post-challenge in this study. 96-well microplates (Sino-American Biotechnology Company) were coated with r*Eg*.P-29 (10 µg/100 µl/well) by overnight incubation in 0.1 M carbonate buffer (pH 9.6) at 4°C. Serum samples were diluted 1:100 in PBST (PBS with 0.05% Tween-20) and tested in duplicate. Bound antibody was detected by HRP-conjugated goat anti-mouse IgG and IgG subclass (Novagen) at a 1:1000 dilution in PBST, and OD values of antibody were read at 490 nm in ELISA reader (Bio-Rad).

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Determination of cytokines

Spleen cells were isolated from mice before and after challenge infection for *in vitro* assays. 5×10^6 lymphocytes per ml were maintained at 37 °C with 5% CO_2 in RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 2 mM L-glutamine, and 25 mM 2-mercaptoethanol. Cells were stimulated with 5 µg/ml concanavalin A. Supernatants 100 µl from 72 h cultures were collected and stored at -20°C.

Optical density value of cytokine was determined by ELISA (Jingmei Biotech Company). Supernatant of lymphocytes cultures was added into the coated wells of 96-well plates, and maintained at 37 °C for 2 h. After washed with PBST, 50 ng of biotin-conjugated antibody was added to each well for another 2 h at 37 °C; then washed with PBST again. 50 ng of peroxidase-labeled streptavidin was then added and incubated for 1.5 h at 37 °C, followed by washing for the third times and substrate addition and incubation for 0.5 h at 37 °C. Finally, the reaction was stopped by the addition of 100 µl of 2 M sulphuric acid. The optical density was measured at 490 nm in ELISA reader (Bio-Rad).

STATISTICAL ANALYSIS

The data were analyzed using SPSS version 16.0, using one-way analysis of variance with Dunnett's test. A value of $p<0.05$ was considered statistically significant and all results were presented as the mean ± SD.

RESULTS

Dynamic investigation on the level of IgG, the isotype of IgG and IgE

OD value profiles of IgGs and IgE in immunized mice before and after infection were shown in **Figure 1.** Different stages were selected for antibodies examination: 0 week (before immunization), 2nd (2 weeks after first immunization), 4th (2 weeks after 2nd immunization), 6th (2 weeks after 3rd immunization), 7th (1 week after infection), 10th (4 weeks after infection) and 31st (25 weeks after infection). Through our study, titer of IgG antibody was increased with times immunized adding **(Figure 1F).** Antiserum collected from immunized mice at 6, 8, 10, 18 and 31st week after immunization contained high titer of IgG antibody compared with IgG antibody response of control mice (*P*<0.01). OD value of IgG1, IgG3, IgG2b of immunized group were all higher than control group at 4, 6, 10, 18th week (P<0.05) **(Figure 1B, 1C and 1E).** In addition, we also found that titer of IgE was higher than control groups compared with other time points (*P*<0.01) **(Figure 1A).** However, OD value of IgG2a did not significantly different between immunized group and control group **(Figure 1D).**

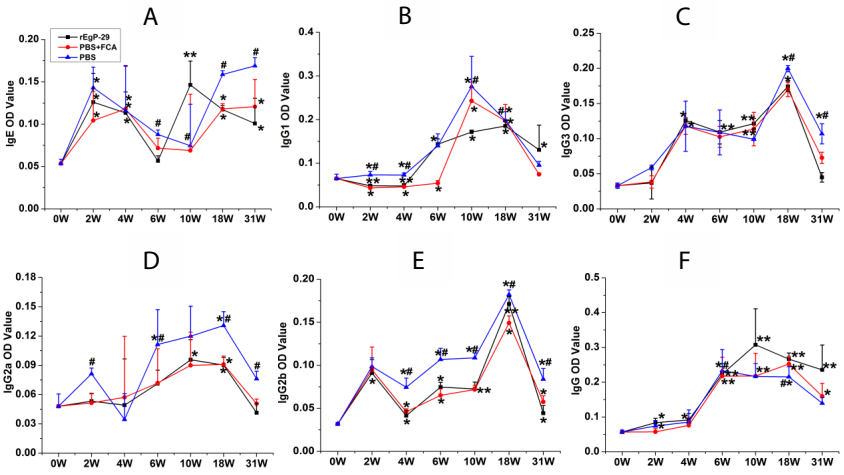

Figure 1. Change of IgG and subclasses of IgG level in mice immunization with rEg.P-29.

Dynamic investigation on splenocyte subsets $CD4^+$ and $CD8^+$ T cell

OD value profiles of IgGs and IgE in immunized mice before and after infection were shown in **Figure 2.** Different stages were selected for antibodies examination as same as **Figure 2.** Both $CD4^+$ and $CD8^+$ increased from 2 weeks after immunized to 18 weeks (after 2 months infected),which were the level of highest. There is serious difference between mice vaccinated with r*Eg*.P-29 PBS+FCA, and PBS.

Dynamic investigation on cytokines

IL-2, IL-4, IL-10, IFN-γ and TNF-α were measured in the supernatant of spleen cells 6 months after the infection as indicators of Th1 and Th2 immune responses, and IL-10 as indicators of immunosuppression **(Figure 3).** Cells taken from r*Eg*.P-29 immunized mice and stimulated with same antigen secreted higher Th1 cytokines (IL-2 and IFN-γ) compared with those from group B or C **(Figure 3A and 3D).** IL-2 secretion in cells of mice immunized with r*Eg*.P-29 significant increased before PSCs challenge (2 weeks after the 3rd immunization). IL-4 and IFN-γ showed an increasing tendency from 0 week to 31st week. IL-10 didn't show

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an increasing before or after infection in r*Eg*.P-29 group compared with control groups **(Figure 3B and 3D).** However, TNF-α was significantly higher in group B and C compared with group A after infection **(Figure 3E).**

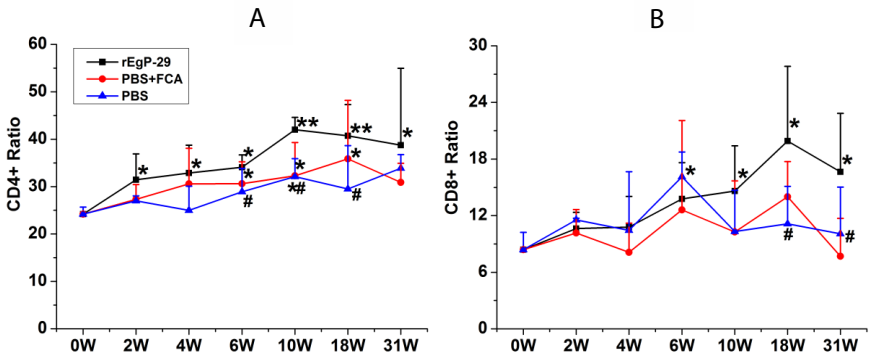

Figure 2. Change of $CD4^+$ and $CD8^+$ ratio in mice immunization with rEg.P-29.

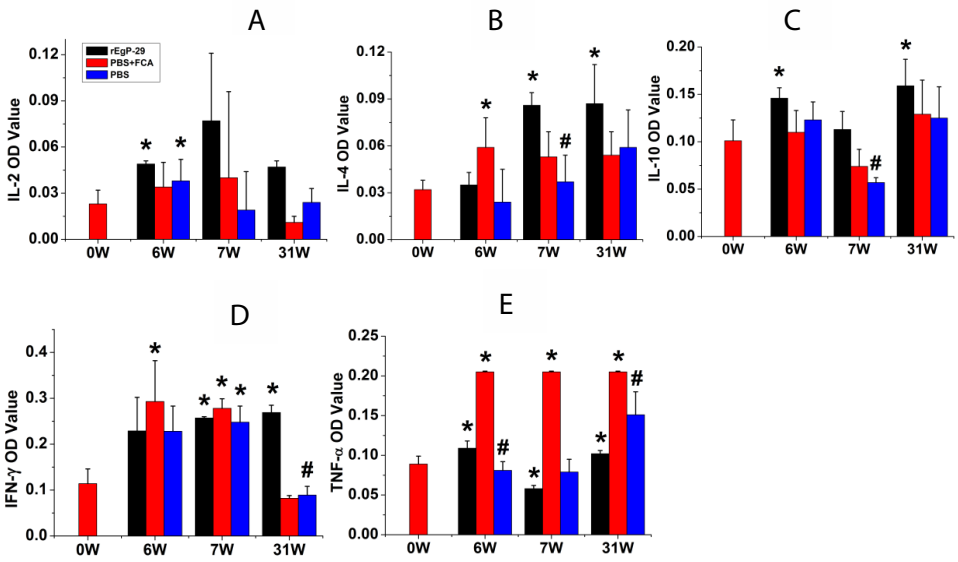

Figure 3. OD value profiles of IL-2, IL-4, IL-10, IFN-γ, and TNF-α in immunized mice before and after infection Different stages were selected for cytokine examination: 0 week (before immunization), 6th week (2 weeks after 3rd immunization), 7th week (1 week after infection), 31st week (25 weeks after infection).

DISCUSSION

Ideal animal model is the basement for *Echinococcus* studying. The factors are as followed: animal type, age and gender. Zhang and Zheng [10] infected with PSCs, and their infectious rates are 94% and 89.28%. That means mice are the suitable host of *Eg* health found that the best animal model for CE is female younger sensitive mice. In this study, ICR mice of 6 weeks and 18-22 g weight were selected, and cyst grew in abdomen after 5 months, that means model of ICR infected by *Eg* successfully, which pave a road for immunologic mechanism investigation later.

Parasite infected host to induce humoral immunity and cell immunity of two aspects. The protective efficacy of humoral immunity in *Eg* was not only correlated with the level of IgG, but also associated with the isotype of IgG. The earliest IgG response to hydatid cyst fluid and on cospheralAgs appears after 2 and 11 week, respectively, in mice and sheep challenged with eggs or oncospheres of *Eg* [15]. As will be described below, these antioncospheral Abs play a major role in parasite killing and are central to the protective immune response against *Eg.* In the chronic phase of CE, elevated levels of Ab, particularly IgG and IgE [16], occur in humans, with IgG1 and IgG4 being predominant [17-19]. IgG1 and IgG3 levels increase significantly at 8 wk post challenge and remain elevated thereafter [20]. In our study, specific IgG, IgG1, IgG3 and IgG2b were significantly elevated in mice immunized with r*Eg*.P-29 compared with the control groups as described above, implying that all or some of these antibodies are relative with immune-protection. OD value of IgE was not signification difference between immunized group and control group except for 10th week (4 weeks after infection). We thought the reason was IgE was increased very early, we missed the optimal time probably, while the reason that it went up rapidly when challenged with PSCs in 10th week was inducing type hypersensitivity.

Information on cellular responses generated by the *Eg* and recombinant vaccines is limited. It is not known whether cell-mediated responses play a role in host protection against *Eg* However, some indirect evidence has shown that these responses are likely to be important. The secondary *Eg.* hydatid cyst mass in mice immunized with the *Eg* vaccine (EG95) coupled to BCG was reduced by nearly 93%, and this was associated with elevated levels of IL-2, IFN-γ, and TNF-α and decreased IL-4,

suggesting that Th1 responses may play a major role against challenge infection in this vaccine model [21] which identified Ags from the parasites' infective larval stage contained within the oncospheres with the potential to induce high levels of protection in vaccinated hosts. Through our experiment, explored the effect of immunization of mice with r*Eg*.P-29 on the outcome of subsequent secondary hydatidosis and the associated immune response. In this way, the present approach referenced and improved not only previous studies that investigated the immune response generated by inoculation of mice with live or dead PSC [22-25] but also the immunoprotection studies of recombinant protein in recent years [21,26]. In our study, IL-2, IL-4 and IFN-showed a significant increase at the 7th week (one week after infection) in r*Eg*.P-29 group. In addition, IL-2 dominated at the first week of post-infection in r*Eg*.P-29 -immunized mice, and this can be seen as an indicator of greater Th1 than Th2 response. IL-4 level was significantly elevated before infection, and it suggests that IL-4 is mainly induced by r*Eg*.P-29 but not pathogens PSCs in this study. IL-4 was reported to be related to stimulating the proliferation and differentiation of mature B cell and assists to drive class switching to IgE which plays a key role in parasite immunity. IL-10 didn't show an increasing before or after infection in r*Eg*.P-29 group compared with control which appeared significantly increasing from early infection to experiment termination. These results are supported by earlier reports about effects of IL-10 in studies of *Eg* infection or other recombinant vaccine candidate tests [27-29]. But how r*Eg*.P-29 inhibits secretion of IL-10 in PSCs challenged mice, need further studies. The results of dynamic investigation on splenocyte subsets $CD4^+$ and $CD8^+$ T cell showed that both $CD4^+$ and $CD8^+$ increased from 2 weeks after immunized to 18 weeks (after 2 months infected),which were the level of highest. There is serious difference between mice vaccinated with r*Eg*.P-29 PBS+FCA, and PBS. So we speculated that both $CD4^+$ and $CD8^+$ T cell were activated by r*Eg*.P-29.

Evidently, immunization with r*Eg*.P-29 leads to effective immunity protection compared with the other two control groups, which there is no significant difference in cyst load reduction. That will provide a foundation for r*Eg*.P-29 to be a valuable vaccine. Humoral and cytokine response played a major role in this protection. However, this immune-protection is due to suppression of the immune escape induced by *Eg* or direct role played by specific antibodies or both need further investigation.

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