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Comparison of Irradiated-Cercaria Schistosome Vaccine Models That Use 15- and 50-Kilorad Doses: the 15-Kilorad Dose Gives Greater Protection, Smaller Liver Sizes, and Higher Gamma Interferon Levels after Challenge

SANDRA R. REYNOLDS* AND DONALD A. HARN

Department of Tropical Public Health, Harvard School of Public Health, and Department of Rheumatology and Immunology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

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The protection and immune response to infection caused by the parasite *Schistosoma mansoni* were studied by comparison of two murine irradiated-vaccine models. Mice were exposed from 1 to 4 times to infective-stage cercariae attenuated with either a moderate dose (15 kilorads) or a high dose (50 kilorads) of radiation. Seven weeks after challenge infection, the mice were assessed for resistance, liver size, and lymphokine responses to parasite antigens. Both vaccine models showed high levels of protection, but the moderate-dose model proved superior in that mice in those groups achieved higher levels of resistance in fewer exposures. Additionally, the mice exposed three times and four times to moderately irradiated cercariae all had significantly lower liver weights independent of worm burden. Assessment of lymphokine production by the spleen cells at the time of perfusion showed that gamma interferon was the only lymphokine of those measured that was differentially produced in the two models and correlated with a decrease in size of in vitro granulomas. The findings suggest that a selected vaccine regimen may lead to greater resistance and decreased liver pathology, the latter of which appears to be mediated by induction of gamma interferon.

The blood vessel-dwelling trematode *Schistosoma mansoni* causes a debilitating parasitic disease affecting more than 300 million people in developing countries. Public health measures such as snail control and drug treatment have had success in a few areas, but the overall prevalence of the disease continues to rise. Therefore, alternative measures of control such as vaccine development remain a high priority.

Mice manifest a schistosomal disease state similar to that seen in humans (25), and high levels of resistance to infection have been attained by exposing mice to radiation-attenuated infective-stage cercariae (for a review, see reference 7). This is generally known as the irradiated-vaccine model. Several aspects of this model have not been adequately defined because of conflicting results reported by different laboratories, notably (i) the dose of radiation which yields the highest levels of resistance; (ii) whether multiple exposures to radiation-attenuated larvae significantly increase resistance, and (iii) whether such a vaccine would aggravate or ameliorate the development of granulomatous egg pathology (26).

In this study, we sought to answer these questions by comparing, post challenge, the effect of earlier exposures of mice to cercariae irradiated with either a moderate dose, 15 kilorads (MDIC), or a high dose, 50 kilorads (HDIC). T cells have been shown to be essential for protection in the vaccine model (23) and are an integral part of granuloma formation (26). Because cytokines and T-helper subsets have recently been the focus of attention as the immune mediators (13, 19, 21, 22), we compared and correlated resistance to infection, liver weight, and in vitro granuloma sizes with the dynamics of lymphokine responses.

* Corresponding author.

MATERIALS AND METHODS

Life cycle. The Puerto Rican strain of *S. mansoni* was maintained in our laboratory by using the snail host *Biomphalaria glabrata* and outbred Swiss-Webster albino mice (Taconic, Germantown, N.Y.).

Mice. C57BL/6J female mice were obtained from Jackson Laboratory (Bar Harbor, Maine). All mice were maintained in accordance with National Institutes of Health guidelines (17). Anesthetized mice (15 per group) were exposed to irradiated (^{60}Co source) and normal cercariae on the abdomen by using the ring method of Smithers and Terry (24). Anesthesia was given intramuscularly as a combination of 80 mg of ketamine HCl (Aveco, Fort Dodge, Iowa) and 5 mg of xylazine (Haver, Shawnee, Kans.) per kg of body weight. Vaccinated and control mice challenged with normal cercariae were perfused 7 weeks after exposure according to the method of Duvall and DeWitt (9). Egg counts were determined after digestion of the livers with KOH as described by Cheever (5) with the modification that the digested material was concentrated by centrifugation before counting. All egg counts were performed in triplicate for each mouse and were within 10% of the mean for the triplicate.

Antigens. Cercarial antigen (Cerc) was prepared by incubation of frozen cercariae obtained from Fred Lewis (Biomedical Research Institute, Rockville, Md.) in 4 mM deoxycholate (Sigma, St. Louis, Mo.) (11). The soluble extract was dialyzed against Dulbecco modified Eagle medium (GIBCO, Grand Island, N.Y.) before use in in vitro experiments. Soluble egg antigen (SEA) was prepared from Percoll (Pharmacia-LKB, Piscataway, N.J.) gradient purified liver eggs as described previously (10).

Lymphokine assays. Interleukin-2 (IL-2) and IL-4 were measured by using the HT-2 bioassay as described by Chang et al. (4). Anti-lymphokine monoclonal antibodies were prepared by cell culture in dialysis tubing (Spectrum, Los

Angeles, Calif.) (14) from the cell lines S4B6.35 (anti-IL-2) and 11B11 (anti-IL-4), a kind gift from DNAX (Palo Alto, Calif.). The gamma interferon (IFN-γ) assay was performed with a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (19). IL-5 was also assayed with a sandwich ELISA according to the method of Schumacher et al. (20), with two separate antibodies from the cell lines TRFK-4 and TRFK-5, also a gift from DNAX. By using serum-free supernatants of antibodies (14), TRFK-4 was biotinylated (19). Recombinant IL-2, IL-4, IL-5, and IFN-γ were all obtained from Genzyme (Boston, Mass.). Units for IL-5 and IFN-γ are as described by the manufacturer. All lymphokine assays were done in triplicate and on two separate occasions. Results for repeated assays were within 10% of their mean.

In vitro granuloma assay. The in vitro granuloma assay was performed and evaluated as described previously (1, 8), with modifications in the type and preparation of beads. One milliliter of Affi-Gel 15 beads (Bio-Rad, Richmond, Calif.) was washed in H_2O and mixed at 4°C for 2 h with either 2 mg of antigens per ml or 0.5 M glycine as a blocked-bead control. After being washed in balanced salt solution, the beads were stored in 0.05% NaN_3 at 4°C until use.

Statistics. Statistical analyses were done by using a one-way analysis of variance and Duncan's multiple range test.

Experimental design. The study began with a large number of age-matched C57BL/6J female mice, with 15 mice in each group. Exposures to irradiated cercariae were at 4-week intervals which started with the 4-times-exposed (4×) group; a new group of mice was added each time. Therefore, the 1× through 4× groups could be infected exactly 4 weeks after their last exposure to irradiated larvae and they would be the same age at that time. The mice were 23 weeks old when they were either exposed to challenge infection with 150 normal cercariae or given boosted injections of Cerc to test for preinfection lymphokine production. To ensure independence of the challenge results, the mice were exposed to the cercariae randomly rather than by group. Mice were perfused within a 3-day interval 7 weeks after challenge infection.

RESULTS

Resistance to challenge infection. The percent reduction of worm burden in the MDIC groups compared with controls ranged from 75.4 to 90.0%, whereas that of the HDIC groups ranged from 40.0 to 79.6%. All of the vaccinated groups showed significantly ($P \leq 0.05$) lower burdens than the infected control group. Most important, as shown in Fig. 1A, each MDIC 1× and 2× group had significantly ($P \leq 0.05$) lower worm burdens and therefore greater resistance to challenge than the corresponding MDIC 1× and 2× groups. The MDIC 3× and 4× groups also had lower (almost significant) worm burdens than the corresponding HDIC groups. Further, only one exposure to MDIC achieved the same level of resistance as three exposures to HDIC. As an additional control to ensure that no worms had survived the lower radiation exposure and thereby induced concomitant immunity or altered the count, we perfused mice which were vaccinated with MDIC but not challenged. No worms, eggs, or granulomas were found in the unchallenged MDIC mice. Our conclusions from these experiments were that both vaccine models led to high levels of protection as measured by worm burdens, but the MDIC model attained these high levels with a single exposure, compared with three in the HDIC model.

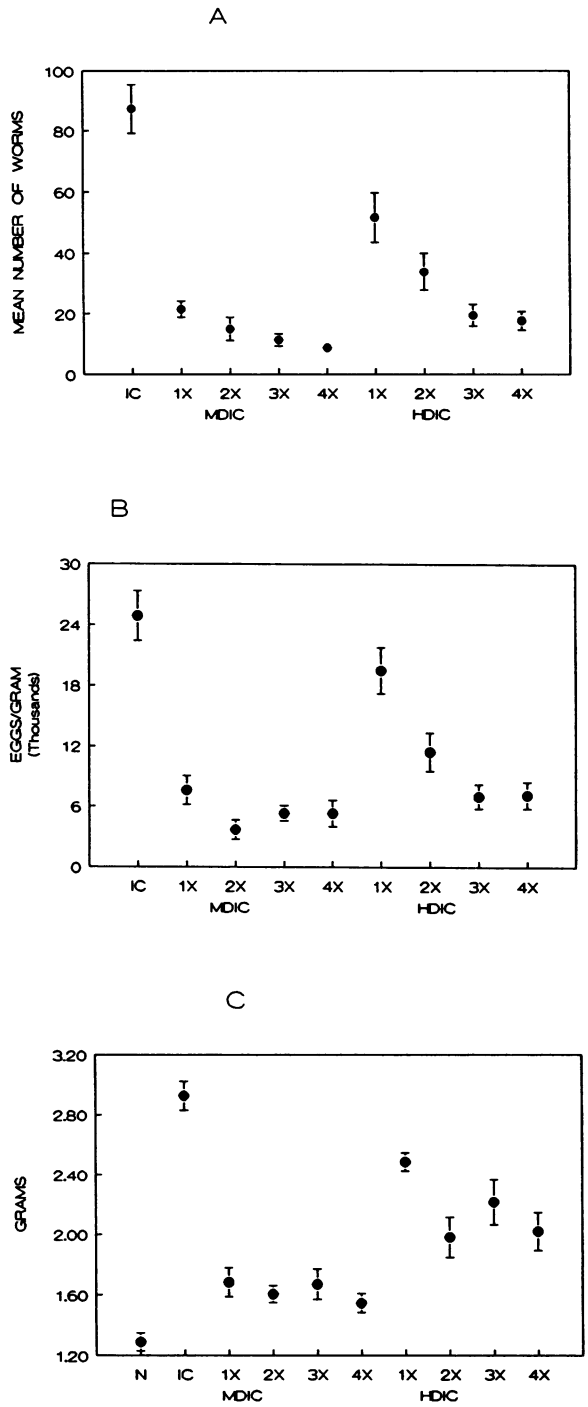

FIG. 1. Worm burdens (A), eggs per gram of liver tissue (B), and liver weights (C) of vaccinated, infected, and perfused mice. The means per group are shown, with the error bars representing standard errors. Statistical significance was calculated as described in the text. IC, infected controls; N, age-matched normal mice.

Egg counts and liver weights. At the time of perfusion, the liver of each individual mouse was weighed and digested to obtain the egg count per gram of tissue. The egg counts shown in Fig. 1B parallel the worm burdens in that fewer eggs correspond to fewer adult worms. Indeed, the two graphs (Fig. 1A and B) look similar. As an additional control

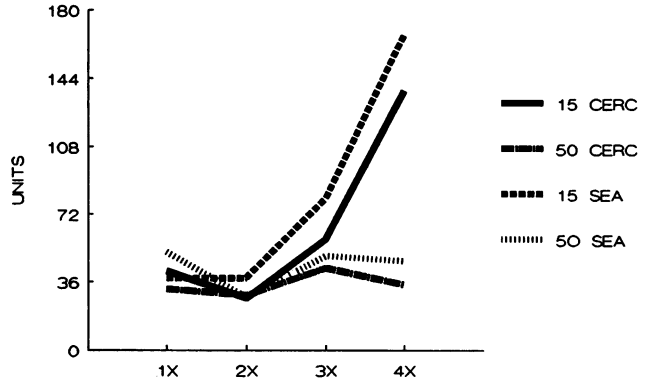

FIG. 2. IFN-γ production of spleen cells after immunization and infection as assessed by ELISA. The response to either Cerc or SEA is shown for all MDIC and HDIC groups. Infected controls showed no IFN-γ production.

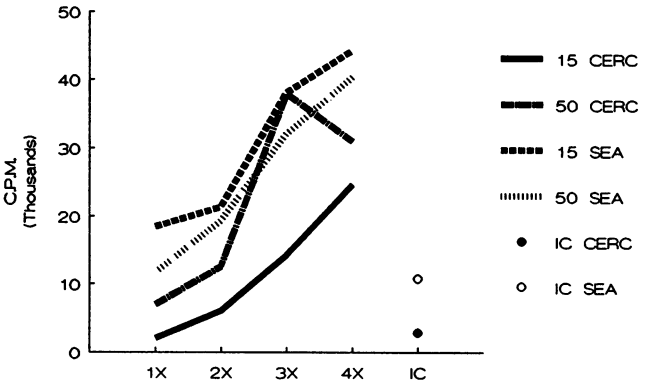

FIG. 3. IL-4 production of spleen cells after immunization and infection was measured by the HT-2 assay in the presence of anti-IL-2. As a control, 100 BRMP units of recombinant IL-4 yielded a mean of 48,280 cpm. The background has been subtracted.

we also determined the mean number of eggs in liver tissue per female worm perfused from the mesenteric veins. None of these counts were significantly different from those from the infected controls, indicating that vaccination did not interfere with egg production in the challenge worms, nor were there any spurious results.

The results for liver weights are shown in Fig. 1C. All of the liver weights from the MDIC mice were significantly lower ($P \leq 0.05$) than those from the HDIC mice even when the groups had equal worm burdens, such as the MDIC 1× and 2× and the MDIC 3× and 4× groups (Fig. 1A). Therefore, liver weights from the MDIC mice were significantly lower, and this result was independent of the number of worms.

Lymphokine production by spleen cells. Spleen cells from each group were also assessed for production of IL-2, IL-4, IL-5, and IFN-γ to two different antigenic preparations. Because vaccinated and challenged mice were exposed to both Cerc and SEA, we assessed the response to both antigens. A lingering response to Cerc might be indicative of cross-reacting epitopes between Cerc and Sea. The results were obtained with a pool of cell from five spleens of each group. Of the four lymphokines, IFN-γ was the most differentially expressed among the MDIC and HDIC groups. Figure 2 shows that the MDIC groups had increasing levels of IFN-γ in response to both Cerc and SEA, which became significantly higher than those of HDIC groups by the third boost and became very high by the fourth. All of the HDIC groups showed only low levels of IFN-γ production, and infected controls showed no IFN-γ production whatsoever.

IL-2, IL-4, and IL-5 production was similar in MDIC and HDIC groups. Figure 3 shows that IL-4 was produced by all groups and to both Cerc and SEA. The amount produced increased as the number of exposures to irradiated cercariae increased. Note that high levels of both IFN-γ and IL-4 were found together in the MDIC groups after the third and fourth exposures. As shown in Fig. 4, the IL-2 responses for both MDIC and HDIC mice were similar, increasing with each additional exposure to irradiated cercariae but only in response to SEA and not to Cerc. Prechallenge assessment showed the mice capable of responding to Cerc (data not shown). Therefore, IL-2 production by the host correlated with antigens currently being seen, which at the time of this postchallenge assessment were SEA rather than Cerc. Figure 5 shows that IL-5 was produced only in response to SEA, with virtually no response to Cerc. There were also significantly higher levels in the HDIC 1× group compared with those in all other groups. Since our prechallenge assessment showed no evidence of IL-5 production, this lymphokine appeared to be associated with the presence of eggs and/or adults.

Granuloma index. Because of the low liver weights observed with the MDIC mice, we assessed the ability of the spleen cells from the vaccinated, challenged mice to form in vitro granulomas. A pool of spleen cells from five mice of the 1× and 4× groups were evaluated for the ability to form granulomas around beads coated with SEA, Cerc, or glycine. The granuloma index is shown in Fig. 6. A higher index corresponds with a larger in vitro granuloma size. Spleen cells from MDIC 4× mice had a significantly ($P \leq 0.05$) reduced granuloma index compared with spleen cells from HDIC 4× mice and infected control cells. This effect was seen with beads coated with either SEA or Cerc. The only lymphokine that correlated with this differential was IFN-γ.

DISCUSSION

There have been differences in results reported by previous investigators as to which radiation dose for cercarial

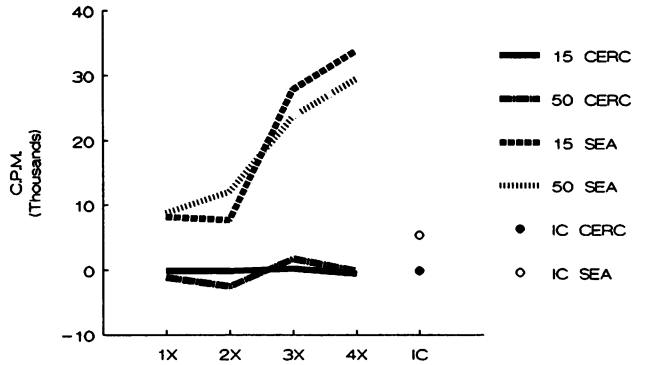

FIG. 4. IL-2 production of spleen cells after immunization and infection was measured by the HT-2 assay in the presence of anti-IL-4. As a control, 50 BRMP units of recombinant IL-2 yielded a mean of 21,390 cpm. The background has been subtracted. IC, infected controls.

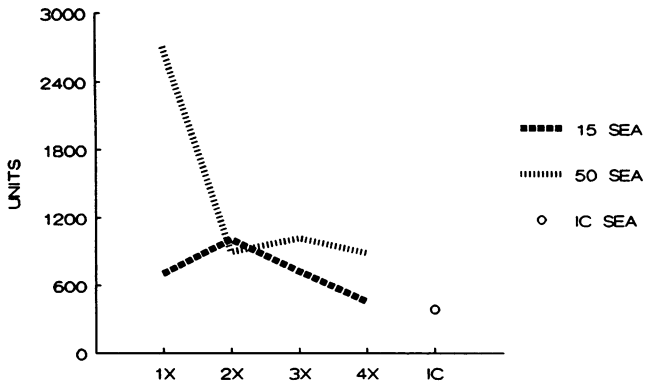

FIG. 5. IL-5 production of spleen cells after immunization and infection assessed by ELISA. See Figure 2 for the legend description. There was no IL-5 response to Cerc in any group or infected controls.

attenuation induces the highest level of resistance to challenge in exposed mice (7). Although high doses of radiation have been used more frequently in studies, our results corroborate the work of Hsü et al. (12) and Bickle et al. (2, 3) in the finding that moderate doses (15 to 20 kilorads) are more protective than high doses (40 to 50 kilorads) when protection is assessed by a decrease in worm burden compared with that in nonimmune mice.

In addition to results for the studies of resistance, a second major finding was that three or four exposures with MDIC were able to induce a significantly higher level of IFN-γ than their HDIC counterparts. Further, an increase in IFN-γ correlated with the formation of smaller in vitro granulomas when cells from MDIC mice were used than when cells from HDIC mice were used. IFN-γ has been previously associated with in vitro killing of schistosomula by macrophages (19). Also, in vivo administration of anti-IFN-γ has been shown to decrease protection in challenge infections (13). To our knowledge, this is the first time that the presence of IFN-γ has been reported to be associated with decreased granuloma size.

With regard to lymphokines assessed in our study, we specifically chose to assess those that are reported to be differentially produced by two different T-helper subsets. Mosmann and coworkers (16) have shown that those cells designated T_h1 produce IL-2 and IFN-γ, whereas those designated T_h2 produce IL-4 and IL-5. Recently, Pearce and coworkers (18) found that T_h2 lymphokine induction was associated with the presence of eggs. We also found IL-4 and IL-5 only after infection and agree that eggs clearly have the ability to induced T_h2 cells. However, we also found high levels of IL-4 in the presence of the T_h1 lymphokine IFN-γ (Fig. 2 and 3). Thus, there may be a third population of cells which are neither T_h1 nor T_h2 that are able to sustain IFN-γ production even when T_h2 lymphokines are present.

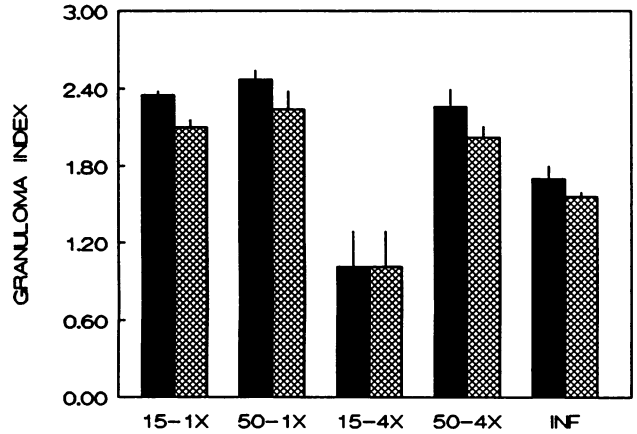

FIG. 6. The granuloma index (by in vitro granuloma assay) of antigen-coated beads mixed with spleen cells. The index of glycine-blocked control beads for each individual group has been subtracted. The error bars represent standard deviations from the means. ■, Cerc; ▩, SEA.

As to which lymphokines are correlated with protection, this study did not focus on the time period in which cells would be activated against the newly incoming worms. However, since IL-4 levels of all the vaccinated mice were much higher than those of infected controls and IFN-γ was completely lacking in the controls, we suspect that these two lymphokines are important in protection. The observation that these responses are to both Cerc and SEA suggests that cross-reacting Cerc and SEA may be important in protection from both incoming worms and granuloma formation.

The findings that MDIC mice had smaller livers than HDIC mice despite the same worm burdens in some groups were encouraging. Liver pathology due to granuloma formation around eggs is immune mediated (26), and it has been previously unclear whether a vaccine would exacerbate the disease. Damian et al. (6) have reported reduced granuloma size in infected baboons previously vaccinated with irradiated larvae. We are currently examining the histopathology and in vivo granuloma sizes found in the two models, and this will be the subject of a future manuscript.

In summary, we have compared two irradiated-vaccine models of protection against *S. mansoni*. The MDIC model was found to be more protective than the HDIC model as assessed by both worm burden reduction and egg numbers found in the liver. The MDIC model resulted in in vitro formation of smaller granulomas, and this was correlated with increased levels of IFN-γ production. Therefore, we propose that MDIC provide a more useful study model and that this should be adopted for future studies. Menson and Wilson (15) have already adopted a model with 20 kilorads for the study of lung-phase immunity. Our findings suggest that it is possible to significantly reduce the pathological effects of schistosomal infections through the use of a vaccine. If this is an antigen-specific phenomenon, then the elucidation of these proteins should provide us with strong vaccine candidates.

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