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INDUCTION OF PROTECTIVE IMMUNITY AGAINST LARVAL *ONCHOCERCA VOLVULUS* IN A MOUSE MODEL

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Abstract. BALB/cBYJ mice were immunized against larval *Onchocerca volvulus* by subcutaneous injection of normal, irradiated, or freeze-thaw–killed *Onchocerca* sp. larvae. The mice received challenge infections of *O. volvulus* third-stage larva (L_3) contained in diffusion chambers implanted subcutaneously. At two-weeks postinfection, the diffusion chambers were removed and larval survival was assessed. When mice were immunized a single time with 35-krad–irradiated or normal *O. volvulus* L_3, there was a significant reduction in the survival of challenge parasites. However, there was little or no reduction in challenge worm survival when mice were immunized a single time with freeze-thaw–killed *O. volvulus* L_3 or fourth-stage larva (L_4), or irradiated *O. lienalis* L_3. When a second dose of freeze-thaw killed *O. volvulus* L_3 or irradiated *O. lienalis* L_3 was administered, there was a significant reduction in parasite survival in immunized mice. Immunization with *O. volvulus* L_4 or a combination of L_3 and L_4 failed to confer protection. These results demonstrate that mice can be immunized against larval *O. volvulus* and that diffusion chambers are an efficient method for studying protective immunity to this parasite in a mouse model.

Studies on the induction and mechanisms of protective immunity to *Onchocerca* spp. have focused largely on responses to microfilariae (mf). It has been shown that mice can be immunized against infection with mf of *O. lienalis* by previous sensitization with normal living mf.[1,2] Resistance to infection with mf can be passively transferred with serum from immunized mice or by transferring a combination of immune T and B cells.[3] Mice immunized against *O. lienalis* mf were also shown to be resistant to infection with mf of *O. volvulus*.[4] While the study of protective immunity to mf may be of importance in understanding the pathogenesis of onchocerciasis, the development of vaccines against the infection will depend on a thorough knowledge of immune responses to the infective third-stage larvae (L_3).[5]

Animal models available for studying immunity to the L_3 of *O. volvulus* are extremely limited. Natural animal reservoir hosts for this infection have not been identified and only rare infections of nonhuman primates in the wild have been reported.[6] Chimpanzees and mangabey monkeys have been experimentally infected with *O. volvulus* L_3.[6–10] Although infections reach patency in chimpanzees and mangabey monkeys, it is unlikely that these animals could be used for large-scale study of protective immunity to *O. volvulus* due to the prohibitive cost and strict regulations associated with their use. Therefore, there is a need for small animal models to study immune-mediated resistance to L_3 of *O. volvulus* and to screen antigens as potential vaccine candidates.

All attempts to recover *Onchocerca* spp. larvae from small laboratory animals following infection have failed.[11–13] However, *Onchocerca* spp. larvae contained in diffusion chambers can be recovered from various rodent hosts for extended periods of time.[14–17] Survival and development of *O. volvulus* L_3 in diffusion chambers implanted in mice were equivalent to that seen in primates.[17] The diffusion chamber technique has been used successfully to contain various filarial worms for immunologic studies in both natural and alternative host systems.[16,18–22] In addition to ensuring accurate recovery of challenge larvae, this method makes it possible to distinguish challenge worms from persisting live immunization worms.

Vaccination against infection using radiation-attenuated parasites has been used successfully against the filarial worms *Brugia malayi*,[21,23,24] *Dirofilaria immitis*,[20,25] *B. pahangi*,[23,26,27] *Acan-*

thocheilonema viteae,[28] and *Litomosoides carinii.*[29] Immunization of mice with irradiated *O. lienalis* L_3 significantly reduced challenge infections of larvae contained within diffusion chambers.[16] The levels of protection elicited with irradiated filarial vaccines were consistently higher than those seen using other parasite preparations; however, the exact reasons for this superiority are unclear.[30] While the radiation dose used to attenuate the immunizing parasites was a critical factor, the optimal radiation dose varied depending upon the parasite system.

The objectives of this study were to demonstrate that protective immunity against *O. volvulus* larvae could be induced in a mouse model and to determine which parasite components are necessary for that induction. It was found that mice can be immunized against larval *O. volvulus* using normal *O. volvulus* L_3 or *O. volvulus* L_3 that were irradiated at a wide range of dosages. Vaccination with dead *O. volvulus* L_3 or irradiated *O. lienalis* L_3 was also effective in inducing immunity when a booster dose was administered. However, preparations containing fourth-stage larva (L_4) failed to confer protection.

MATERIALS AND METHODS

Animals

Male BALB/cByJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were between six and 12 weeks of age at the start of the experiment.

Antigen preparations

Cryopreserved L_3 were prepared in Liberia. Briefly, pupae of *Simulium yahense* were collected in the field and reared to adults in the laboratory. The flies were fed on *O. volvulus*–infected donors and after seven days, L_3 were collected and cryopreserved in dimethyl sulfoxide and sucrose using Biocool II computerized freezing equipment (FTS Systems Inc, Stone Ridge, NY). Cryopreserved *O. lienalis* L_3 were prepared as previously described.[16] The L_3 were defrosted as previously described[16, 17] and placed in a 1:1 (v/v) mixture of National Cancer Institute Tissue Culture Medium 135 and Iscove's modified Dulbecco's media containing 100 U/ml of penicillin/streptomycin and 10 μg/ml of gentamicin (Sigma, St. Louis, MO). Larvae were irradiated at different doses by exposure to x-rays using an orthovoltage x-ray unit (Siemens, Erlangen, Germany). Fourth-stage larvae were prepared by implanting subcutaneously in mice diffusion chambers containing *O. volvulus* L_3 for nine days. It was previously demonstrated that *O. volvulus* L_3 molt to L_4 3–7 days after implantation in vivo in diffusion chambers.[17] Dead L_3 or L_4 were prepared for immunization by freezing and thawing larvae five times on dry ice and in a 37°C water bath.

Experimental protocol

Mice were immunized by injecting 50 larvae subcutaneously into the nape of the neck for the primary immunization, followed two weeks later by 25 larvae for the second immunization, unless otherwise noted. Three weeks after the initial immunization, animals received challenge infections consisting of 25 *O. volvulus* L_3 implanted subcutaneously within diffusion chambers. Experiments were terminated two weeks postchallenge to allow sufficient time for challenge L_3 to develop into L_4, thereby giving the host two larval targets for the immune response to attack.[17] Mice were killed by exsanguination under methoxyflurane (Pitman-Moore Inc., Mundelein, IL), diffusion chambers were removed, and larvae were recovered. Actively motile larvae with good structural integrity were counted as live and were then fixed in 70% alcohol containing 5% glycerine at 60°C. Larval lengths were measured using projected images in the Macmeasure image analysis system (Research Services Branch, National Institute of Mental Health, Bethesda, MD).

Statistical analysis

All data were analyzed by multivariate general linear hypothesis multifactorial analysis of variance using Systat 5.2 (Systat, Evanston, IL). Probability values of less than 0.05 were considered significant. The percent reduction was calculated as follows: % Reduction = [(Mean control mouse worm survival − Mean immunized mouse worm survival) ÷ Mean control worm survival] × 100.

RESULTS

Onchocerca volvulus L_3 were x-irradiated in doses ranging from 5 to 85 krad and implanted

TABLE 1

In vivo survival and development of x-irradiated Onchocerca volvulus *infective third-stage larvae (L_3) contained in diffusion chambers that were implanted in mice for two weeks**

Radiation dose (krad)	No. of mice	% live recovery (mean ± SD)	No. of worms	Length, μm (mean ± SD)†	Stage		
					% L_3	% L_3/L_4	% L_4
0	3	24 ± 14	15	509 ± 86	7	40	53
5	3	23 ± 8	16	478 ± 66	0	25	75
15	3	24 ± 14	14	536 ± 71	14	43	43
25	3	21 ± 13	12	595 ± 49	0	0	100
35	3	28 ± 24	13	496 ± 61	23	46	31
45	2	22 ± 3	8	470 ± 58	0	63	37
55	2	36 ± 28	14	454 ± 70	7	50	43
65	3	27 ± 6	12	488 ± 67	8	67	25
75	3	12 ± 14	8	576 ± 99	13	38	50
85	2	6 ± 3	3	472 ± 19	0	67	33

* Larvae that had formed the fourth-stage (L_4) cuticle, but had not yet shed the L_3 cuticle, were classified as L_3/L_4.
† Length of L_3 = 497 ± 96 μm.

in diffusion chambers in naive mice to assess the development of radiation-attenuated worms in vivo. After two weeks, the diffusion chambers were removed and larval survival and development were assessed. Larval survival of approximately 25% was found in all groups, except for larvae irradiated with 75 and 85 krad, for which survival rates decreased to 12% and 6%, respectively (Table 1). The lengths of recovered worms were not significantly different among groups; however, the percentage of larvae completing the molt to L_4 varied. By two weeks after implantation, all worms irradiated with 25 krad had completed the molt to L_4, while approximately one-quarter of the surviving 35-krad–irradiated larvae remained L_3. Overall, regardless of the irradiation dose, the majority of larvae recovered after two weeks had either synthesized the L_4 cuticle but had not yet completed the molt to L_4 (L_3/L_4) or they had successfully molted to the L_4 (Table 1).

TABLE 2

Effect of immunization of BALB/cBYJ mice with Onchocerca volvulus *infective third-stage larvae (L_3), x-irradiated at various dosages, on the survival of challenge larvae implanted in diffusion chambers subcutaneously*

	Dose of irradiation (krad) per L_3	No. of mice	% live recovery (mean ± SD)	% reduction
Experiment 1	Control	5	38 ± 11	–
	5	5	18 ± 12	53*
	15	5	13 ± 2	66*
	25	3	25 ± 12	34
	35	5	7 ± 7	82*
	45	4	21 ± 5	45*
	55	5	14 ± 11	63*
	65	3	16 ± 11	58*
	75	5	18 ± 5	53*
	85	5	23 ± 7	39*
Experiment 2	Control	5	34 ± 8	–
	25	5	22 ± 8	35*
	35	5	14 ± 9	59*
	45	5	9 ± 9	74*

* $P < 0.05$.

Onchocerca volvulus L_3 were x-irradiated in dosages ranging from 5 to 85 krad and were then used to vaccinate mice using a single immunization of 50 L_3 per mouse. Immunization resulted in reductions in challenge parasite survival ranging from 34% to 82% in all groups of immunized mice (Table 2, experiment 1). The greatest reduction was seen in mice vaccinated with 35-krad–irradiated L_3, while the least reduction was found with 25-krad–irradiated L_3 vaccination. Mice were then immunized with either 25-, 35-, or 45-krad–irradiated larvae. Significant reductions in challenge larval survival was observed in all of the immunized groups (Table 2, experiment 2). Because the 35-krad–irradiated L_3 immunization consistently induced a significant level of protective immunity, this immunization protocol was chosen for further study.

Mice were immunized with initial doses of 10–150 35-krad–irradiated L_3, followed in some cases by a second immunization of 25 irradiated L_3 (Table 3). A minimum of dose of 25 L_3 was needed to confer a significant level of protection. The greatest reduction in worm survival was seen in mice receiving an initial dose of 50 irradiated larvae, followed by a second dose of 25 L_3 two weeks later. Increasing the initial immunizing

TABLE 3

Determination of the optimal primary dose and the effect of a booster inoculation on the induction of protective immunity in mice by 35-krad–irradiated Onchocerca volvulus *infective third-stage larvae (L_3)*

Primary dose	Booster dose	No. of mice	% live recovery (mean ± SD)	% reduction
Control	–	11	40 ± 9	–
10	–	6	45 ± 7	0
25	–	6	23 ± 13	43*
50	–	4	21 ± 8	48*
50	25	5	14 ± 8	65*
100	25	5	26 ± 8	35*
150	25	5	28 ± 11	30*

* $P < 0.05$.

dose above 50 L_3 did not result in a further reduction in challenge worm survival (Table 3).

At the termination of the irradiated larvae vaccine trials, surviving larvae were collected from diffusion chambers and their lengths were determined. There was no consistent difference in length between worms recovered from immunized animals compared with those collected from control animals.

Various methods of vaccine preparation from *Onchocerca* spp. larvae were compared with 35-krad–irradiated *O. volvulus* L_3 for their ability to induce protection against larval *O. volvulus*. Immunization with a single dose of normal, unirradiated *O. volvulus* L_3 induced a significant level of protection. Immunization with a single dose of dead *O. volvulus* L_3 or 15-krad–irradiated *O. lienalis* L_3 had no significant effect on challenge worm survival. However, when booster doses of dead *O. volvulus* L_3 or 15-krad–irradiated *O. lienalis* L_3 were given, there was a significant decrease in challenge worm survival. Immunizations using killed *O. volvulus* L_4 or a combination of L_3 and L_4 were not effective in inducing protective immunity (Table 4).

TABLE 4

Effect of immunization of mice with normal, irradiated, and killed Onchocerca volvulus *infective third-stage larvae (L_3) and/or fourth-stage larvae (L_4) and irradiated* Onchocerca lienalis *L_3 on the survival of* O. volvulus *L_3 implanted in diffusion chambers. Mice were immunized either a single time or received a booster immunization**

Treatment, species, and stage of immunizing larvae	No. of mice	No. of doses	% reduction
O. volvulus			
Normal L_3	8	1	54†
35 krad L_3	9	1	60†
	16	2	67†
Dead L_3	5	1	14
	15	2	67†
Dead L_4	4	1	3
Dead L_3/L_4	5	1	0
	3	2	0
O. lienalis L_3			
15 krad	5	1	17
	9	2	42†

* Data are the combined results from four experiments; values listed are the percent reductions obtained when the immunized groups were compared with the control group in a particular experiment.
† $P < 0.05$.

DISCUSSION

In the present study, radiation-attenuated larvae were chosen for initial attempts at immunization of mice against larval *O. volvulus* because of the successes of irradiated vaccines against filaria in the past.[16, 20, 21, 23–29] Dogs have been protected against infection with *D. immitis* by immunization with 20-krad–irradiated L_3. This dose of radiation caused the larvae to develop into sterile, stunted adults.[25] If greater than 20 krad was used to attenuate the immunizing larvae, the immunizing worms died prematurely; immunization with these larvae failed to protect dogs against the infection. This finding suggests that the development of immunizing larvae to L_4 or adult worms is a requirement for effective immunization of dogs against *D. immitis*. For successful immunization of rats against *L. carinii*, immunizing larvae must be attenuated by radiation dosages greater than 40 krad. If immunizing *L. carinii* L_3 were irradiated with less than 40 krad, they failed to confer protection.[29] Irradiation with 40 krad prevented L_3 from molting to L_4. This finding suggests that in the *L. carinii* system, prolonged exposure to L_3 is required for the induction of protective immunity. Therefore, to develop immunity against some parasites, such as *D. immitis*, it is important for the host to be exposed to more than one stage, whereas for others, such as *L. carinii*, prolonged exposure to a single stage is crucial.

In the present experiments, protective immunity was seen in mice vaccinated with irradiated L_3, regardless of the radiation dose used to attenuate the larvae. In vivo, irradiated *O. volvulus* larvae in diffusion chambers survived and developed to approximately the same degree

regardless of the radiation dosage used. These findings suggest that for *O. volvulus*, unlike the examples mentioned above, the dose of radiation used to irradiate immunizing larvae is not a critical factor in determining whether a vaccine will induce a significant level of parasite killing. This successful immunization of mice with irradiated larvae appears to conflict with a study in which chimpanzees immunized with 45-krad–irradiated *O. volvulus* failed to develop protective immunity.[31] The experimental protocols used in the chimpanzee study and in the present mouse study differ in the timing and dosage of the immunizations, making comparisons between these two studies difficult. The criterion used for vaccine efficacy in the chimpanzee study was the absence of mf in the skin of immunized animals. It is possible that protective immunity would have been recognized in the chimpanzee study if the presence or absence of live larvae or adults had been used as the criteria for vaccine success.

Killed parasite vaccines have induced protective immunity in other filarial systems.[18, 32, 33] In the present experiments, a single injection of 50 killed *O. volvulus* L_3 did not produce immunity to challenge. However, two immunizations with killed *O. volvulus* L_3 were able to confer resistance to infection comparable with that seen with irradiated larvae. Immunization with killed L_4, however, did not induce protective immunity. Therefore, exposure to larval excretory/secretory antigens or L_4-specific antigens does not appear to be required for the development of protective immunity against larval *O. volvulus* in the mouse. Induction of protective immunity was dependent upon the presence of L_3 antigens and the dose and timing of the immunizations.

Immunization of mice in the present study with irradiated *O. lienalis* L_3 was capable of producing a significant reduction in the survival of challenge *O. volvulus* larvae. The dose of irradiation used on the immunizing *O. lienalis* larvae was based on the optimal dose found for homologous immunization and challenge.[16] Cross-species protection between filarial parasite species has been demonstrated with *Onchocerca* spp. mf[4] and *Brugia* spp. L_3.[33]

Growth retardation of challenge larvae has been reported in studies of protective immunity in *D. viteae*,[19] *B. malayi*,[21] and *D. immitis*.[34] Immunity induced against *O. volvulus* by 35-krad–irradiated *O. volvulus* L_3 was not associated with a decrease in the length of challenge worms. A possible explanation for this finding is that there was great variability in the lengths of the L_3, as has been previously reported.[35] This variability may have overshadowed any differences caused by the immune response.

In conclusion, these studies demonstrate that protective immunity to larval *O. volvulus* can be induced in mice using live, dead or radiation-attenuated *O. volvulus* L_3 or irradiated *O. lienalis* L_3. The model developed in the present study can be a valuable tool for the screening of antigens for use in vaccines against *O. volvulus*.

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