PROTECTIVE IMMUNITY ELICITED BY ULTRAVIOLET-IRRADIATED THIRD-STAGE INFECTIVE HOOKWORM (*NECATOR AMERICANUS* AND *ANCYLOSTOMA CANINUM*) LARVAE IN MICE AND HAMSTERS

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Abstract. The protective immunity elicited by ultraviolet-irradiated third-stage infective larvae of *Necator americanus* (UV-NaL3) and *Ancylostoma caninum* (UV-AcL3) was evaluated in laboratory mice (a non-permissive model) and hamsters (a permissive model). After optimizing the time of exposure to UV-irradiation, both oral and subcutaneous vaccination routes with UV-AcL3 in mice were explored. Oral vaccination was more effective at reducing the number of challenge AcL3 entering the lungs, whereas subcutaneous vaccination was more effective at blocking muscle entry. When UV-irradiated NaL3 and non-irradiated AcL3 were used as vaccines in hamsters, both of them were effective at reducing adult hookworm burdens. However, the length of protection afforded by UV-irradiated L3 was substantially greater than that resulting from immunization with non-irradiated L3. A single dose was less effective than multiple doses. The protective immunity elicited by UV-irradiated NaL3 given once every other week for a total of three immunizations was similar to that elicited by non-irradiated AcL3 given during the same schedule. Protection was not significantly affected by administering the L3 on a weekly basis for a total of three immunizations, even though the antibody titers were reduced using this schedule. These studies will facilitate the elucidation of the mechanisms underlying larval protection.

INTRODUCTION

Human hookworm infection is highly endemic in Sub-saharan Africa, East and South Asia, China and the Americas (Hotez *et al*, 2005). In China alone, there were an estimated 194 million cases determined by the first nationwide parasite survey conducted between 1988 and 1992 (Yu *et al*, 1994; Hotez *et al*, 1997). In the second nationwide parasite survey (2001-2004), the prevalence of hookworm declined significantly after adoption of a series of intervention measures, and the number of estimated cases were 39.3 million (Anonymous, 2005). Both major anthropophilic hookworm species are represented in China, with *Necator americanus* predominating in South China, especially in Hainan and Yunnan (Liu *et al*, 1999; Zhan *et al*, 2000; Gandhi *et al*, 2001; Bethony *et al*, 2002), and *Ancylostoma duodenale* predominating in more northerly latitudes, such as in Anhui and Jiangsu Provinces (Sun *et al*, 1998; Wang *et al*, 1999). In many parts of China, both hookworm species occur sympatrically (Hotez *et al*, 2005).

Endemic hookworm persists in the world

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despite the widespread availability of albendazole and other anthelminthic drugs. The failure of anthelmintic drugs to control hookworm in many countries is a consequence of high rates of reinfection following treatment and specific behavioral practices, such as indiscriminate defecation or the employment of human feces as nightsoil fertilizer. These observations have prompted research efforts to develop anti-hookworm vaccines as an alternative biotechnology control measure (Hotez *et al*, 1999, 2004).

Proof-of-concept that it is feasible to develop a hookworm vaccine is based on the observation that multiple doses of living third stage infective hookworm elicit protection in laboratory animal hosts (Hotez *et al*, 1999). Previously, L3 of the dog hookworm *Ancylostoma caninum* were shown to elicit high levels of protective immunity in mice when administered in doses of 500 L3 once every 2 weeks for a total of 3 immunizations (Xiao *et al*, 1998a). Although *A. caninum* L3 do not develop to adult hookworms in mice (mice are considered non-permissive hosts), they undergo extra-intestinal migrations analogous to those in permissive hosts (Xiao *et al*, 1998a). Reductions in host worm burden can be determined by recovery of L3 from the lungs and muscles following challenge infections (Xiao *et al*, 1998a; Ghosh and Hotez, 1999). The protection is robust with more than 90% reduction in hookworm burdens noted after larval challenge compared to non-vaccinated controls (Xiao *et al*, 1998b). Studies on the mechanisms of protection afforded by living L3 vaccinations reveal that antibody responses of the Th2 type are required for hookworm burden reduction (Xiao *et al*, 1998a; Ghosh and Hotez, 1999). However, host cellular immunity is also required with murine studies demonstrating that protection requires leukocyte adhesion, mast cell degranulation, cutaneous and subcutaneous granulomata formation, and pulmonary inflammation (Xiao *et al*, 1998b, 2001; Yang *et al*, 1998, 1999).

Although protection associated with live L3 vaccines is robust in terms of worm burden reductions, it is also short lived. Vaccine protection is maximal for the first two weeks following immunization, but it subsequently declines precipitously (Xiao *et al*, 1999). In order to optimize L3-induced protection, it is worthwhile to explore whether the length of protection can be improved by first attenuating the L3 with ionizing radiation (Hotez *et al*, 1999). An improved live irradiated L3 vaccine would then be a first step towards identifying specific antigens linked to immunity. Here we report that L3 attenuated with ultraviolet irradiation offers advantages over non-irradiated L3. UV-irradiation is effective for attenuating both *Ancylostoma caninum* and *Necator americanus*. The attenuated L3 are effective vaccines for both mice challenged with *A. caninum* (non-permissive model) and hamsters with *N. americanus* (permissive model).

MATERIALS AND METHODS

Effect of ultraviolet on *Ancylostoma caninum* and *Necator americanus* L3

A Shanghai strain of *A. caninum* was maintained in hybrid dogs, and *N. americanus* in hamsters, as previously described (Xiao *et al*, 1998a; Xue *et al*, 2003). Infected third-stage larvae of *A. caninum* (AcL3) and *N. americanus* (NaL3) were collected by the glass tube-filter paper method (Ren *et al*, 1994).

For ultraviolet (UV) irradiation, 100 and 1,000 L3 were suspended in 0.15 ml and 0.25 ml of distilled water was placed in a well of a glass slide, respectively. The slide was placed on a smooth surface under an ultraviolet lamp (15 W, 1537 nm) at a distance of 5 cm. After the larvae were exposed to UV-irradiation for 1, 2, 4, 6 and 8 minutes, their activity was examined under an inverted microscope equipped with a temperature chamber at 37ºC.

To test the viability of the UV-irradiated

L3, they were placed on a sieve layered with cotton paper immersed in water at 45ºC. Thirty minutes. after penetration, the larvae detected in the water were counted. Non-irradiated L3 served as the controls.

Mice infections with UV-irradiated AcL3

Outbred male Kunming strain mice, weighing 18-22 g, were purchased from the Shanghai Animal Center, Chinese Academy of Sciences (Shanghai, China), and maintained on commercially obtained rodent food and water ad libitum. Approximately 1,000 AcL3 suspended in water and exposed to UV light (15W, 1537 nm, distance: 5 cm) for 5-60 seconds were administered orally or subcutaneously to the mice. Based on previous kinetics established by infecting mice with AcL3 and determining that lung entry is maximal at 48 hours and muscle entry at 96 hours, these time periods were selected to harvest these organs, respectively (Xiao *et al*, 1998a). At 48 hours post-infection, the lungs and trachea were removed from sacrificed mice and minced with scissors in a 9 cm diameter culture dish. The minced lung was suspended in distilled water and maintained in an incubator for 2 hours at 37ºC. The number of AcL3 released from the lung tissue were counted under a stereoscope. To measure hookworm burden in the muscles, mice were sacrificed at 96 hours post-challenge infection, and the muscles were removed, minced with scissors, and the muscle pieces were then placed in a metal sieve padded with two layers of cotton paper. The sieve was immersed in a culture dish (9 cm in diameter) containing water at a temperature of 45ºC for 30 minutes. During this period, the larvae migrating through the cotton paper and sieve were counted under a stereoscope.

Mice vaccinations with UV-irradiated AcL3

Approximately 2,000 AcL3 suspended in 0.15 ml of distilled water were placed in a well of the slide, which was placed on a smooth surface under an ultraviolet lamp (15 W, 1537 nm) at a distance of 5 cm. The larvae were exposed to UV light for 30 seconds before they were used for immunization. Groups of 10 mice were immunized three times either orally or subcutaneously with 2,000 UV-irradiated AcL3 once every two weeks. One week after final immunization, the mice were challenged orally or subcutaneously with 1,000 non-attenuated (non-UV-exposed) AcL3. At the time of maximal larval entry into the lungs and muscles, at 48 hours and 96 hours post-challenge, respectively, the AcL3 were counted as described above. Protection was determined by comparing the mean number of larvae recovered from the immunized mice compared with non-immunized mice. To determine the length of protection associated with UV-irradiated L3 immunizations, the times of challenge following vaccinations were varied.

Hamster infections and vaccinations with UV-irradiated L3 or AcL3

Male golden hamsters (*Mesocricetus auratus*) aged 8 weeks were supplied by the Shanghai Animal Center, Chinese Academy of Sciences (Shanghai, China), and maintained on commercially obtained rodent food and water ad libitum. Groups of hamsters were immunized subcutaneously by injection in the abdominal skin with NaL3 exposed to UV light (UV-NaL3) for 30 seconds, or non-irradiated AcL3, once every second week for one and three times, or once every week for three weeks. Subsequent challenge with 150 non-irradiated NaL3 was performed by injecting the vaccinated hamsters subcutaneously (in the leg) one week after the final immunization. All groups of hamsters were sacrificed at either 25 or 28 days post-challenge in order to recover the adult worms from intestine. Vaccine protection was evaluated by determining the mean worm number relative to the non-immunized control hamsters.

Serologic response in hamsters

Antibody levels to adult *N. americanus*

antigens were measured by ELISA in two different experiments: 1) in two groups of 5 hamsters, blood samples were drawn from the retroorbital sinus of each hamster for separation of serum just before each vaccination with 500 or 2,000 UV-irradiated NaL3 once every week for three weeks, one week following the last immunization prior to challenge with 150 NaL3 (non-irradiated) and then every week post-challenge for 12 weeks; a total of 16 weeks from the beginning of the test. A non-vaccinated group of 5 hamsters served as the control group. Specific antibody levels against adult hookworm antigens in the sera with a dilution of 1:100 were then determined by enzyme-linked immunosorbent assay (ELISA) using goat anti-hamster IgG HRP conjugate as the second antibody (Xiao *et al*, 1998a; Xue *et al*, 1999); 2) Four groups of 10 hamsters were vaccinated with 2,000 UV-irradiated NaL3 or non-irradiated AcL3 once every week or every other week for a total of three immunizations. Two groups of 10 hamsters received only a single vaccination with UV-irradiated NaL3 or non-irradiated AcL3. Another non-vaccinated group of 10 hamsters served as the control group. Blood samples were drawn from each hamster just before each vaccination, one week after the last vaccination, prior to challenge with 150 normal NaL3, and four weeks post-challenge. Specific antibody levels againsts adult hookworm antigen in the sera with a dilution of 1:100 were measured using ELISA (Xiao *et al*, 1998a; Xue *et al*, 1999).

RESULTS

Larval activity following UV-irradiation

As shown in Table 1, the ability of L3 to penetrate cotton filter paper was unaffected by UV irradiation lasting one minute. However, reduction in larval penetration activity began to decline following 2 minutes of exposure and was nearly abolished after 3 minutes of UV irradiation exposure. The reduction in larval penetration after 2 minutes of exposure was consistent with their appearance under light microscopy. L3 irradiated with UV light for 1 minute exhibited no differences in their visible behavior relative to non-irradiated L3. In contrast, after UV irradiation for 2 minutes the L3 appeared stiff, although they still exhibited spontaneous movement. After 4 and 6 minutes of light exposure, the larvae coiled and showed only slight movements. At 8 minutes after exposure no larval movements were noted (data not shown).

Table 1
In vitro activity of Ac-L3 following UV-irradiation[c].

Exposure time (min)	Number of samples tested	Mean AcL3 number after penetration(x ± SD)	Reduction rate of penetration(%)
0	11	1,104 ± 82	-
1	5	1,071 ± 58[a]	0
2	11	694 ± 185[b]	37.1
3	7	170 ± 85[b]	84.6
4	10	15 ± 7[b]	98.6
8	5	0.4 ± 0.9[b]	99.9

[c]AcL3 in water were exposed to UV-irradiation (15 W, 1537nm, distance: 5 cm) for 1-8 minutees. Each sample contained 1,000-1,200 AcL3. The L3 penetrating 3 layers of cotton paper were counted. [a]$p>0.05$, [b]$p<0.01$ *vs* the control.

Mouse infectivity experiments with UV-irradiated L3

Following oral or subcutaneous infection in mice, non-irradiated AcL3 enter the lungs at 48 hours and subsequently leave the lungs before they begin to appear in the muscles (Ghosh *et al*, 1996; Xiao *et al*, 1998). At 96-hour post-infection, non-irradiated AcL3 enter the skeletal muscles in maximal numbers. AcL3 do not molt to the L4 stage in mice. Table 2 shows three different AcL3 infectivity experiments in which larvae were exposed to UV-irradiation for different periods of time and administered either orally or subcutaneously to mice. Relative to non-irradiated AcL3, the AcL3 exposed for 10 seconds or less successfully entered into the lungs by 48 hours after oral infection. There was, however, a significant reduction in the number of AcL3 entering the lungs following UV exposure lasting 30 seconds. When AcL3 exposed for 10-15 seconds were administered orally, there was no apparent impact on the number of larvae migrating into the muscles at 96-hour post-infection (relative to non-irradiated AcL3). However, after 20 seconds of exposure to UV-irradiation, the number of AcL3 recovered from muscles was reduced relative to the control AcL3 ($p<0.01$). When the route of infection was changed from oral to subcutaneous, the reduction in the number of AcL3 that reached either the lungs or muscles was reduced following only 10 seconds of exposure ($p<0.01$). Therefore AcL3 entering via the subcutaneous route were more sensitive to UV-irradiation.

Mice vaccinations with UV-irradiated L3

Table 3 summarizes three different experiments in which mice were immunized either orally or subcutaneously with UV-irradiated L3 prior to challenge with non-irradiated L3. AcL3 were exposed to UV irradiation for 30 seconds. In each experiment, mice immunized via the oral route exhibited greater hookworm burden reductions in the lungs compared to subcutaneoulsy vaccinated mice 48 hours post-challenge. However, subcutaneously vaccinated mice exhibited greater hookworm burden reductions in the muscles 96 hours post-challenge.

As shown in Table 4, the length of protection with respect to lung hookworm burdens remained significant up to 4 weeks after completing the oral immunizations series. However, the length of protection with respect to muscle hookworm burdens remained sig-

Table 2
In vivo migration of AcL3 in mice following UV-irradiation[a] exposure.

Administration route of AcL3	Harvesting AcL3 from											
	Lung (48 hours) Exposure time of AcL3 to UV (seconds)							Muscle (96hours) Exposure time of AcL3 to UV (seconds)				
	0	5	10	15	25	30	60	0	10	15	20	25
Oral	80	69	84	-	-	3	0	273	223	226	115	-
	98	75	90	-	-	0	2	282	223	263	202	-
	104	105	116	-	-	0	2	288	334	293	212	-
Subcutaneous	175	-	31	4	0	-	-	603	258	10	-	1
	182	-	62	8	0	-	-	612	392	53	-	2
	201	-	87	33	0	-	-	667	429	126	-	2

[a]15W, 1573 nm, distance: 5 cm. Each mouse infected with 1,000 UV-irradiated AcL3.

Table 3
Protective immunity in mice immunized orally or subcutaneously with UV-irradiated AcL3[d].

Group	Challenge route	Larva recovery from lung			Challenge route	Larva recovery from muscle		
		N	MNL	LRR		N	MNL	LRR
NIM	Oral	5	113 ± 54	-	SC	5	474 ± 95	-
OIM	Oral	4	50 ± 21[b]	56	SC	4	315 ±103[b]	34
SIM	Oral	5	88 ± 30[a]	22	SC	5	159 ± 129[c]	57
NIM	Oral	5	73 ± 31	-	SC	5	347 ± 46	-
OIM	Oral	6	11 ± 7[c]	85	SC	6	157 ± 84[c]	55
SIM	Oral	5	31 ± 10[b]	58	SC	5	100 ± 24[c]	71
NIM	Oral	5	150 ± 44	-	SC	5	225 ± 22	-
OIM	Oral	5	31 ± 15[c]	76	SC	5	139 ± 69[b]	38
SIM	Oral	3	51 ± 30[c]	66	SC	4	91 ± 50[c]	60

[d]AcL3 were exposed to UV-irradiation for 30 seconds; mice were immunized either orally or subcutaneously with 2,000 UV-irradiated AcL3 once every 2 weeks for 3 times. Mice were then challenged either orally or subcutaneously with 1,000 non-irradiated AcL3 one week after the final immunization. NIM, non-immunization; OIM, oral immunization; SIM, subcutaneous immunization; N, number of mice; MNL, mean number of larva (x±SD); LRR, Larva reduction rate (%).[a]p>0.05, [b]p<0.05, [c]p<0.01 *vs* the corresponding control.

nificant up to 8 weeks after completing the subcutaneous immunization series.

Hamster infections and vaccinations with UV-irradiated *Necator americanus* third-stage infective larvae (NaL_3) or AcL3

Unlike mice, which are non-permissive hosts for both *A. caninum* and *N. americanus*, hamsters are only permissive for *N. americanus* (Xue *et al*, 2003); *ie,* the NaL3 can develop to egg laying adult hookworms in the small intestine of hamsters. Using this animal model, protective immunity elicited by UV-irradiated NaL3 and non-irradiated AcL3 were studied. In the first and second experiments, groups of 4-5 hamsters were immunized subcutaneously with 500 or 2,000 UV-irradiated NaL3 (NaL3 were exposed to UV-irradiation for 30 seconds at a distance of 5 cm) once each week for 3 times, then challenged subcutaneously with 150 non-irradiated NaL3 one week after the last vaccination. In the group immunized with 500 UV-irradiated NaL3, the mean worm number was significantly lower than that of the non-vaccination group with a worm reduction rate of 84.7% (Table 5). In the hamsters immunized weekly with 2,000 UV-irradiated NaL3, all hamsters were protected and no worms were found in the small intestines (Table 5).

In the third experiment, groups of 10 hamsters were vaccinated with 2,000 UV-irradiated NaL3 or 2,000 non-irradiated AcL3 once every second week for 1 or 3 times, or vaccinated with the same number of UV-irradiated NaL3 or non-irraduated AcL3 once every week for 3 consecutive weeks and challenged with 150 non-irradiated NaL3 one week after the last vaccination. As shown in Table 6, the mean worm burden derived from the non-vaccinated group was 19.7±10.8. The levels of hookworm burden reduction were significantly improved by increasing the number of doses of UV-irradiated NaL3 or non-irradiated AcL3 from one to three times. The protective immunity elicited by these two kinds of larvae were similar, with worm reduction rates of

Table 4
Length of protection afforded by oral or subcutaneous immunization with UV-irradiated AcL3 in mice.

Group(n=10)	Time after immunization (weeks)	Mean number of AcL3 (x ± SD)	Larva reduction rate (%)
Orally		Lung[d]	
NIM	1	123 ± 50	-
IM	1	4 ± 6[c]	97
NIM	2	188 ± 21	-
IM	2	30 ± 34[c]	84
NIM	4	128 ± 20	-
IM	4	58 ± 33[c]	55
Subcutaneously		Muscle[c]	
NIM	1	445 ± 45	-
IM	1	61 ± 46[c]	86
NIM	2	431 ± 82	-
IM	2	84 ± 60[c]	81
NIM	4	332 ± 128	-
IM	4	130 ± 54[b]	61
NIM	8	355 ± 162	-
IM	8	209 ± 66[b]	41

[d]Mice (n=10 per group) were immunized orally with 2,000 UV-irradiated AcL3 once every two weeks for 3 times and were then challenged orally with 1,000 non-irradiated AcL3 at different intervals after final immunization and sacrificed 48 hours after challenge for recovering larvae from lung. [c]Mice (n=10 per group) were immunized subcutaneously with 2,000 UV-irradiated AcL3 once every two weeks for 3 times and then challenged subcutaneously with 1,000 normal AcL3 at different intervals after final immunization and sacrificed 96 hours after challenge for recovering larvae from muscles. IM, immunization; NIM, non-immunization. [b]$p<0.05$, [c]$p<0.01$ *vs* the corresponding control.

Table 5
Protective immunity in hamsters immunized subcutaneously with UV-irradiated NaL3 (UV-NaL3)[a].

Group[b]	Species of L3	No.hamser per group	No. L3 immunized	Mean worm number (x ± SD)	Worm reduction rate (%)
NIM-1	-	4	-	23.5 ± 13.2	-
IM-2	UV-NaL_3	5	2000	0	100
IM-3	UV-NaL_3	5	500	3.6 ± 8.0[a]	84.7
NIM-4	-	5	-	28 ± 24	-
IM-5	UV-NaL_3	5	2000	0	100

[a]NaL3 were exposed to UV-irradiation for 30 seconds. [b]Hamsters were challenged subcutaneously with 150 non-irradiated NaL3 and were sacrificed at 25 days (NIM-1, IM-2 and IM-3) or 28 days (NIM-4, IM-5) after challenge; [c]$p<0.05$ *vs* the corresponding control.

Table 6
Protective immunity elicited by UV-irradiated NaL3 (UV-NaL3) or non-irradiated AcL3 (AcL3) in hamsters[a].

Number of immunization	Species of larvae	No.hamster	Mean worm number[e] (x ± SD)	Worm reduction rate (%)	p-value *vs* control
Control	-	10	19.7 ± 10.8	-	-
1[b]	UV-NaL3	10	10.3 ± 4.8	47.7	<0.05
	AcL3	10	11.0 ± 6.3	44.2	<0.05
3[c]	UV-NaL3	10	4.2 ± 8.6	78.7	<0.01
	AcL3	10	2.9 ± 2.7	85.3	<0.01
3[d]	UV-NaL3	10	1.9 ± 3.8	90.4	<0.01
	AcL3	10	6.1 ± 2.7	69.0	<0.01

[a]Protective immunity in hamsters was elicited by subcutaneous immunization with 2,000 UV-irradiated NaL3 (exposed to UV-irradiation for 30 seconds) or 2,000 non-irradiated AcL3; [b]single vaccination; [c]once every second week for 3 times; [d]once every week for 3 times; [e]hamsters were each challenged subcutaneously with 150 NaL3 one week post-immunization and sacrificed 25 days post-challenge.

47.7%-78.7% and 44.2%-85.3%, respectively. In the group of hamsters vaccinated with the same number of UV-irradiated NaL3 once every week for three weeks, the worm reduction rate was 90.4%, which is higher than that elicited by the same number of UV-irradiated NaL3 given once every second week for 3 times. The difference in the mean worm burdens between these two groups was not statistically significant ($p<0.05$). The protective immunity obtained by the vaccination with UV-Irradiated NaL3 given once every week for 3 weeks was higher than that of non-irradiated AcL3 given at the same schedule. The mean worm burdens between these two groups were statistically significant ($p<0.05$).

Serologic response

Hamsters vaccinated with UV-irradiated NaL3. With the first vaccination in hamsters with 500 or 2,000 UV-irradiated NaL3, no apparent increase in serum specific IgG levels as measured by ELISA (expressed by OD value) was seen. After the second vaccination, the IgG levels increased significantly in the group of hamsters vaccinated with 2,000 UV-irradiated NaL3. The specific antigen level continued to increase after challenge with normal NaL3 and reached its peak two weeks later. Afterwards, the IgG levels showed some fluctuation and reached the lowest level at 7 weeks post-challenge. Afterwards, the specific IgG levels showed some recovery up to the end of the observation period. Similar patterns of specific IgG levels were also seen in the group of hamsters vaccinated with 500 UV-irradiated NaL3, but the specific IgG levels were lower than those of the hamsters vaccinated with the higher dose of UV-irradiated NaL3. As for the non-vaccinated group of hamsters, a significant increase in specific IgG levels was seen 9-14 weeks post-infection with 150 normal NaL3, which is similar to the two groups of hamsters vaccinated with UV-irradiated NaL3 (Fig 1).

Hamsters vaccinated with UV-irradiated NaL3 and non-irradiated AcL3. As shown in Fig 2, the specific IgG levels were higher in the groups vaccinated with UV-irradiated NaL3 and non-irradiated AcL3, once every second week for 3 times, than those of the groups

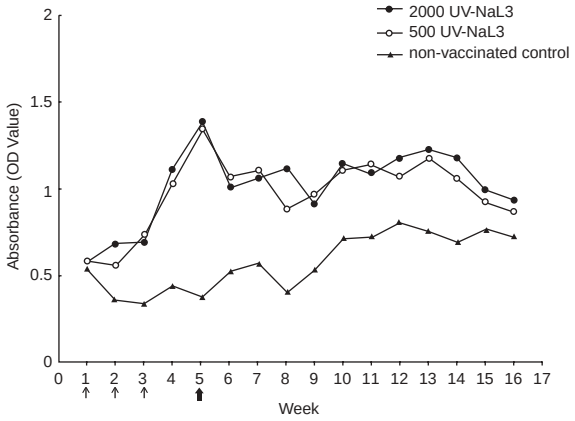

Fig 1–Specific IgG levels (OD value) against NaL3 antigen in the serum of hamsters vaccinated with 500 or 2,000 UV-irradiated NaL3 once every week for three weeks.↑, vaccination; ⬆, challenge with 150 NaL3.

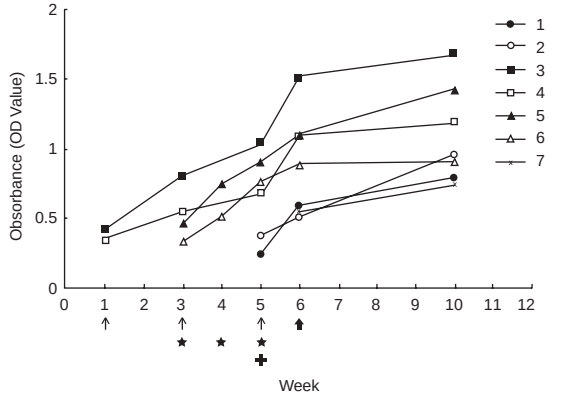

Fig 2–Specific IgG level (ELISA, OD value) against adult *N. americanus* antigen in the serum of hamsters vaccinated with 2000 UV-irradiated NaL3 or 2000 non-irradiated AcL3 giving singly, once every week for 3 weeks or once every second weeks for 3 times.↑, vaccination once every second week; ★, vaccination once every week; ✚, single vaccination; ⬆, challenge with 150 NaL3; 1, 3, 5, UV-irradiated NaL3; 2, 4, 6, non-irradiated AcL3; 7, non-vaccination control.

vaccinated with the same antigens once every week for 3 consecutive weeks. Meanwhile the following features were also evident: 1) the specific IgG levels elicited by UV-irradiated NaL3 were usually higher than those elicited by non-irradiated AcL3, and 2) only a slight increase in specific IgG levels was seen in hamsters vaccinated singly with UV-irradiated NaL3 or non-irradiated AcL3, although the protective immunity was significant.

DISCUSSION

The protective immunity elicited by UV-irradiated NaL3, UV-irradiated AcL3 and non-irradiated AcL3 was evaluated in laboratory mice and hamsters. It was determined that the viability of AcL3 diminishes significantly *in vitro* if they are exposed to UV irradiation for more than one minute, but that infectivity of mice *in vivo* diminished after greater than 15 seconds of irradiation. Both oral and subcutaneous vaccination routes with UV-irradiated AcL3 were explored. Oral vaccination was more effective at reducing the number of challenge AcL3 entering the lungs, whereas subcutaneous vaccination was more effective at blocking muscle entry. In a previous study we found that when mice were vaccinated with non-irradiated AcL3, vaccine protection was only observed for two weeks following completion of immunization (Xiao *et al*, 1999). In this study, protection afforded by UV-irradiated AcL3 lasted 4-8 weeks following completion of oral or subcutaneous immunization series.

When used as vaccines in hamsters, both UV-irradiated NaL3 and non-irradiated AcL3 were effective at reducing adult hookworm burdens. The protective immunity of non-irradiated AcL3 given once every second week was significantly higher than that given once every week. A single dose was also found to be effective, but less than multiple doses. Specific IgG determinations indicate that the IgG levels in the serum of hamsters that received a single vaccination with UV-irradiated NaL3 or non-irradiated AcL3 were much lower than that those of hamsters that received multiple doses. It appears that protective im-

munity may be related to specific antibody levels. Similar results were seen when hamsters were vaccinated with non-irradiated AcL3 once every week or every second week. No such relationship was seen between the IgG level and protective immunity in the groups of hamsters that received UV-irradiated NaL3 once every week or every second week. This suggests that protective immunity elicited by UV-irradiated NaL3 and non-irradiated AcL3 is not only dependent on humoral immunity, but cellular immunity may also be important.

The immunological basis by which irradiated hookworm L3 elicited robust protection in laboratory animals is still unknown. Recently, the immunological basis of effector immunity in dogs vaccinated with X-irradiated *A. caninum* L3 was explored (Fujiwara *et al*, 2006). They determined that Th2 type responses were critical, and noted that an L3 secreted protein ASP-2 is an immunodominant secretory antigen associated with larval protective immunity. Studies are in progress to determine if similar Th2 and anti-ASP-2 antibody responses occur following UV-NaL3 immunizations, and if *N. americanus*-derived ASP-2 may represent a potential vaccine antigen for human hookworm infection (Goud *et al*, 2005; Hotez *et al*, 2005).

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