***Schistosoma mansoni* egg granuloma size reduction in challenged baboons after vaccination with irradiated cryopreserved schistosomula**

(schistosomiasis/immunopathogenesis/modulation)

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ABSTRACT Young male baboons born in captivity were immunized with an attenuated, cryopreserved schistosomular vaccine derived from γ -irradiated (50 krad) cercariae of the Puerto Rican strain of *Schistosoma mansoni*. Protection against a heterologous Kenyan strain of *S. mansoni*, after percutaneous infection, was assessed. Partial protection (33–53% reduction in worm burden) was obtained in three of six vaccinated–challenged baboons, an unremarkable result. Of greater interest was the fact that all six unvaccinated–challenged control baboons, but only one of the six vaccinated–challenged baboons, had macroscopic egg granulomas on their liver surfaces. This difference in granuloma size was substantiated by measuring hepatic and colonic granulomas. The mean ($\pm$ SEM) hepatic and colonic granuloma diameters for the six unvaccinated baboons were $406 \pm 38 \mu\text{m}$ and $313 \pm 27 \mu\text{m}$, respectively, and for the five “typical” vaccinated–challenged baboons the mean diameters were $283 \pm 27 \mu\text{m}$ and $202 \pm 23 \mu\text{m}$, respectively. Both hepatic and colonic granulomas were significantly smaller in the five typical vaccinated–challenged baboons. Not only did the exceptional vaccinated–challenged baboon have very large hepatic and colonic granulomas, but also it was the only one of its group whose mesenteric lymph node cells were not suppressed in their *in vitro* proliferative response to a schistosome antigen. These results strongly suggest that granuloma size reduction in the majority of the vaccinated baboons was the result of immunoregulation—i.e., the small postvaccination granulomas were “modulated.” Despite their small size, hepatic granulomas in the typical vaccinated baboons were apparently as effective in sequestering egg toxins and preventing hepatocyte damage as the larger granulomas of the control baboons. Smaller, less obstructive granulomas are thought to be more beneficial to the host than large, vigorous granulomas, with respect to lessening chronic disease. The present results give encouragement that a vaccine to ameliorate disease in human schistosomiasis is possible. This effect should add to the attractiveness of partial protection against challenge infections conferred by attenuated larval vaccines, as reported by others, to yield a dually beneficial vaccine for human use.

It is well established from studies in the murine model for chronic hepatointestinal schistosomiasis that the eggs of *Schistosoma mansoni*, and the granulomas that they induce and elicit, are the main agents of pathogenesis (reviewed in ref. 1). Moreover, the granulomas produced around newly laid eggs are smaller in chronic than in acute infections (2). This phenomenon, termed spontaneous modulation of gran-

uloma formation (3), appears to impede the disease process, at least in mice (4). Some evidence exists for immune response involvement in granuloma production and modulation in human schistosomiasis (5, 6).

The possibility of a vaccine to prevent immunopathogenesis in this disease was proposed over a decade ago (7). Except for one report (8) of granuloma size reduction in mice after pretreatment with soluble egg antigen (SEA) and another report (9) after hydridoma (idiotypic) sensitization, little practical progress toward its realization has been made, despite the great advances that have come in understanding the immunoregulatory events that underlie granuloma formation and modulation in murine models (10–13).

This report describes a vaccination experiment in baboons (*Papio cynocephalus*) using irradiated cryopreserved larvae (schistosomula) of *Schistosoma mansoni*. Baboons are excellent experimental models for human schistosomiasis (14–17), including vaccine testing (18–21). We found a reduction in the size of hepatic and intestinal granulomas in baboons infected after having been vaccinated, yet these smaller granulomas appeared to be fully effective in sequestering toxic egg products in the liver. Cellular immunological assays conducted on the animals suggested that the granulomas were modulated in vaccinated–challenged baboons. This finding may point the way to development of the elusive anti-pathogenesis vaccine for human schistosomiasis.

MATERIALS AND METHODS

Baboons. Fourteen young male baboons (*Papio cynocephalus*) born in captivity were purchased from the Southwest Foundation for Research and Education (San Antonio, TX). They were 13–23 months of age and 3.5–5.3 kg in weight at the start of the experiment. The animals were individually caged, with free access to water, and were regularly fed with Purina Monkey Chow and fresh fruits. Animal care and experimentation practices at the University of Georgia are periodically evaluated by the University's Animal Care Committee and other agencies in order to ensure compliance with established federal regulations and guidelines.

Experimental Design. The baboons were divided into three groups: six for vaccination and subsequent parasite challenge infection (V–C group), six as unvaccinated challenge infection controls (C group), and two as vaccinated uninfected

Abbreviations: A, acute infection; C, unvaccinated–challenged controls; V, vaccine only; V–C, vaccinated–challenged; LTC, long-term chronic infection; PBMC, peripheral blood mononuclear cell(s); SC, spleen cell(s); MLNC, mesenteric lymph node cell(s); AINC, axillary and inguinal node cell(s); Con A, concanavalin A; SWAP, schistosome worm antigen preparation.

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ed controls (V group). The experiment was designed to allow a matched pair analysis, with three variables being matched as closely as possible: baboon weight and age and age of the challenging parasite (cercariae) pool. Matched pair-mates (one V-C and one C animal) differed from each other by no more than 0.3 kg in weight and 6 months in age.

Parasitology. V-C and V group baboons each received a single injection of the vaccine, which consisted of irradiated cryopreserved larvae (schistosomula) of the NMRI strain of *S. mansoni* (Puerto Rican origin). The vaccine was prepared at the Biomedical Research Institute (Rockville, MD) as detailed elsewhere (22). Briefly, cercariae were irradiated with 50 krad (1 rad = 0.01 gray) at a rate of 2 krad/min from a bilateral ^{60}Co source at the Armed Forces Radiobiological Research Institute (Bethesda, MD). The irradiated cercariae were mechanically transformed to schistosomula, quick frozen, and stored and transported to Athens, GA, in liquid N_2 . Storage time before use was 3 months. Vivification was by quick thawing, and viability was determined by published criteria (23). Eighty-six percent of the thawed schistosomula were scored as "normally active." Another characteristic of this particular lot of vaccine was 90% tail retention after mechanical treatment. Other studies have established the schistosomular nature of such tailed larvae (24). Each vaccinated baboon received a single intramuscular dose, in a thigh, adjusted to contain 10,000 active schistosomula. The vaccine was administered while the animal was restrained unsedated in a false-back "squeeze" cage.

Two months after vaccination, V-C and C group animals were infected percutaneously with 325 (aliquot-counted) cercariae from a single pool of a Kenyan strain (KEB) of *S. mansoni* (16), while under ketamine and sodium pentobarbital anesthesia.

Four months after the time of challenge infection, all baboons were humanely killed for parasitologic, histologic, and immunologic analyses. Worms were recovered by perfusion, careful inspection of the mesenteric vessels, and dissection therefrom of any worms remaining after perfusion. Percent worm recovery was compared between matched pair-mates by the following equation:

$$\Delta\% \text{ worm recovery} = \frac{\text{worm no. in C baboon} - \text{worm no. in V-C baboon}}{\text{worm no. in C baboon}} \times 100.$$

Hematoxylin/eosin-stained sections of large intestines and livers were searched for granulomas. The diameters of selected colonic granulomas of all infected baboons were measured at $\times 100$ magnification with a calibrated ocular micrometer. Comparability was assured by measuring only nonconfluent, submucosal granulomas around single eggs containing nearly mature (type III) embryos, as defined by Prata (25). At least 12 suitable colonic granulomas were found and measured for each infected baboon in the vaccination experiment. To relate granuloma size in V-C group animals to the normal evolution of *S. mansoni* egg granulomas in baboons, colonic granulomas from two other infected groups of baboons were similarly selected and measured. One group, A, included four baboons with acute infections of short duration (47–66 days) and the other, LTC, included eight baboons with long-term chronic infections (348–1406 days). Five to 19 granulomas were measured from each baboon in these additional groups. The six C group baboons of the vaccination experiment also served as representatives of intermediate-duration (131–135 days) infections.

Stage comparability of measured hepatic granulomas was impossible to attain because of the limited preserved tissue available and the scarcity of granulomas with any one developmental egg stage. Therefore, all sections were searched and the 12 largest granulomas from each liver were measured.

Immunology. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood after dilution with GIBCO RPMI 1640 medium, by layering and centrifugation on Sigma Histopaque 1.077. Interfacial cells were washed and diluted with this medium containing 25 mM Hepes, gentamicin, and 10% heat-inactivated, pooled normal baboon serum. Lymphoid organs were removed from the animals at necropsy and teased apart in culture medium to dissociate cells. Three cell preparations were produced: SC (spleen cells), MLNC (mesenteric lymph node cells), and AINC (axillary and inguinal node cells). Cells were strained through a mesh and washed with culture medium. Erythrocytes in the SC preparation were hypotonically lysed. Viabilities ranged from 88% to >99% in the organ preparations and 96% to >99% for the PBMCs, by erythrosin B exclusion. For use in proliferation assays, these cell preparations were adjusted with culture medium to yield final concentrations of 2×10^5 viable cells per well. The assays were conducted in 96-well flat-bottom Linbro microtiter plates, using 100 μl of cell suspension and 100 μl of mitogen [concanavalin A (Con A)] or schistosome worm antigen preparation (SWAP) in culture medium per well. All stimulant concentrations and all cell controls were tested in triplicate. The plates were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air, for 4 (Con A) or 6 (SWAP) days. Eighteen hours before harvesting, 50 μl of culture medium containing 0.8 μCi (1 Ci = 37 GBq) of tritiated thymidine (6.7 Ci/mmol, New England Nuclear) was added to each well. Cells were harvested onto filter paper discs with a Titertek cell harvester, and radioactivity incorporation, as a measure of cell proliferation, was determined with a Beckman LS 8000 counter. Data are expressed as Δcpm per culture, obtained by subtracting the triplicate mean of the unstimulated controls (background) from that of the stimulated cells. Mean background levels for the four cell populations tested were 1395 cpm (SC), 892 cpm (MLNC), 605 cpm (AINC), and 2060 cpm (PBMC).

Lymphocyte Stimulants. Con A (lyophilized in NaCl; Calbiochem-Behring) was rehydrated with GIBCO RPMI 1640 medium, sterilized by filtration, adjusted to a concentration of 1 mg/ml, and stored in 1-ml aliquots at -20°C . Prior to assay, the Con A was diluted to the desired concentrations with culture medium (lacking serum) and sterilized by filtration.

SWAP was prepared from KEB strain worms raised in mice. Worms were homogenized in a Sorvall Omni-Mixer with about 5 vol of extraction buffer (20 mM phosphate-buffered 0.5 M NaCl, pH 7.0, containing 1 mM L-cysteine, 2 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at $40,000 \times g$ for 30 min and the pellets were resuspended in extraction buffer, rehomogenized, and recentrifuged twice more. After the final centrifugation, surface lipids were removed by skimming. The skimmed supernatants were pooled and recentrifuged at $100,000 \times g$ for 1 hr. More floating lipids were skimmed off and the remainder of the lipid material was removed by low vacuum filtration through Whatman no. 1 qualitative filter paper. After concentration by use of Calbiochem Aquacide IV and protein determination by the Bradford assay (ref. 26, using Bio-Rad reagents), the protein concentration was adjusted to a desired value and the SWAP preparation was divided into aliquots and frozen at -70°C for storage.

Statistics. Worm burdens in V-C and C baboons were compared by means of a one-tailed *t* test for matched pairs. Granuloma diameter and lymphocyte stimulation (Δcpm) data were analyzed for significance (Δcpm data were $\log_{10}$ transformed) by a two-level nested analysis of variance and, where appropriate, a Student-Newman-Keuls (SNK) multiple comparison test (27).

Table 1. Worm recoveries and presence of macroscopic hepatic granulomas in V-C and C baboons

Group	Baboon no.	Pair no.	Treatment		Worm recovery*	$\Delta\%$ worm recovery	Macroscopic hepatic granulomas
			Vaccine	Challenge			
C	C-422	1	—	+	125	NA	+
	C-455	2	—	+	198	NA	+
	C-458	3	—	+	107	NA	+
	C-468	4	—	+	128	NA	+
	C-503	5	—	+	123	NA	+
	C-546	6	—	+	115	NA	+
V-C	C-451	1	+	+	216	+72.8	—
	C-430	2	+	+	132	-33.3	—
	C-494	3	+	+	64	-40.2	—
	C-467	4	+	+	144	+12.5	—
	C-507	5	+	+	138	+12.2	—
	C-497	6	+	+	54	-53.0	+
V	C-440	NA	+	—	0	NA	NA
	C-525	NA	+	—	0	NA	NA

NA, not applicable.

*Difference in mean worm recoveries of C ($\bar{x} = 132.7$) and V-C ($\bar{x} = 124.7$) groups is not significant.

RESULTS

Granulomas and Histopathology. Upon necropsy, infected baboons were obviously divisible into two clear-cut groups: the first ($n = 7$) had macroscopic granulomas on the uncut liver surfaces, the second ($n = 5$) had only tiny, inconspicuous hepatic granulomas. As shown in Table 1, six of the seven baboons with large hepatic granulomas were C group animals, while the seventh (C-497) had received the vaccine before being challenged. This dichotomy in granuloma size was quantitatively confirmed and extended by measuring circumoval granulomas in the large intestines. The results (Table 2) confirmed the initial observation. The six C group baboons with macroscopic hepatic granulomas also had larger colonic granulomas [mean ($\pm$ SEM) diameter $313 \pm 27 \mu\text{m}$] than did the five "typical" V-C baboons [mean ($\pm$ SEM) diameter $202 \pm 23 \mu\text{m}$]. This difference is highly significant ($P < 0.001$).

In comparing granuloma sizes of C and V-C group baboons to acute and chronic infection granulomas (Table 2), it was noted that C group granulomas, in baboons with infections of intermediate duration, were intermediate in size between acute and chronic infection granulomas. The five typical V-C animals had the smallest granulomas, these being significantly smaller than what might be considered the normally modulated granulomas of long-term chronic infections. Granulomas whose sizes closely approximate the group means are illustrated in Fig. 1.

It was impossible to precisely stage hepatic granulomas in numbers large enough to yield significance, since little liver tissue was saved for fixation (the bulk having been digested with KOH to yield data on tissue egg burdens, to be reported elsewhere), and granulomas were scarce in the extant samples. As an alternative, the 12 largest granulomas were identified and measured from each liver sample after many sections had been cut (Table 3). The results were consistent with both the macroscopic dichotomy and the measurements of staged colonic granulomas. The mean ($\pm$ SEM) diameter of hepatic granulomas of the six control baboons was $406 \pm 38 \mu\text{m}$, whereas the five typical V-C baboons' mean ($\pm$ SEM) hepatic granuloma diameter was $283 \pm 27 \mu\text{m}$, a difference that was highly significant ($P < 0.001$). Hepatic granulomas from V-C and C baboons were compared with respect to possible adjacent parenchymal damage. Little difference between the two groups in the condition of hepatocytes at the

periphery of granulomas was noted (Fig. 2).

Worm Burdens. Protection, on an individual animal basis, was erratic and no overall group protection was evident (Ta-

Table 2. Colonic granuloma diameters in V-C and C baboons, compared with those in baboons with acute and chronic infections

Group	Baboon no.	Duration of infection, days	<i>n</i>	Granulomas		
				Diameter, μm		Group mean* $\pm$ SEM
				$\bar{x}$	$\pm$ SD	
A	2507	49	11	581	± 182	490 ± 68
	N-95	56	12	486	± 127	
	2818	66	17	423	± 69	
	2947	47	7	468	± 76	
C	C-422	134	24	266	± 30	313 ± 27
	C-455	133	14	302	± 58	
	C-458	135	12	329	± 65	
	C-468	131	14	288	± 39	
	C-503	132	14	321	± 76	
	C-546	135	14	370	± 82	
V-C	C-451	133	15	220	± 40	202 ± 23
	C-430	133	14	156	± 50	
	C-494	135	16	220	± 28	
	C-467	131	13	221	± 39	
	C-507	132	19	191	± 66	
	C-497	134	13	449	$\pm 104^\dagger$	
LTC	S-628 [‡]	1406	13	278	± 64	251 ± 20
	B-300 [‡]	1233	12	279	± 59	
	1368 [‡]	1405	7	248	± 40	
	A-868 [‡]	1405	7	247	± 59	
	N-235	564	13	212	± 51	
	N-240	1001	14	227	± 31	
	AUG-3	348	12	274	± 30	
	B-156 [‡]	1405	5	245	± 39	

*All group means are significantly different from each other at $P < 0.05$ or less.

[‡]Exceptional, not included in statistical analysis (see text).

[‡]Animal infected several times, time from last infection to termination 133–175 days. All other baboons had single exposures.

FIG. 1. Colonic submucosal granulomas representative of group mean diameters. Bars represent 100 μ m. (A) C group, infection of intermediate duration; (B) V-C group; (C) A group; and (D) LTC group.

ble 1). Three baboons (C-430, C-494, and C-497) had moderate worm burden reductions (33.3–53.0%) as compared to their unvaccinated counterparts. On the other hand, baboon C-451 had a worm burden that was increased by 72.8% (much of this increase was represented by unpaired, immature female worms). Two baboons were little changed, with approximately 12% increases over their partners.

Lymphocyte Responses. Lymphoid cells from the spleens (SC), mesenteric lymph nodes (MLNC), and axillary and inguinal lymph nodes combined (AINC) were taken at necropsy and stimulated with Con A and SWAP in proliferation assays *in vitro* (Tables 4 and 5). Also shown in these tables are similar assays performed on peripheral blood mononuclear cells (PBMCs) sampled approximately 1 week before necropsy. The MLNC Con A response of C group baboons was marginally higher than that of V group baboons ($P < 0.05$), but other differences were insignificant. Con A responses for SC, AINC, and PBMC were not different among

Table 3. Hepatic granuloma diameters in V-C and C baboons

Group	Baboon no.	Granuloma diameter, μ m	
		$\bar{x} \pm$ SD	Group mean* $\pm$ SEM
C	C-422	444 $\pm$ 83	406 $\pm$ 38
	C-455	426 $\pm$ 88	
	C-458	340 $\pm$ 70	
	C-468	304 $\pm$ 56	
	C-503	478 $\pm$ 24	
	C-546	441 $\pm$ 64	
V-C	C-451	305 $\pm$ 41	283 $\pm$ 27
	C-430	283 $\pm$ 27	
	C-494	323 $\pm$ 91	
	C-467	242 $\pm$ 34	
	C-507	260 $\pm$ 38	
	C-497	561 $\pm$ 58 [†]	

The 12 largest granulomas from each baboon were measured (see text).

*Group mean difference highly significant at $P < 0.001$.

[†]Exceptional, not included in statistical analysis (see text).

FIG. 2. Hepatic granulomas and adjacent hepatocytes from C group (A) and V-C group (B) baboons. Bars represent 50 μ m.

the three groups of baboons. With respect to stimulation by SWAP, the mean peak responses of SC, MLNC, AINC, and PBMC are shown in Table 5. No statistically significant between-group differences were obtained in comparisons of SC, AINC, and PBMC anti-SWAP responses. However, C group MLNC responses were significantly higher ($P < 0.025$) than were those from V-C group baboons. Table 5 shows that one of the V-C baboons, C-497, had an anti-SWAP MLNC response very much higher than did its other group members ($P < 0.001$ in each case).

DISCUSSION

Our results demonstrate, in a good primate model for human schistosomiasis, that vaccination with irradiated cryopreserved larvae can alter the expression of granulomatous hypersensitivity to the parasite's eggs. The vaccinated baboons, with one clear exception, developed hepatic and colonic granulomas that were considerably smaller than those formed in the unvaccinated controls. The smaller hepatic granulomas in vaccinated baboons were nevertheless effective in sequestering toxic egg products, as evidenced by the lack of damage to adjacent hepatocytes (Fig. 2). The exceptional baboon (C-497) had large, vigorous granulomas, and it was also the only one of its group whose draining (MLNC) lymphocytes were not suppressed in their responsiveness to a schistosome antigen. These cells, moreover, had a good proliferative response to Con A. Because of the coincidence of vigorous granulomas and unsuppressed MLNC in baboon C-497, we feel justified in suggesting that the small granulomas in the majority of the V-C baboons were probably the result of immunoregulation, i.e., their granulomas were modulated. In fact, their colonic granulomas were even smaller than those found in baboons with chronic, long-standing experimental infections, suggesting, if their granu-

Table 4. Proliferative responses to Con A by lymphocytes taken from various compartments of vaccinated and/or challenged baboons

Group	Proliferative response, peak Δ cpm $\times 10^{-3}$			
	AINC	MLNC	SC	PBMC
C	376 (5)	449* (5)	222 (5)	368 (6)
V-C	312 (5)	341 (5)	247 (5)	354 (6)
V	317 (2)	284* (2)	374 (2)	400 (2)

Group means of triplicate culture means. Numbers in parentheses indicate number of baboons tested.

*Values significantly different ($P < 0.05$) from each other. All other differences are insignificant.

Table 5. Proliferative responses to SWAP by lymphocytes taken from various compartments of vaccinated and/or challenged baboons

Group	Baboon no.	Proliferative response, peak Δ cpm $\times 10^{-3}$			
		AINC	MLNC	SC	PBMC
C	C-422	0.5	12.6	21.0	3.6
	C-455	0.1	24.8	111.8	21.2
	C-458	ND	ND	ND	9.6
	C-468	0.1	3.0	10.1	0.8
	C-503	0.3	12.9	37.8	0.4
	C-546	0.2	28.3	61.4	3.3
V-C	C-451	ND	ND	ND	6.0
	C-430	0.5	0.2	58.8	53.0
	C-494	0.4	0.6	2.6	2.0
	C-467	0.2	0.7	68.8	2.5
	C-507	0.3	0.4	2.1	0.6
	C-497	0.1	21.8*	4.3	11.9
V	C-440	6.4	2.4	168.2	6.4
	C-525	0.1	0.3	192.6	51.0

ND, not done.

*Value significantly different ($P < 0.001$) from each of the other V-C group members.

lomas were indeed modulated, that the modulation performance in vaccinated baboons may even exceed that in "natural" infections.

Using the lung model of granuloma formation, in which schistosome eggs are injected intravenously into mice and the resulting egg granulomas in the pulmonary microvasculature are measured, Warren and Domingo (28) found no evidence for granuloma size reduction after sensitization with irradiated cercariae. Whether this discrepancy between their results and ours stems from differences in the vaccines themselves, in their modes of presentation, in the model used (eggs produced by a challenge infection versus injected eggs), or in the hosts used remains to be determined.

This study provided no convincing evidence for a good and/or consistent protective effect of the irradiated, cryopreserved schistosomular vaccine used. However, it should be noted that recent studies using mice have shown that attenuating radiation doses lower than the 50 krad used herein give more consistent and higher protection (29).

The exceptional baboon, C-497, was instructive in another way, in that it showed a dissociation between protection against challenge infection and granuloma modulation. Two other partially protected animals (C-430 and C-494) had concurrently reduced colonic granulomas. This decoupling of the two effects of the vaccine reinforces the concept of dual, anti-worm and anti-egg, immunity in schistosomiasis (reviewed in ref. 30).

If amelioration of the chronic disease process through granuloma modulation in human schistosomiasis exists, and if the baboon is an appropriate model for this aspect of the disease, then the present results should give credibility to the idea that an anti-immunopathogenesis vaccine for human use is a realistic goal (7, 31). This effect could be an added benefit to protection against infection, which so far has never been absolute after using attenuated schistosome larval vaccines in animal models. The possibility of dual benefits should make these vaccines more attractive for human use.

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