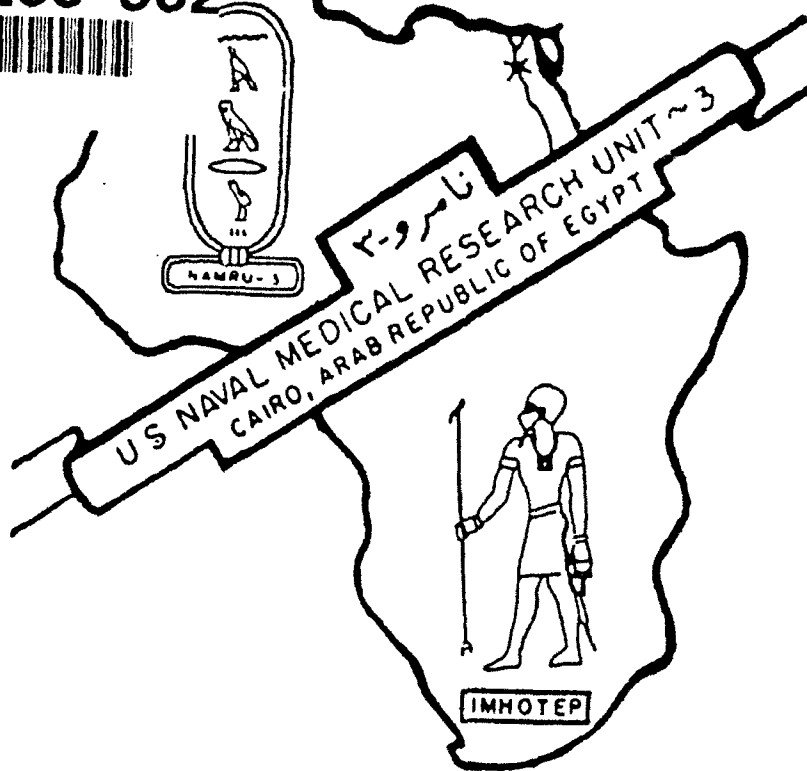AD-A265 562

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EVIDENCE THAT BOTH NORMAL AND IMMUNE ELIMINATION OF
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MIGRATION PRIOR TO PARASITE DEATH

BY

David A. Dean and Beverly L. Mangold

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U.S. NAVAL MEDICAL RESEARCH UNIT NO. 3
(CAIRO, ARAB REPUBLIC OF EGYPT)
PSC 452, BOX 5000
FPO AE 09835-0007

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EVIDENCE THAT BOTH NORMAL AND IMMUNE ELIMINATION OF *SCHISTOSOMA MANSONI* TAKE PLACE AT THE LUNG STAGE OF MIGRATION PRIOR TO PARASITE DEATH

DAVID A. DEAN AND BEVERLY L. MANGOLD

U. S. Naval Medical Research Unit No. 3, Cairo, Egypt

Abstract. The number and distribution of autoradiographic foci observed in this and previous studies following percutaneous infection with ^{75}Se-labeled *Schistosoma mansoni* cercariae indicate that the lungs are the principal site of worm elimination in both normal mice and mice immunized with irradiated cercariae. It was observed in the present study, however, that the intensities of the autoradiographic foci produced in the lungs during both the normal (early) and immune (late) phases of elimination were identical to those of foci produced in the livers of the same mice by larvae shown to be alive. In contrast, foci produced in the lungs by heat-killed, intravenously injected, lung schistosomula became smaller and fainter with time, disappearing completely between seven and 10 days after injection in normal mice and between four and six days in immunized mice. These results indicate that although the targets of both normal and immune elimination do not proceed beyond the lung stage of migration, they do not die in the lungs. A possible explanation for this paradoxical situation, for which there is some experimental evidence, is that unsuccessful migrators leave the blood stream, enter alveoli, pass up the trachea, and are eventually digested in the gastrointestinal tract or eliminated from the body intact.

In the first applications of the autoradiographic tracking method of Georgi[1] to the study of schistosome migration, it was observed that nearly all *Schistosoma mansoni* larvae that penetrated the skin of either naive mice (C57Bl/6J) or mice immunized with irradiated cercariae eventually migrated to the lungs.[2-3] It was therefore concluded that the failure of half or more of skin penetrants to survive to adulthood in control mice, and the additional loss of worms in immunized mice, could not be explained by the death of larvae in the skin. In the immunized mice, migration to the lungs was delayed for several days relative to controls. At two and three weeks after challenge infection, the number of *S. mansoni* detected in the livers of irradiated cercaria–immunized mice by autoradiography was lower than in control mice, and this reduction was accounted for by increased retention in the lungs. This observation did not prove that immune elimination took place in the lungs, however, because it did not rule out the possibility that migration to the liver or other sites was only delayed, with elimination taking place in these sites at some later time.

In this study, we extended the period of autoradiographic quantification of migrating schistosomula to five weeks; low temperature exposure of film was used to improve the sensitivity of larval detection.[4] In addition, we attempted to distinguish between autoradiographic foci produced by live and dead schistosomula by comparing the optical densities of individual foci with those produced by known live and dead schistosomula. These observations were carried out in C57Bl/6J mice that had received no, one, or three immunizing exposures to gamma-irradiated cercariae. The results, together with those of previous studies, enabled us to distinguish temporally between the phase of worm elimination during which half or more of the worms in the body are eliminated from naive and immunized mice, and the phase during which half or more of the remaining worms are eliminated from immunized mice. The results of the optical density studies indicated that most, if not all, of the schistosomula detected in the lungs by autoradiography during the periods of both normal and immune elimination were alive. Microscopic observations carried out as part of this study[7] as well as those reported previously[8,9] (except one[10]) failed to detect damage to lung schisto-

TABLE 1
Experimental protocols used in this study

Exp no.	Age of mice (weeks)[*]	Immunization[†]						Challenge	
		1st		2nd		3rd			
		Day	No.	Day	No.	Day	No.	Day	No.
1	7	0	1,000	91	520	119	710	144	104‡
2	9	0	513	28	533	56§	511	84	116‡
3	8	0	1,000	87	551	122	539	151	135‡
								158	120¶
4	12	0	539	101	553	129	572	154	153‡
								162	150¶
5	6	0	1,047					50	158‡

* Age of mice at the beginning of the experiment.
† Average number of 50 kr-irradiated *Schistosoma mansoni* cercariae used for a 1-hr exposure by tail immersion. Penetration ranged from 79% to 96%.
‡ Average number of live ⁷⁵Se-labeled cercariae that penetrated the skin during a 1-hr exposure by tail immersion. Penetration ranged from 91% to 99%.
§ Mice immunized once in experiment 2 received only the third immunizing exposure.
¶ Approximate number of heat-killed ⁷⁵Se-labeled schistosomula injected into the tail vein.

somula. In addition, it has been observed that after some time in the lungs, a significant proportion of schistosomula are found in alveoli.[9, 11-18] As a possible explanation for the simultaneous relocation to air spaces, absence of detectable damage, and disappearance of schistosomula, we favor the hypothesis that an important mechanism of schistosome elimination from both normal and immune hosts is the expulsion of intact, possibly live, schistosomula from the lungs via airways into the gastrointestinal tract.[9]

MATERIALS AND METHODS

Host and parasite

Female C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. A Puerto Rican–derived strain of *Schistosoma mansoni*[19] was used. Cercariae used for immunization were exposed to 50 kilorads of gamma radiation from a cesium-137 source at a rate of 1,300 rads/min. Both immunizations and challenge infections were administered by immersing the tails of restrained unanesthetized mice in 4.3-ml suspensions of cercariae for approximately 1 hr. The number of penetrant cercariae was estimated for each group by subtracting the average number of cercarial bodies left in the exposure tubes from the average number of cercariae in the original suspension. The age of mice and the time and size of immunizing and challenge exposures in the various experiments are outlined in Table 1.

Autoradiography

Radiolabeled cercariae were collected from snails exposed four to seven days earlier to a 5-hr pulse with 20 µCi of ⁷⁵Se-L-selenomethionine.[1, 4, 5, 20] Macroautoradiographic scoring of migrating worms was performed by exposing x-ray film (XAR-5; Eastman Kodak, Rochester, NY) in the presence of Lightning-Plus intensifying screens (Du Pont de Nemours, Wilmington, DE) to squashed dried preparations of mouse tissue for six weeks in the dark.[1, 4, 5] Exposures were carried out at room temperature in all experiments except number 2, in which they were carried out at –196°C. After development, foci of reduced silver were counted with the aid of a light box and magnifying lens.

Preparation of heat-killed lung schistosomula

Lung-stage schistosomula were obtained by exposure of mice to 3,000–4,000 radiolabeled cercariae and recovery of schistosomula seven days later by mincing and incubation of lungs.[21] The schistosomula were killed by suspending them in 1.0 ml of Earle's lactalbumin hydrolyzate medium (Gibco, Grand Island, NY) containing 5% normal mouse serum in a 12-ml glass conical tube and immersing the tube in a swirling 50°C bath for 5 min. They were then diluted with the same medium and 120–150 schistosomula in 0.4 ml were introduced into each mouse by tail vein injection.

TABLE 2

Detection of Schistosoma mansoni by autoradiography and portal perfusion[a]

Exp no.	Days after challenge	No. of mice	No. of irradiated cercaria exposures	No. of adult worms recovered by portal perfusion (mean ± SEM)	No. of autoradiographic foci (Mean ± SEM)			Total recovered plus foci (mean ± SEM)[†]
					Skin	Lungs	Liver	
1	21	7	0	25.1 ± 4.0	1.9 ± 0.3	4.7 ± 0.6	1.7 ± 0.5	33.4 ± 4.5
		8	3	4.6 ± 0.9	4.4 ± 1.1	19.9 ± 3.0	1.9 ± 0.6	30.8 ± 3.7
	43	8	0	39.8 ± 1.4	ND	ND	ND	ND
		8	3	10.5 ± 1.2	ND	ND	ND	ND
2	21	5	0	36.0 ± 2.5	ND	8.4 ± 2.3	1.0 ± 0.8	45.4 ± 3.0
		5	1	13.6 ± 2.5	ND	26.4 ± 2.1	1.4 ± 0.4	41.4 ± 3.1
		5	3	5.6 ± 1.1	ND	29.8 ± 2.5	1.0 ± 0.4	36.4 ± 2.5
	28	5	0	43.0 ± 2.7	ND	5.6 ± 0.9	0.8 ± 0.5	49.4 ± 2.7
		5	1	14.8 ± 1.4	ND	15.8 ± 2.4	0.8 ± 0.6	31.4 ± 3.6
		5	.3	7.8 ± 1.6	ND	25.4 ± 2.0	1.0 ± 0.0	34.2 ± 3.1
	35	6	0	45.8 ± 5.2	ND	3.3 ± 0.9	2.5 ± 0.4	51.7 ± 5.3
		6	1	14.5 ± 1.9	ND	12.3 ± 1.8	0.5 ± 0.5	27.3 ± 2.8
		6	3	5.5 ± 1.6	ND	12.8 ± 1.6	0.3 ± 0.2	18.7 ± 2.0

[a] ND = not determined.

[†] No. adult worms recovered by portal perfusion plus the total no. of autoradiographic foci detected in the tail skin, lungs, and liver after perfusion.

Adult worm recovery

Worms were recovered from the liver and mesenteric veins by portal perfusion. At three, four, and five weeks after infection, the total perfusate was collected in tubes and erythrocytes were lysed with saponin.[22] At six weeks or later, worms were collected on 135-μ Nitex screens (Tetko, Elmsford, NY).[23]

Optical density measurement

Optical densities of individual autoradiographic foci were measured with a Model TD502 Macbeth transmission densitometer (Macbeth, Newburgh, NY). Each focus was centered over a 1-mm aperture and visible light from a tungsten-halogen lamp was passed through the film to a detector supplied with a digital readout. The net optical density for each focus was obtained by subtracting the mean of 20 background readings taken from areas between foci on the autoradiogram of the same tissue sample.

Statistical analysis

Differences between the mean worm and autoradiographic focus counts of control and immunized groups of mice were evaluated for statistical significance with the two-tailed Student's *t*-test. Differences producing P values less than 0.05 were considered significant.

RESULTS

Numbers and distribution of challenge S. mansoni

The numbers and timing of immunizing (irradiated) and challenge (unirradiated, radiolabeled) cercarial exposures for all five experiments are outlined in Table 1. At three weeks after challenge infection, similar total numbers of worms were detected in control and immunized mice by a combination of portal perfusion and autoradiography of the skin, lungs, and liver (Table 2). The distribution of worms was different in the two groups, however; immunized mice had more worms in the lungs and fewer in the liver. The recovery of worms from immunized mice by portal perfusion was reduced relative to controls ($P < 0.001$); the total number detected by recovery and autoradiography was not significantly reduced (Table 3).

After three weeks, the total number of worms detectable in the lungs and liver by recovery and autoradiography remained the same or increased slightly in control mice, but decreased progressively in immunized mice (Table 2). The decrease observed in immunized mice was entirely accounted for by a decrease in the number of autoradiographic foci in the lungs. As in control mice, only one or two foci were retained in the liver after portal perfusion, and all of the worms recovered were alive.

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TABLE 3

Reduction in the number of worms detected in immunized mice compared with controls

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Exp no.	Days after challenge	Irradiated cercaria exposures	% reduction			
			Portal perfusion recovery	P	Total recovered plus foci*	P
1	21	3	81.7	<0.001	7.8	>0.5
	43	3	73.6	<0.001		
2	21	1	62.2	<0.001	8.8	0.3 < P < 0.4
		3	84.4	<0.001	19.8	0.05 < P < 0.01
	28	1	65.6	<0.001	36.4	0.001 < P < 0.005
		3	81.9	<0.001	30.8	0.005 < P < 0.01
	35	1	68.3	<0.001	47.2	0.001 < P < 0.005
		3	88.0	<0.001	63.8	<0.001
3	64	3	65.9	<0.001		
4	41	3	75.3	<0.001		
5	28	1	60.8	<0.001		

* No. of adult worms recovered by portal perfusion plus the total no. of autoradiographic foci detected in the tail skin, lungs, and liver after perfusion.

Changes in number and optical density of autoradioaraphic foci produced by heat-killed, intravenously injected lung schistosomula

The ^{75}Se-labeled schistosomula recovered from the lungs of normal (unimmunized) mice were killed by heating at 50°C for 5 min and injected intravenously into control mice and mice immunized three times (experiments 3 and 4). The lungs of these mice were then removed at two-day intervals and autoradiographed. In control mice, the number of autoradiographic foci detected did not change significantly for four days and then decreased steadily thereafter until day 10, at which time nearly all had disappeared (Figure 1). The average optical densities decreased by approximately half over each two-day interval for the first six days, and then leveled off at the minimum detectable level. In immunized mice, foci were eliminated three to four times more rapidly: the number and average optical density on day 2 were comparable with the values of control mice on days 6–8.

Optical densities of autoradioaraphic foci produced following a percutaneous infection

The frequency distribution patterns of optical densities were determined for autoradiographic foci in the lungs and livers of percutaneously infected mice during the periods of normal and immune elimination. To facilitate presentation of data and comparison of groups, the optical densities obtained in these studies were arbitrarily divided into ranges and assigned to the series of categories defined in Table 4.

Period of normal elimination. In experiment 3, focus optical densities were determined for control mice at seven, 14, and 21 days after infection and compared with the densities of foci produced by intravenously injected, heat-killed lung schistosomula derived from the same batch of labeled cercariae. The killed lung schistosomula were injected approximately 2 hr before the day 7 autopsy. As observed in experiment 4 (Figure 1), by day 7 after injection, most foci produced by heat-killed schistosomula had disappeared in control mice, and all had disappeared in mice immunized three times (Figure 2). In control mice, most of the remaining foci were faint, with 86% belonging to optical density categories 1 and 2 (Table 4). In contrast, on both day 14 and day 21 after infection (seven and 14 days, respectively, after injection of killed schistosomula), less than 5% of the foci produced by schistosomula from a percutaneous infection were in categories 1 and 2.

Period of immune elimination. In two experiments, the percentage distributions of foci among the various optical density categories were compared 21 days after a percutaneous challenge infection in control mice and mice immunized once (experiment 5) or three times (experiment 4). In both experiments, the optical density distribution patterns for the lungs of control and immunized mice were indistinguishable (Figures 3 and 4). In addition, the optical density distribution patterns for the lungs of both control and immunized mice were indistinguishable from those for the livers of the same mice. In experiment 4, optical densities were also determined
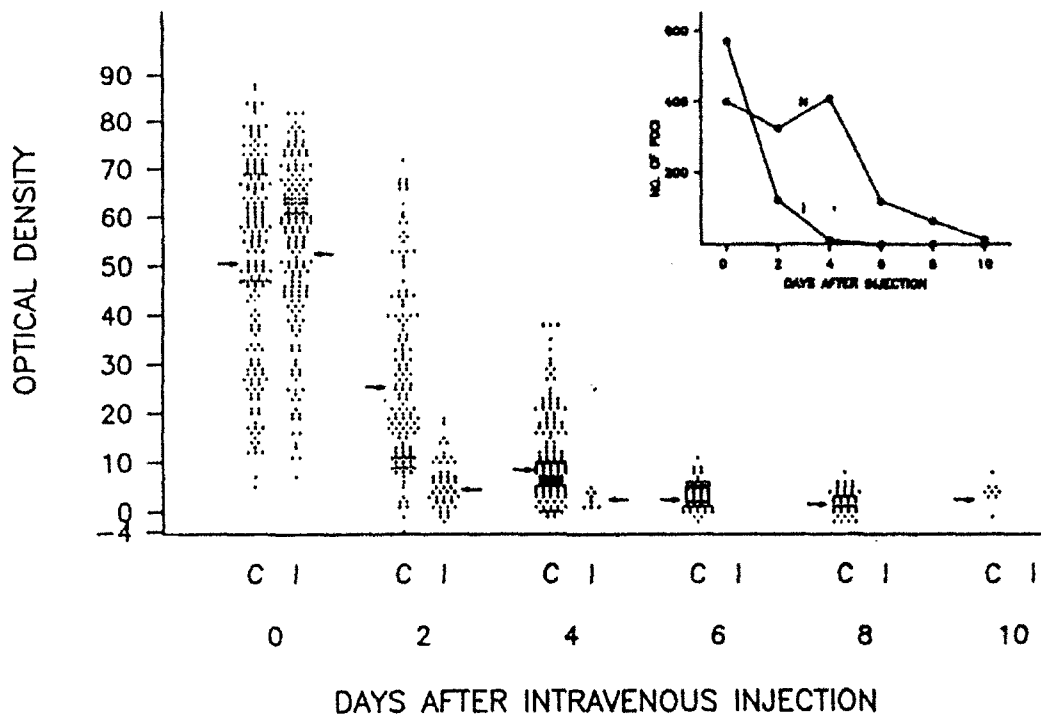
FIGURE 1. Number and optical densities of autoradiographic foci detected in the lungs of control (C) mice and mice immunized three times with irradiated cercariae (I) at various times after intravenous injection of approximately 250 ⁷⁵Se-labeled *Schistosoma mansoni* lung schistosomula killed by heating at 50°C for 5 min (experiment 4). Arrows indicate the mean optical densities. The number of optical density determinations was less than the total number of foci (inset, N = control, I = immunized), particularly at early points, since optical densities were not determined for foci sufficiently close to each other to produce convergence. The combined data for two mice are presented for each point. (Negative optical densities were obtained for a few foci that were less dense than the average background, but more dense than the background in their immediate vicinity.)

28 days after challenge. Again, there was no detectable shift towards lower optical densities in the lung or liver foci of immunized mice, or in lung foci in comparison with liver foci of the same mice (Figure 3).

DISCUSSION

In the following discussion, a distinction will be made between death and elimination of schistosomes. Death will be used in the usual sense, to indicate the disappearance of vital signs such as morphologic integrity and motility. Elimination will be used in a restricted sense, to indicate the disappearance of autoradiographic foci, either through the dispersion of radioisotope from the neighborhood of disintegrating schistosomes or the loss of intact larvae from the body. On the basis of previous observation,[4, 5, 24, 25] it will be assumed that the assay procedures used are

sufficiently sensitive to allow detection of all living worms.

The results of this study confirm previous autoradiographic findings demonstrating that half

TABLE 4
Categories of optical densities used for comparisons in Figures 2–4

Category	Optical density range*
1	−4–2
2	3–5
3	6–10
4	11–20
5	21–30
6	31–40
7	41–50
8	51–60
9	61–70

* Negative optical densities were obtained for a few foci that were less dense than the average background but more dense than the background in their immediate vicinity.
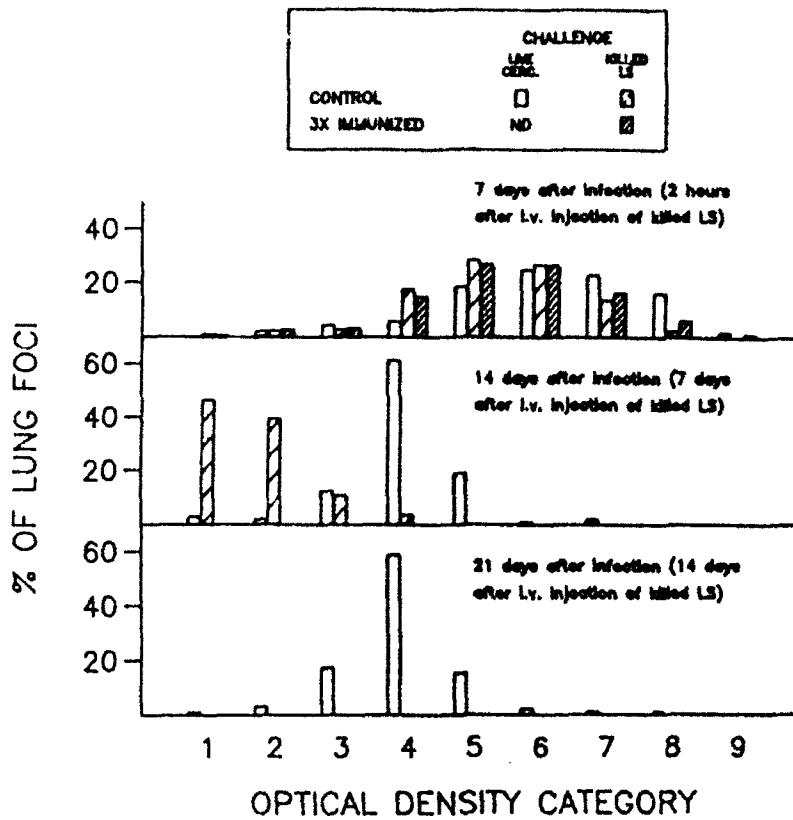
FIGURE 2. Optical density frequency distribution patterns of autoradiographic foci produced in the lungs of control mice and mice immunized three times (3X) with irradiated cercariae (CERC.) at various times after infection with live ^{75}Se-labeled *Schistosoma mansoni* cercariae or intravenous (i.v.) injection with lung schistosomula (LS) killed by heating at 50°C for 5 min (experiment 3). The optical density categories are defined in Table 4. Values represent the average data for three or four mice.

or more of the larvae from a percutaneous *S. mansoni* infection are eliminated from both control mice and mice immunized with irradiated cercariae between seven and 21 days after infection.[2-5, 24, 25] In addition, they demonstrate that after day 21, normal mice show no further decrease in worm burden while immunized mice continue to lose worms at about the same rate until some point after day 35. Similar results have recently been reported by Wilson and others,[6] although they found that the normal phase of elimination persisted somewhat longer.

At the time the normal phase of worm elimination ends and the immune phase begins (day 21), there is a striking difference between the distribution of worms in control and immunized mice: most worms had migrated to the liver in control mice, while most remained in the lungs in immunized mice (Figure 5). Thereafter, the

disappearance of worms from immunized mice is accounted for by their disappearance from the lungs. There is no indication that the missing worms migrate to the liver before elimination; essentially all of the worms detectable in the liver from day 21 onwards can be recovered as live worms by portal perfusion (Table 2). Although sites other than the lungs and liver were not examined in this study, other autoradiographic studies have shown that *S. mansoni* larvae do not selectively accumulate in tissues other than the lungs and liver in either normal mice or mice immunized with irradiated cercariae between weeks 3 and 5 after infection.[5, 25, 26] It seems likely, therefore, that the worms that disappear from the lungs are eliminated from the body.

Histopathologic examination of immunized challenged mice has provided indirect evidence of schistosome elimination in the lungs, in the
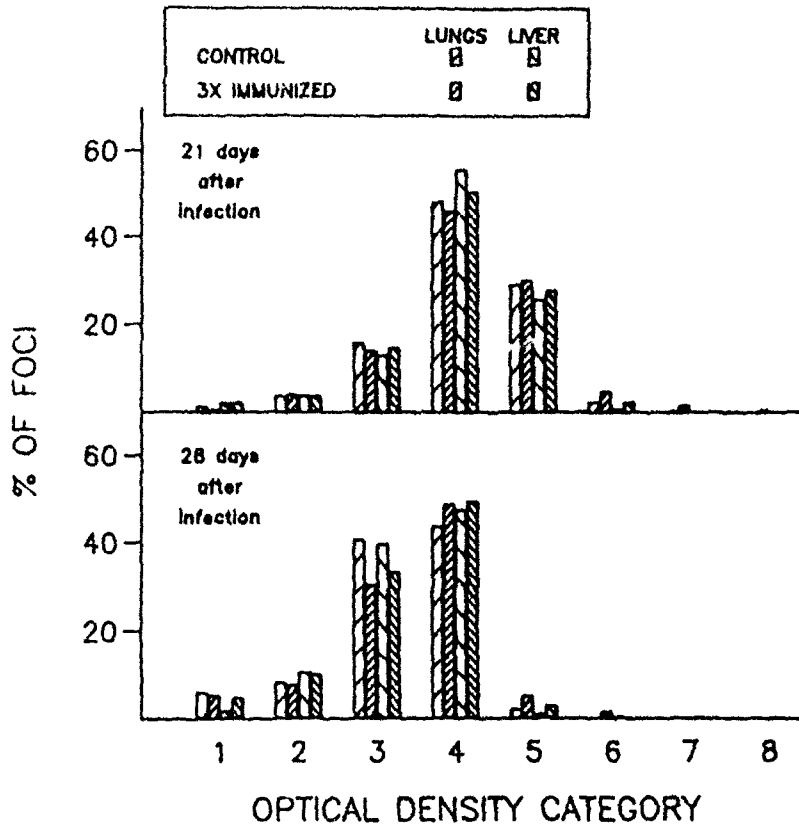
FIGURE 3. Optical density frequency distribution patterns of autoradiographic foci produced by ^{75}Se-labeled *Schistosoma mansoni* larvae in the lungs and livers of control mice and mice immunized three times (3X) with irradiated cercariae. Mice were evaluated 21 and 28 days after infection (experiment 4). The optical density categories are defined in Table 4. Values represent the average data for three or four mice.

form of parasite-free inflammatory foci that are believed to represent sites previously occupied by schistosomula.[a] Similar to elimination in naive mice,[27] however, efforts to demonstrate damaged and dead worms in immunized mice have been frustrating.[7-9] Since heat-killed schistosomula continued to produce autoradiographic foci for several days in the lungs, we were hopeful that analysis of the optical densities of lung foci would provide a means of studying worms in the elusive stage between death and elimination. This analysis produced rather surprising results. During both the normal (early) and immune (late) phases of elimination, the optical density frequency distribution patterns obtained for schistosomula in the lungs were indistinguishable from those obtained for live larvae in the livers of the same mice. In contrast, the foci produced by heat-killed injected schistosomula faded steadily be-

fore disappearing. Although it must be considered that larvae dying in situ may be cleared somewhat differently from larvae killed by heating at 50°C, the striking similarity between the optical densities of lung and liver populations provides compelling evidence that most, if not all, of the larvae being detected in the lungs are alive.

With the information available from studies in mice and rats, it is possible to construct a hypothesis for the process of *S. mansoni* elimination that contains the following elements.

1) Schistosomula are at greatest risk of being eliminated from the body during the period they are in the lungs. This is supported by evidence from normal and immune mice[2-6, 26] and rats[28] indicating that the number of larvae in the body decreases by half or more during the lung phase of migration. Also, the period of larval sensitiv-
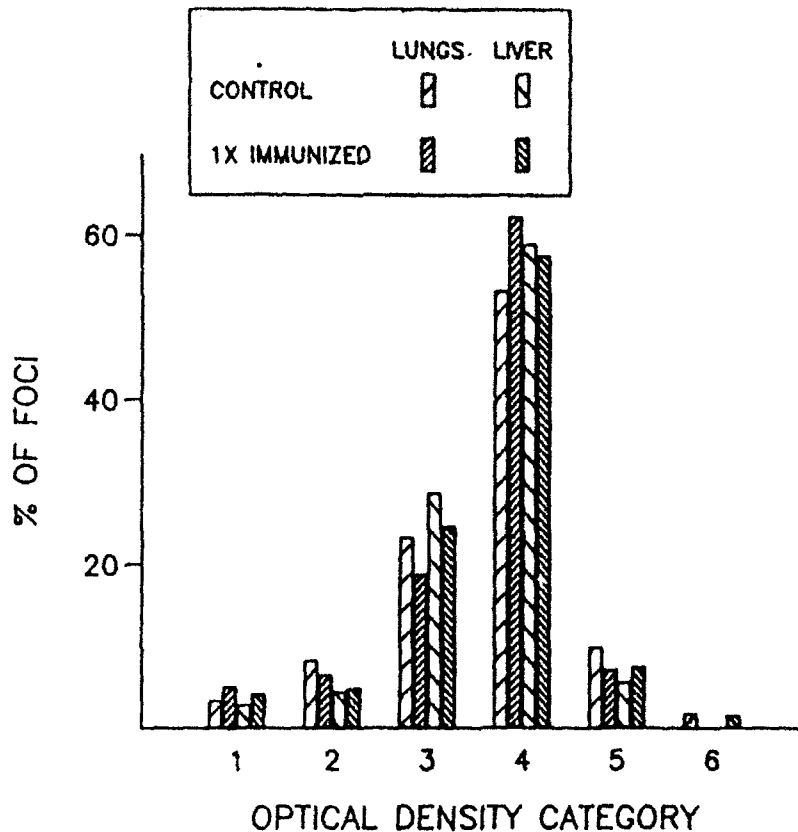
FIGURE 4. Optical density frequency distribution patterns of autoradiographic foci produced by ^{75}Se-labeled *Schistosoma mansoni* larvae in the lungs and livers of control mice and mice immunized once (1X) with irradiated cercariae. Mice were evaluated 21 days after infection (experiment 5). The optical density categories are defined in Table 4. Values represent the average data for three or four mice.

ity to passively transferred antibody from irradiated cercaria-immunized mice[29] and rats[30] and previously infected rats[31] coincides with the period of residence in the lungs.

2) Schistosomula are exposed to the same risk per unit of time in normal and immunized animals. This is supported by evidence from studies in irradiated cercaria-immunized mice[2, 5, 6] and previously infected rats,[28] which indicates that the rate of worm disappearance from the body is similar in normal and immunized animals, and proportional to the size of the lung population in both cases. The cumulative risk of elimination is greater in immunized hosts possibly only because the period of lung residence is extended.

3) Schistosomula that are eliminated leave the blood stream, enter alveoli, and pass via the trachea into the gastrointestinal tract. This idea was first suggested to us by Donato Cioli before we were aware of experimental evidence for it, and has been proposed by Crabtree and Wilson.[9] Since that time, we have found reported observations of schistosomula of *S. mansoni*,[9, 12, 14-18] *S. japonicum*,[11, 14, 15] and *Schistosomatium douthitti*[13] within alveoli. Recently, schistosomula have been demonstrated by autoradiography in the trachea and lumina of the esophagus, stomach, and intestines.[32] In an electron microscopic study, Crabtree and Wilson found that in both normal mice and mice immunized with irradiated cercariae, *S. mansoni* schistosomula gradually shift from vascular to alveolar locations after arriving in the lungs.[9] By the third week of infection, more than half of the larvae observed were partially or wholly within alveoli.

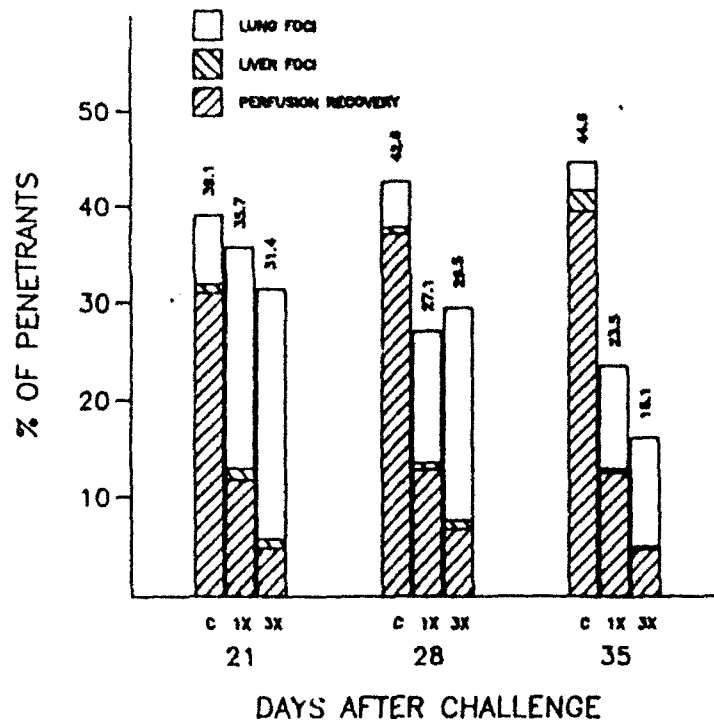4) Schistosomula are eliminated from the lungs while still alive. The optical density data reported

LUNG FOCI
LIVER FOCI
PERFUSION RECOVERY

% OF PENETRANTS

50

40

30

20

10

C 1X 3X   C 1X 3X   C 1X 3X
21   28   35

DAYS AFTER CHALLENGE

FIGURE 5. Percentage of the estimated number of skin-penetrating ^{75}Se-labeled *Schistosoma mansoni* cercariae accounted for by portal perfusion and autoradiography (experiment 2). C = control mice; 1X = mice immunized by one exposure to irradiated cercariae; 3X = mice immunized by three exposures to irradiated cercariae. Numbers above bars indicate the total percentage of perfusion recovery and autoradiographic foci. The numbers of mice are reported in Table 2.

here and the microautoradiographic data from the same study[7] support this conclusion. Also, in the electron microscopic observations of Crabtree and Wilson on schistosomula in pulmonary blood vessels and alveoli, no evidence of larval damage was found.[9]

If it is demonstrated directly that the bulk of schistosomula that are eliminated are coughed up while still alive, then it is probable that only two possible sites of larval killing will need to be seriously considered. The first possibility is that they are destroyed in the gastrointestinal tract. It is difficult to imagine that this developmental stage of such a highly specialized trematode would have mechanisms for surviving the effects of digestive enzymes. A second, less likely, possibility is that they are eliminated from the body via the gastrointestinal tract while still alive, to face once again the challenges presented by the outside world.

The results of this study indicate that resistance in irradiated cercaria–immunized mice, al-though immunologically specific,[33, 34] is a consequence of the diversion of larval migration at the lung phase, rather than the direct killing of challenge organisms. The observation of delays in the lung migration of challenge infection larvae in irradiated cercaria–immunized rats, irradiated cercaria–immunized guinea pigs, and previously infected rats, as well as the direct relationship among schistosome species between speed of migration through the lungs and survival to adulthood in naive mice[24, 35] suggest that this may be a generally important mechanism of schistosome elimination in both normal and immune hosts.

It must also be pointed out that there are three well-documented examples of schistosome elimination in sites other than the lungs: skin killing in rhesus monkeys repeatedly immunized with irradiated *S. japonicum* cercariae,[36] skin killing in CBA/Ca mice immunized with irradiated *S. mansoni* cercariae of the Mill Hill strain,[37-40] and the additional skin and liver killing seen in guin-

ea pigs immunized with irradiated *S. mansoni* cercariae.[38,41] Other examples may exist in nature.

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6. AUTHOR(S)
Dean, David A., and Mangold, Beverly L.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Naval Medical Research Unit No. 3
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The number and distribution of autoradiographic foci observed in this and previous studies following percutaneous infection with ^{75}Se-labeled Schistosoma mansoni cercariae indicate that the lungs are the principal site of worm elimination in both normal mice and mice immunized with irradiated cercariae. It was observed in the present study, however, that the intensities of the autoradiographic foci produced in the lungs during both the normal (early) and immune (late) phases of elimination were identical to those of foci produced in the livers of the same mice by larvae shown to be alive. In contrast, foci produced in the lungs by heat-killed, intravenously injected, lung schistosomula became smaller and fainter with time, disappearing completely between seven and 10 days after injection in normal mice and between four and six days in immunized mice. These results indicate that although the targets of both normal and immune elimination do not proceed beyond the lung stage of migration, they do not die in the lungs. A possible explanation for this paradoxical situation, for which there is some experimental evidence, is that unsuccessful migrators leave the blood stream, enter alveoli, pass up the trachea, and are eventually digested in the gastrointestinal tract or eliminated from the body intact.

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