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CRYOPRESERVATION OF SCHISTOSOME LARVAE.(U)
SEP 79 M A STIREWALT, F A LEWIS N00014-78-C-0081
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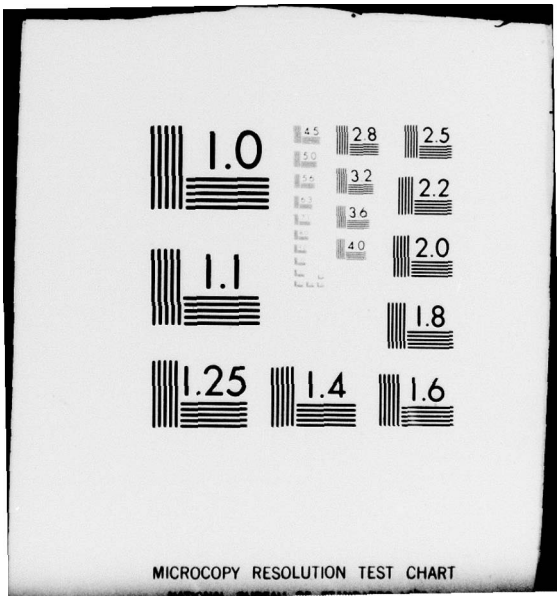
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MICROCOPY RESOLUTION TEST CHART

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by

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Optimal conditions for freezing and thawing schistosomules were set up. Thirty to 60% of the schistosomules stored at -196C and quick thawed were normal in appearance and activity. Partial protection of mice by intramuscular injection of cryopreserved irradiated schistosomules as shown by challenge worm reductions of 50 to 60% below that of non-immunized controls.

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Background

Significant protective immunity to Schistosoma mansoni develops in several mouse strains (a) during a patent chronic infection, and (b) following immunization with highly irradiated viable cercariae. Immunity in the latter develops without the complications arising as a result of the pathologic changes in a chronic patent infection. Since injection of highly irradiated cercariae does not lead to a patent schistosomal infection this type of immunization shows potential for human vaccine use. Non-viable preparations have been almost universally unsuccessful in inducing protective immunity.

Deployment of a live vaccine on a large scale however is immediately beset with numerous problems. Foremost among these are the complexities of maintaining a Schistosoma mansoni life cycle in endemic areas and a facility for irradiating the freshly emerged cercariae. By using cryopreserved schistosomules these and many other problems could be effectively circumvented. Work supported by this contract showed that irradiated schistosomules could be effectively frozen and stored in liquid nitrogen. Furthermore, upon thawing they served as effective immunizing agents against a challenge infection when injected into mice.

METHODOLOGY AND RESULTS

Much of the investigation supported by this contract necessitated an empirical approach. Methods developed for successful freezing and thawing large quantities of viable S. mansoni schistosomules are outlined below. All cooling procedures were handled in a biological freezing unit (Cryo-Med, St. Clemens, Michigan) capable of providing reproducible, predetermined cooling rates. Except where indicated all schistosomules frozen were derived by the shear pressure technique of Colley and Wikel (1974).

(1) Table I shows that aluminum weighing dishes proved to be the best vessels for cooling and thawing samples. Even dispersion of heat throughout the suspension was insured by covering the surface with an aluminum disc. High percentages of lively organisms could not be recovered when glass tubes, vials, and Petri dishes were used as cooling vessels.

(2) Among the various cryoprotectant media tested, 17.5% methanol in Earle's salts with lactalbumin hydrolysate (ELAC) was the freezing medium which reproducibly yielded the highest percentage of lively organisms. Dehydration time for schistosomules suspended in 17.5% methanol at 0C could be extended as long as 1 hour (Table II). Longer dehydration periods invariably reduced recovery of highly active organisms.

(3) The rate of cooling schistosomules and the optimal temperature for quenching samples in liquid nitrogen were interdependent (Figure I). Increases in cooling rate depressed the optimal quenching temperature. Highest percentages of

normally active schistosomules were obtained with cooling rates of 0.4 C/min and 0.8 C/min, and corresponding quenching temperatures of -32 C and -35 C, respectively.

(4) Table III demonstrates that schistosomules could be stored for at least 2 1/2 months in liquid nitrogen without appreciable loss in activity.

(5) When normal, unattenuated schistosomules were frozen and thawed, approximately 1% of them developed to maturity when injected into mice. Maturation was highly dependent on the route of injection, the subcutaneous route being inferior to intramuscular.

(6) Schistosomules attenuated by ^{60}Co-irradiation showed comparable percentages of recovery of lively organisms to those unattenuated by radiation.

(7) Schistosomules prepared by vortex and differential temperature methods, or by penetration of dried rat skin did not survive cryopreservation as well as the shear pressure organisms did.

(8) Cryopreserved schistosomules derived from ^{60}Co-irradiated cercariae were used in experiments to immunize mice against a challenge infection with normal cercariae. The results demonstrated that irradiated, frozen and thawed schistosomules induced protective immunity in the recipients comparable to that using nonfrozen irradiated controls (Tables IV and V).

DISCUSSION

Cryopreservation of relatively large organisms, such as multicellular parasites, presents a challenging problem for optimizing cooling and thawing procedures. Various features of the problem contribute to this challenge. A schistosomule is composed of many different cell types, each probably with different cooling requirements. Therefore, it may be impossible to select a single cooling rate, quenching temperature, or concentration of cryoprotective additive which will support survival of all the component cells. The conditions adopted to cryopreserve multicellular organisms in a viable state may, at best, be a compromise. It follows that establishing the best procedures for cryopreservation of schistosomules requires close attention to detail, and a tedious empirical approach. Nevertheless, it is worthy of maximal effort since conditions have not been found to cryopreserve the cercarial stage successfully.

The potential for the development of a live vaccine against schistosomiasis has, until now, been seriously limited because of the problems inherent in the storage and transporation of viable, attenuated organisms. Research funded by this contract has led to the development of techniques for successful cryopreservation and recovery of attenuated schistosomules which can serve as effective immunizing agents against a challenge *S. mansoni* infection.

SIGNIFICANT ACCOMPLISHMENTS

1) Cryopreservation of ^{60}Co-irradiated schistosomules with recovery after thawing of 30 to 60% normal in activity and appearance.

2) Partial protection of mice was provided by intramuscular injection of cryopreserved irradiated schistosomules as shown by challenge worm reductions of 50 to 60% below that of non-immunized controls.

TABLE I

Types of Freezing Vessels Used in Cryopreservation Studies with Schistosomules of *Schistosoma mansoni*[a]

Freezing vessel	Size	Volume of schistosomule suspension in vessel	% recovery of normally active schistosomules ($\bar{X}$ or $\bar{X} \pm$ S.E.)
Glass tubes	75 X 100 mm	200 ul	less than 1
Glass tubes	75 x 100 mm	2 ml	0
Glass vials	30 X 15 mm	200 ul	less than 1
Petri dishes (glass)	60 x 10 mm	2 ml	0
Aluminum weighing dishes	57 mm dia.	3 ml	26 ± 10
Aluminum weighing dishes + aluminum cover	57 mm dia.	3 ml	47 ± 14

[a]Cooling rate of 0.4 C/min and quenching temperature of -32 C. Cryoprotective additive was methanol (17.5%) in ELAC.

TABLE II

Effect of Dehydration Time in Cryoprotective Additive on Recovery of Normally Active Schistosoma mansoni Schistosomules Following Cryopreservation[a]

Dehydration time	% recovery of normally active schistosomules (X ± S.E.)[b]
10 min	40 ± 2
30 min	41 ± 7
1 hr	42 ± 5
2 hr	28 ± 6

[a]Results of four experiments. Cryopreservative additive was 17.5% methanol in ELAC.

[b]Cooling rate of 0.4 C/min and quenching temperature of -32 C.

TABLE III

Effect of Storage Time in Liquid Nitrogen on Recovery of Normally Active *Schistosoma mansoni* Schistosomules[a]

Storage time at -196 C	% recovery of normally active schistosomules ($\bar{X} \pm$ S.E.)
24 hours	31 ± 13
1 week	33 ± 5
2 weeks	31 ± 1
3 weeks	37 ± 2
4 weeks	29 ± 6

[a]Results of three experiments. Cooling rate of 0.4 C/min and quenching temperature of -32 C.

TABLE IV

Results of Immunization of Mice with Cryopreserved Irradiated Schistosoma mansoni Schistosomules[a]

Experiment	Group[b]	No. of adult challenge worms recovered $\bar{X} \pm$ S.D.	% reduction	$P \leq$ (vs. Gr. 3)
I[c]	1 Unfrozen controls	24 ± 7	60	0.001
	2 Cryopreserved	33 ± 18	45	0.003
	3 Non-immunized controls	60 ± 15	—	
II[d]	1 Unfrozen controls	41 ± 10	45	0.001
	2 Cryopreserved	45 ± 17	40	0.001
	3 Non-immunized controls	75 ± 14	—	

[a]Experimental mice received two immunizations each of 1000 of either unfrozen control schistosomules or cryopreserved schistosomules. Cryopreserved schistosomules were held in liquid nitrogen for 60 min before thawing and injecting into mice. Mice were challenged percutaneously with 150 normal cercariae.

[b]Fifteen mice/group.

[c]Groups 1 and 2 immunized on Days 1 and 14, and challenged on Day 70.

[d]Groups 1 and 2 immunized on Days 1 and 14, and challenged on Day 56.

TABLE V

Comparison of One Versus Two Immunizations of Irradiated Cryopreserved *Schistosoma mansoni* Schistosomules on Induction of Resistance in NIH/Bmri Mice[a]

Group[b]	Schistosomule treatment	No. of schistosomules injected on day 1	No. of schistosomules injected on day 25	Adult worm recovery $\bar{X} \pm$ S.D.	% reduction[c]
1	Cryopreserved	1500	—	28 ± 14	59
2	Unfrozen controls	1500	—	24 ± 12	65
3	Cryopreserved	1500	2000	35 ± 15	50
4	Unfrozen controls	1500	1500	28 ± 12	59
5	Non-immunized controls	—	—	69 ± 11	—

[a]Mice were challenged percutaneously with 150 normal cercariae on Day 56 of the experiment.

[b]Fifteen, 6-week old-mice/group.

[c]All reductions significant at $P \leq 0.001$.

FIG. I Comparative effects of cooling rate and quenching temperature on recovery of normally active Schistosoma mansoni schistosomules ($\bar{X} \pm$ S.E.). Cooling rates: □—□ , 0.2 C/min.; ○—○, 0.4 C/min; ○—○ , 0.8 C/min; □—□, 1.2 C/min. At 0.4 C/min and 0.8 C/min cooling rates, each curve represents the results from six experiments. At 0.2 C/min and 1.2 C/min each curve represents the results from three experiments. The percentage of schistosomules dead at the peak for each rate is: 0.2 C/min, 80%; 0.4 C/min, 44%; 0.8 C/min, 28%; 1.2 C/min, 67%. All experiments used methanol (17.5%) as the cryoprotective additive.

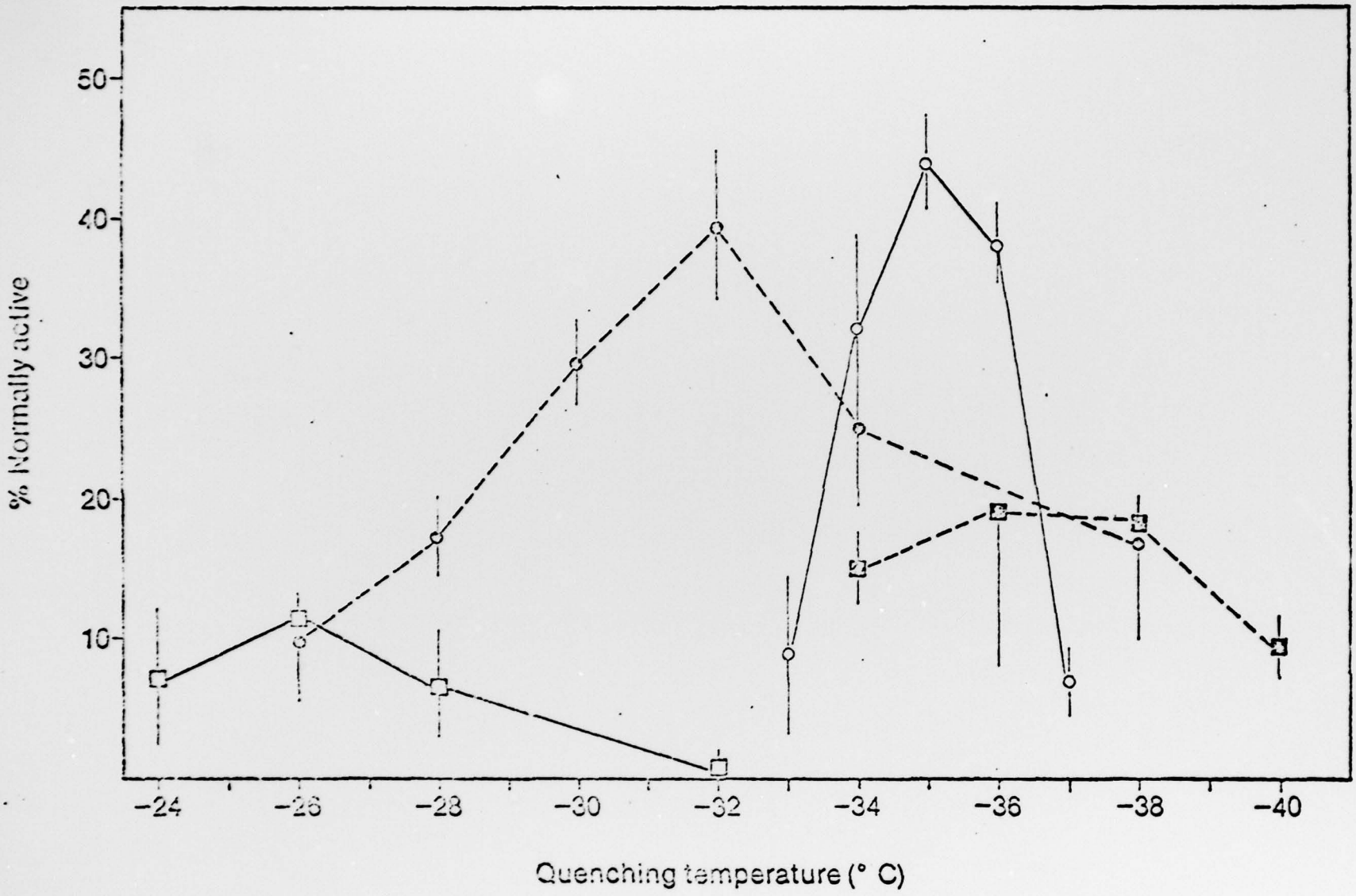
50
40
30
20
10
% Normally active
−24
−26
−28
−30
−32
−34
−36
−38
−40
Quenching temperature (° C)

Figure I

PUBLICATIONS

1) Stirewalt, M.A., Lewis, F.A., and Murrell, K.D. 1979. *Schistosoma mansoni*: Cryopreservation of Schistosomules. *Exp. Parasit.* (in press).

2) Murrell, K.D., Stirewalt, M.A. and Lewis, F.A. 1979. *Schistosoma mansoni*: Vaccination of mice with cryopreserved irradiated schistosomules. *Exp. Parasit.* (in press).