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Cryopreservation and long-term in vitro maintenance of second-stage larvae of *Toxocara canis*

T. Ramp, J. Eckert, and B. Gottstein
Institute of Parasitology, University of Zurich, Winterthurerstr. 266a, CH-8057 Zürich, Switzerland

Abstract. Second stage larvae of *Toxocara canis* were isolated from developed eggs, frozen in Eagle's Minimal Essential Medium with 5% dimethyl sulfoxide or 10% glycerol as cryoprotectants according to two cooling schedules and maintained in liquid nitrogen for 1 week. After thawing, the previously frozen larvae (FL) and unfrozen controls (CL) were maintained in a chemically defined medium in vitro for 35 weeks. While CL had motility rates around 95% to 97% throughout the experiment, previously frozen larvae (FL) exhibited rates of 48%–58% at the beginning and of 19%–39% at the end of the 35 week in vitro maintenance period. The surviving FL and CL larvae proved to be infective for mice. Excretory/secretory (ES) antigens isolated from several batches of culture medium in which FL and CL had been maintained reacted in the ELISA with human sera containing antibodies against *Toxocara*. Antigens from FL and CL separated by SDS-PAGE and silver-stained showed some differences in polypeptide patterns. Western-blot analysis revealed that these differences were not related to antigenic polypeptides but were most likely caused by substances without antigenic properties originating from dead and/or degenerating larvae. It can be concluded that ES antigens produced by previously frozen larvae are essentially the same as those derived from unfrozen controls.

The value of cryopreservation of *T. canis* larvae for routine production of ES antigens will be further evaluated.

In vitro maintenance of *Toxocara canis* second-stage larvae, the main causative agent of human "internal (visceral) larva migrans" (human toxocariasis), in a chemically defined medium was described by de Savigny (1975). In cultures the larvae release excretory/secretory (ES) antigens which are of proven value in the serodiagnosis of human toxocariasis (de Savigny and Tizard 1977; de Savigny et al. 1979; Smith et al. 1983; van Knapen et al. 1983; Maizels et al. 1984; Speiser and Gottstein 1984; Glickman and Schantz 1985).

The production of ES antigens for routine serology needs a continuous supply of second-stage larvae (L2). They can be isolated from developed *T. canis* eggs (Annen et al. 1975) which survive for many months in 1% formaldehyde (final concentration) at +4° C. Another possibility is the storage of L2 already isolated from the eggs. In this paper we describe studies on the storage of *T. canis* second-stage larvae in liquid nitrogen (lN_2), their subsequent maintenance in a chemically defined medium and the analysis of antigenic properties of ES products released in vitro.

Materials and methods

1. Experimental design. In our experiments second-stage larvae of *T. canis* were submitted to different experimental conditions: (a) cryopreservation using two different cryoprotectants: "frozen larvae" (FL) (Table 1, experiments 1, 2 and 4, 5); (b) larvae treated with cryoprotectants but not frozen: "control larvae" (CL) (Table 1, experiments 3 and 6); (c) untreated larvae, as a reference: "reference larvae" (RL) (Table 1, experiment No. 7).

After storage in liquid nitrogen for 1 week the frozen larvae (FL) were thawed and maintained in vitro in a chemically defined medium. For comparison CL and RL were also kept in vitro. The motility of the larvae was determined in weekly intervals and at the end of the experiment infectivity was tested in mice. Furthermore, the excretory-secretory products released by the larvae into the culture medium were analysed for their antigenic properties.

2. Preparation of larvae. After treatment with mebendazole (Telmin^R) or pyrantel pamoate (Banminth^R) *Toxocara canis* worms

Reprint requests to: J. Eckert

Table 1. Experimental design and cooling rates from the schedules used for the cryopreservation of *Toxocara canis* second-stage larvae

Experiment[a]	Cryoprotectant	Cooling: schedule[a]	Step	Cooling rate overall[b]	(° C min^{-1}): linear[c]	Number of in vitro cultures maintained for 35 weeks
1 (FL)	DMSO (5%)	4 step	1:precooling	0.8	–	3
			2:cooling	1.1	2.8	
			3:cooling	1.7	4.8	
			4:liquid nitrogen	52.0	189.7	
2 (FL)	DMSO (5%)	3 step	1:precooling	0.8	–	3
			2:cooling	4.7	23.4	
			3:liquid nitrogen	–	–	
3 (CL)	DMSO (5%)	not frozen	control larvae			3
4 (FL)	Glycerol (10%)	4 step	1:precooling	0.8	–	3
			2:cooling	1.0	2.9	
			3:cooling	1.2	4.0	
			4:liquid nitrogen	62.9	189.0	
5 (FL)	Glycerol (10%)	3 step	1:precooling	0.7	–	3
			2:cooling	4.7	27.1	
			3:liquid nitrogen	–	–	
6 (CL)	Glycerol (10%)	not frozen	control larvae			3
7 (RL)	none	not frozen	reference larvae			13/6[d]

[a] see materials and methods;
[b] cooling rates are expressed as means of three runs each and calculated from starting time until transfer to the next step;
[c] these values indicate initial, maximal linear rates;
[d] weeks 1–16 of in vitro maintenance: 13 cultures; weeks 17–35: 6 cultures.
– not determined

were collected from 5 week old puppies. The isolation of eggs from female gravid worms was performed as described by Annen et al. (1975) with the following modification: instead of dissecting the worms individually they were immersed in sodium hypochlorite solution (final concentration of 0.15% active chlorine) and then gently squashed in a mortar to release the eggs. The resulting suspension was washed 3 times in sodium hypochlorite solution (0.15%) by a sedimentation-decanting process and finally passed through a sieve (200 µm mesh size) to eliminate large tissue fragments. The incubation of eggs and the isolation of second-stage larvae were performed according to Annen et al. (1975).

3. Cooling and thawing procedure. For cryopreservation L2 were pooled from two untreated reference cultures initiated 1 week before starting the freezing experiment. Aliquots of about 6,000–7,000 L2 suspended in 0.1 ml of HMEM (see section 4) were transferred to 1.8 ml round bottom, screw cap cryotubes (Nunc, DK-Roskilde) and mixed thoroughly with 0.9 ml HMEM containing dimethyl sulfoxide (DMSO, analytical grade; Fluka, CH-Buchs/SG) or glycerol (anhydrous; Fluka, CH-Buchs/SG) in quantities to give final concentrations (v/v) of 5% and 10%, respectively.

The larvae of the FL batch were submitted to the following cooling schedules: (a) *4 step schedule*: step 1: precooling phase from initially +22° C to +2° C in a refrigerator for 30 min; step 2: cooling for 30 min in a freezer at −28° C; step 3: cooling for another 30 min in a freezer at −80° C; step 4: final transfer to liquid nitrogen at −196° C; (b) *3 step schedule*: step 1: as in a; step 2: cooling for 2 h in the gas phase of liquid nitrogen (about −180° C); step 3: final transfer to liquid nitrogen.

The temperature curves resulting from cooling schedules (a) and (b) were recorded by a Pt-100 temperature probe connected to a Philips PM 8222 recorder (Philips, NL-Eindhoven). The calculated cooling rates (decrease of temperature in ° C min^{-1}) are summarized in Table 1. The control larvae (CL) were also treated with cryoprotectants as described above but submitted to precooling only. The latter treatment was prolonged to 1 h to compensate for handling time.

The frozen samples were stored for 1 week in liquid nitrogen using a Chronos 80 storage system (Messer, D-Griesheim). Subsequently they were thawed by immersion for approximately 3 min in a waterbath at +37° C until only a small piece of ice was left in each of the cryotubes. For quick dilution of the cryoprotectant the content of each tube was poured into 10 ml of HMEM (see section 4) prewarmed to +37° C and centrifuged at 600 × g for 5 min at room temperature. The cryoprotectant was finally removed by two additional runs of the same procedure. The elimination of DMSO and glycerol in the cryoprotectant-treated nonfrozen controls (CL) was carried out in the same way. After the third centrifugation the larvae were resuspended in 2 ml HMEM (see section 4) and maintained in vitro as described below.

4. In vitro maintenance of larvae. The second-stage *T. canis* larvae were maintained under axenic conditions in vitro as described by de Savigny (1975) in a chemically defined medium (Eagle's Minimal Essential Medium with Hank's Salts = HMEM; KC Biological, Lenexa, Kansas, USA) supplemented with antibiotics (penicillin G potassium salt and streptomycin sulfate at 100 IU and 250 µg per ml medium respectively; Fluka, CH-Buchs/SG) with air as gas phase at +37° C at an initial pH of 7.2. However, in our experiments the density of

Table 2. Infectivity of *Toxocara canis* second-stage larvae for mice after cryopreservation and subsequent 35 weeks in vitro maintenance in a chemically defined medium

Experiment[a]	Cryopreservation:		Infectivity test:	
	cryoprotectant added	Cooling schedule[a]	Dose[b]	Number of mice inoculated: infected[c]
1 (FL)	5% DMSO	4 step	420	3:1
2 (FL)	5% DMSO	3 step	870	3:3
3 (CL)	5% DMSO	not frozen	790	3:3
4 (FL)	10% glycerol	4 step	420	3:3
5 (FL)	10% glycerol	3 step	700	3:3
6 (CL)	10% glycerol	not frozen	750	3:3
7 (RL)	none	not frozen	750	3:3
	none	not frozen	370[d]	2:1

[a] see materials and methods;
[b] number of motile larvae administered by intraperitoneal injection per mouse;
[c] 3 weeks post infection. Of the 23 mice 18 contained larvae in the brain, 5 in the liver, 6 in the musculature;
[d] infected with L2 newly hatched from infective eggs stored for 35 weeks at +4° C in 1% formaldehyde

larvae in the culture medium was lower than described by de Savigny (1975).

The reference larvae (RL) were maintained in 10 ml of medium in 25 cm^2 plastic tissue culture flasks (No. 25100, Corning Glass Works, Corning New York, USA) at a density of 6,000–8,000 L2 ml^{-1}. The flasks were kept horizontally, except for an approximately $^1/_2$–2 h sedimentation time before and during the weekly change of medium which was performed by aspirating the supernatant. Control larvae (CL) and L2 thawed after cryopreservation (FL) were maintained in 3 ml of medium in flat-sided culture tubes (Nunc, DK-Roskilde). The density of larvae in these cultures was 2,000–2,300 ml^{-1}. Cultures of FL and CL were set up in triplicates, resulting in a total of 18 cultures. The larvae were maintained in vitro for 35 weeks (Table 1).

The supernatant obtained at the weekly medium changes containing the ES products was filtered through a 0.22 µm filter membrane (Millex-GS: Millipore, F-Molsheim; or Val-Filt: Van Leer Medical, F-Irigny) and stored at −28° C. After thawing, the supernatants obtained from cultures of experiments 1–6 (Table 1) during the 35 weeks culture period were separately pooled and used for analysis (see section 6).

5. Viability assays in vitro and in vivo. The viability of the larvae maintained in vitro was judged by their motility. All vessels were examined once per week by random microscopic counting of 100 L2 in each tube or flask. The percentage of active, motile larvae and of stretched, immotile larvae was recorded in that manner over 35 weeks. Statistical analysis was performed with Student's t-test (alpha levels Bonferroni-corrected).

Further, the viability of the parasites was tested after 35 weeks of in vitro maintenance by an infectivity trial in mice (Table 2). For each test two to three mice were used (strain ZUR-ICR, 12 week old SPF females bred at the Laboratory Animal Division, Faculty of Veterinary Medicine, University of Zürich) each of which received between 420 and 870 motile larvae by intraperitoneal injection. For comparison, two additional mice were infected in the same way with 370 L2 freshly hatched from developed eggs which had been stored conventionally for 35 weeks in 1% formaldehyde at +4° C.

The mice were kept at +21° C (±1° C) under a 12 h/12 h light/dark rhythm. They received pelleted standard food (Nr. 890-185: Nafag, CH-Gossau/SG) and water ad libitum. All animals were killed in a chloroform jar 3 weeks after infection. Three 0.05–0.1 g pieces of brain, lung, liver and hind leg muscle tissue were examined by the squashing technique used in trichinoscopy (Boch and Supperer 1983).

6. Analysis of Toxocara ES products. The ES products released by the *Toxocara* larvae into the culture medium (see section 4) were used for an enzyme-linked immunosorbent assay (ELISA) (Speiser and Gottstein 1984). Analysis of ES products was performed by SDS-PAGE, silver-staining and Western-blot (Gottstein et al. 1986). For ELISA and Western-blot investigations a pool of sera from 30 patients was used. In these patients clinical symptoms and antibody detection with *Toxocara* ES antigens in the ELISA were suggestive for toxocariasis. Another pool of sera from 30 healthy blood donors which did not react with *Toxocara* ES antigens in the ELISA was a negative control. The ELISA was performed according to the alkaline phosphatase system (Speiser and Gottstein 1984). Optimizing titrations of the ES antigen batches from the 6 groups of cultures (Table 1: CL and FL, experiments 1–6) for coating ELISA microplates resulted in protein concentrations varying from 0.03 to 0.35 µg per ml coating buffer solution. The protein amounts of these six ES antigen batches were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, D-München) using bovine plasma gamma globulin as a standard.

Results

1. Motility of Toxocara larvae in vitro

As demonstrated in Fig. 1 reference larvae (RL) (Table 1, experiment 7) and unfrozen control larvae (CL) treated with 5% DMSO or 10% glycerol (experiments 3 and 6) contained 96%, 95% and 96% motile larvae at the beginning of in vitro maintenance. Some fluctuations of motility were observed during 35 weeks. At the end of this period motility in all 3 groups was essentially unchanged (96%, 97%, 97%, respectively) as compared with the initial values. In contrast, previously frozen larvae (FL, experiments 1, 2, 4 and 5) had significant-

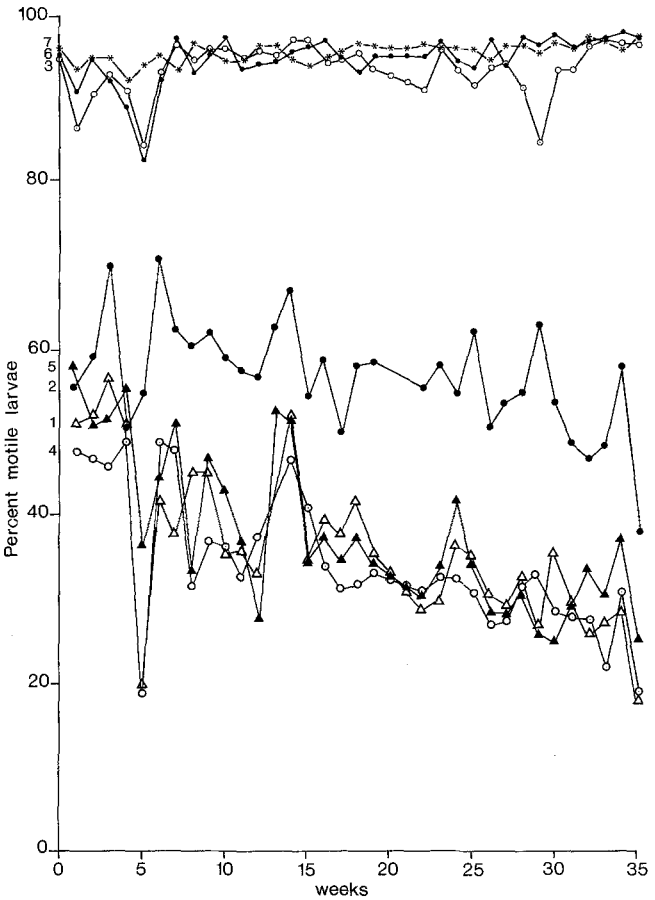

Fig. 1. Motility rates of second-stage *Toxocara canis* larvae in vitro. *1, 2, 4* and *5:* previously cryopreserved larvae; *3* and *6:* unfrozen control larvae treated with cryoprotectants and submitted to precooling only; *7:* untreated reference larvae (for details see Table 1 and text)

ly ($P<0.001$) lower motility values at the beginning of in vitro maintenance: 51%, 56%, 48% and 58%, respectively. Moreover, the final values after 35 weeks (18%, 39%, 19% and 26%, respectively) were also significantly ($P<0.001$) lower than in the CL and RL groups. Some losses of larvae occurred during the culture period due to accidental removal of larvae at the medium changes.

2. *Infectivity of larvae for mice*

Results of the in vitro viability tests are summarized in Table 2. In each group 2–3 mice were inoculated with larvae and 1–3 animals contained L2 at necropsy. This proved that previously cryopreserved larvae were still infective to mice after maintenance for 35 weeks in a defined medium in vitro. Of the 23 infected mice 18 contained L2 in the brain, 5 in the liver, and 6 in muscle. Quantitative differences in the worm burden of mice in the various groups could not be evaluated as the squash technique is unreliable for exact determination of parasite numbers.

3. *Analysis of ES products*

ES antigens isolated from pooled medium of CL and FL larvae in experiments 1–6 (Table 1) reacted in the ELISA with human sera containing *Toxocara* antibodies. In these cases the optical density (OD) values varied from 0.51 to 0.56 at 404 nm whereas the reactions with negative control sera from people without *Toxocara* resulted in OD values between 0.08 to 0.14 at 404 nm. The OD values for the six antigen batches derived from FL and CL larvae were not significantly different from each other. The SDS-PAGE pattern of silver-stained polypeptides and the demonstration of their antigenicity by Western-blot is shown in Fig. 2. Qualitative and quantitative differences in the silver-stained polypeptide pattern of the six ES antigen batches separated by SDS-PAGE were seen in the relative molecular mass range between 66 K and 8 K. The Western-blot analysis revealed that these differences were not related to antigenic polypeptides as no qualitative differences in antigenic fractions were seen in the comparison of the six ES antigen batches.

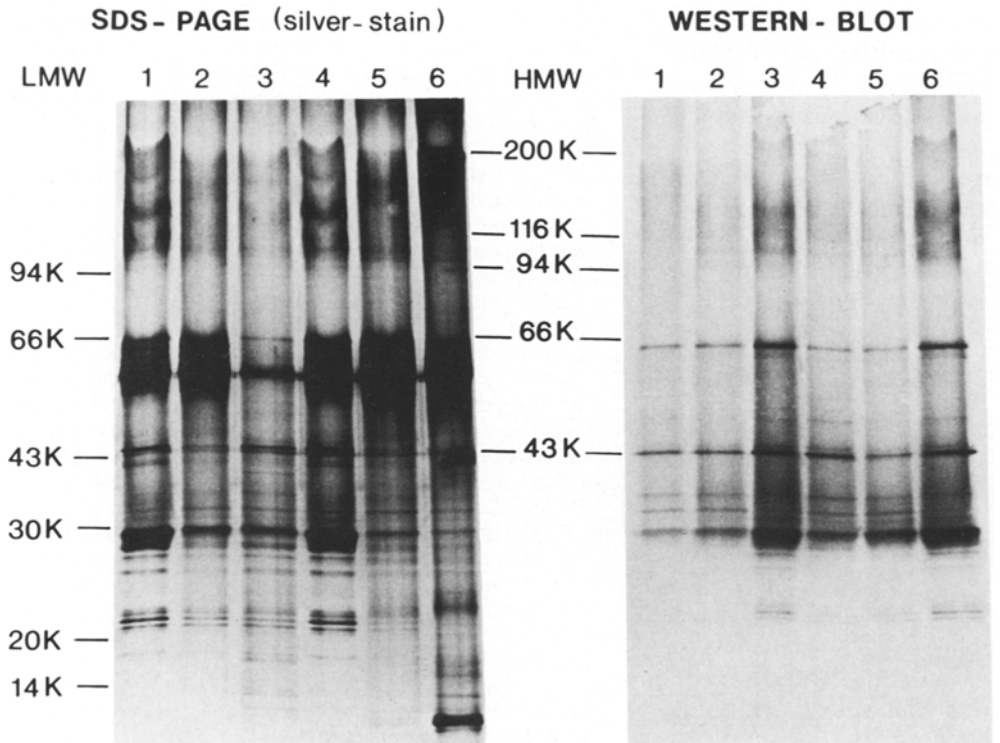

Fig. 2. SDS-PAGE and Western-blot analysis of various ES antigen batches from 6 groups of cultures. *1, 2, 4* and *5:* previously cryopreserved frozen larvae; *3* and *6:* unfrozen control larvae treated with cryoprotectants and submitted to precooling only. *LMW* and *HMW:* positions of low and high molecular size standards. Western blot was performed with half of the same gel run for silver-staining (for details see Table 1 and text)

Discussion

Deep-freezing and storage in liquid nitrogen is an technique increasingly used to preserve parasites in a viable state (see James 1980; Leef et al. 1981; Eckert and Ramp 1985; James 1985). In our experiments it was demonstrated that storage of viable *Toxocara canis* second-stage larvae in liquid nitrogen is possible. During the precooling phase from initially +22° C to +2° C and the subsequent cooling to −80° C, −180° C or −196° C, respectively, the larvae tolerated a range of overall cooling rates between 0.7° C and 62.9° C min^{-1} with higher values in the linear part of the cooling procedure (Table 1). As the larvae can be preserved for 1 week in liquid nitrogen in a viable state it can be assumed that prolonged cryopreservation for months or years and the establishment of a "bank" with such larvae should be also possible.

It has to be recognized, however, that the cryopreservation schedules (involving freezing and thawing) used in our experiments damaged about 40–50% of the larvae, judged from motility rates after thawing. Moreover, previously cryopreserved larvae had shorter survival times in vitro than control and reference larvae (CL and RL) (Fig. 1). The damaging factor is not the cryoprotectant. This can be deduced from the fact that L2 treated with cryoprotectants and remaining unfrozen (CL) had similar high motility rates in vitro than normal reference larvae (RL). It appeared that the concentration of larvae in the medium within the indicated limits of our experiments was not critical for their survival in vitro. Therefore, it has to be concluded that the cooling and/or thawing rates are the decisive factors for the survival rates of *T. canis* larvae; this also applies to other organisms (Ashwood-Smith and Farrant 1980; Leef et al. 1981).

Although the in vitro survival rate was reduced after cryopreservation, a proportion of the larvae survived for 35 weeks in a chemically defined medium and proved to be infective for mice. Our results did not reveal differences in the degree of infection (i.e. proportion of inoculated and infected mice) with previously frozen larvae (FL) in comparison to control (CL) and reference larvae (RL).

It is important to know the antigenic properties of excretory/secretory products released in vitro to determine the value of the cryopreservation

technique for the production of *Toxocara* ES antigen. Our results have shown that ES products from FL, CL and RL larvae exhibit antigenic properties in the ELISA. Further analysis of the ES products by SDS-PAGE separation and silver staining demonstrated quantitative and qualitative differences in the polypeptide patterns of the batches. However, the Western-blot technique revealed that these differences were not related to antigenic antibody-binding polypeptides. It is assumed that these substances may originate from dead larvae and represent degradation products without antigenic properties. On the other hand, the pattern of antigenic polypeptides demonstrated by Western-blot was constant through all experimental groups. This indicates that ES antigens produced by previously frozen larvae are most likely of similar quality to antigens released by unfrozen control larvae. Further studies to evaluate the comparative value of antigens from frozen and unfrozen larvae in the routine ELISA are planned. The general serological qualities of *Toxocara* ES antigens obtained from our culture system have been described (Speiser and Gottstein 1984).

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