

Activation of Mouse Peritoneal Cells to Kill *Listeria monocytogenes* by T-Lymphocyte Products

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An *in vitro* system has been used to demonstrate that glass-adherent mouse peritoneal cells can be activated to kill intracellular *Listeria monocytogenes* by antigen-stimulated T-lymphocytes derived from immunized mice. The soluble products of such stimulated lymphocyte cultures could only be shown to similarly activate peritoneal cells if the antigen used in both the immunization and lymphocyte stimulation was also present on the target intracellular organism.

The macrophage response to intracellular bacterial infection has been studied in animal systems both *in vivo* and *in vitro* (7,8,10), and it has been shown that the cellular immune reaction mediating the killing of bacteria in such systems involves both lymphocytes and macrophages (6-8).

Recently, the thymus-derived lymphocyte has been demonstrated to play an essential role in the expression of cellular immunity to the intracellular bacterium *Listeria monocytogenes* in an *in vivo* mouse system (2), supporting the hypothesis that thymus-derived lymphocytes activate macrophages to kill intracellular bacteria. In an *in vitro* system, spleen cells from guinea pigs chronically infected with *Toxoplasma gondii* when stimulated with this antigen were found to activate normal guinea pig macrophages to kill *L. monocytogenes*. The cells responsible were shown to be thymus derived by abrogation of this activating effect after prior treatment of the spleen cells with antiserum prepared against fetal guinea pig thymus cells (5). Furthermore, supernatant fluid from cultures of *Toxoplasma*-stimulated guinea pig spleen cells, derived from such chronically infected animals, were also able to confer resistance to *L. monocytogenes* on normal macrophages *in vitro* (4).

In mice it has been reported (3) that supernatant fluids from *Listeria*-immune lymphocyte cultures stimulated with *Listeria* antigen *in vitro* activate mouse macrophages to resist the identical target intracellular organism, but in this case significant activation was also produced by the supernatant fluid from antigen-stimulated non-immune cells. In view of the latter observation, it was felt necessary to

examine the activating potential of supernatant fluid from antigen-stimulated mouse lymphocyte cultures in a system in which this antigen was not present on the target intracellular organism within the normal glass-adherent peritoneal cells.

MATERIALS AND METHODS

Animals. Specific-pathogen-free female BALB/c mice (12 weeks old) (Olac Southern, Bicester, Oxon) were used in all experiments.

Immunization. Mice were immunized with two live microorganisms and used for experiments not less than 3 weeks after the final immunizing injection.

(i) **Bacillus Calmette-Guérin (BCG).** A 0.1-ml volume of a freeze-dried preparation of vaccine (Glaxo) was reconstituted in 0.1 ml of saline and injected intraperitoneally into each mouse on three occasions at 2-week intervals.

(ii) ***L. monocytogenes*.** A virulent derivative of strain NCTC 5214, obtained by a modification of continuous passage in mice, was used to immunize mice in the same manner with 10⁸ living cells in 0.2 ml of saline obtained from a 16-h culture in tryptose soya broth (Oxoid, London).

Medium and serum. Medium 199 (Wellcome Research Laboratories, Beckenham, Kent) with bicarbonate buffer was made up from the same batch of powder for all experiments involving peritoneal cell culture. RPMI 1640 medium (GIBCO) was used with bicarbonate buffer for lymphocyte cultures. The same batch of heat-inactivated fetal calf serum from Flow Laboratories, Inc., Rockville, Md., was used in a concentration of 20% (we initially screened it for *Listeria*-agglutinating properties with negative results). Penicillin, at a concentration of 1 µg/ml, was used in all cultures except where stated otherwise. The final enriched culture medium for peritoneal cell cultures was adjusted to pH 7.3 to 7.4.

Peritoneal cell cultures. The peritoneal cavities of normal BALB/c mice, sacrificed by cervical dislocation, were lavaged with 5 ml of medium 199 contain-

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ing 20% fetal calf serum and 5 U of preservative-free heparin per ml at 4 C. The cells obtained were pooled, centrifuged at 800 rpm for 10 min at 4 C, and suspended in medium-serum at a concentration of 2×10^6 cells per ml. A 1-ml volume of this cell suspension was added to each flying glass cover slip in Leighton tubes, and the cells were then allowed to settle for 15 min at 37 C in a moist atmosphere of 5% carbon dioxide in air. The medium was then removed, the cover slip was washed once in buffered Hanks balanced salt solution, and 1 ml of fresh medium-serum was added. After a further 2 h of incubation, the cultures were exhaustively washed with buffered Hanks balanced salt solution to remove nonadherent cells. The cultures were incubated in fresh medium-serum overnight and then used for experiments.

Infection of peritoneal cell cultures. Inocula of the virulent derivative of *L. monocytogenes* NCTC 5214 were prepared from 16-h cultures in tryptose soya broth, centrifuged at $3,000 \times g$ for 20 min, and washed three times in phosphate-buffered saline. Quantitation was achieved by optical density measurements at 650 nm, with confirmation by pour plate dilution assay in tryptose soya agar read at 36 h. The organisms were suspended in medium 199 containing 20% fetal calf serum without antibiotic to achieve a multiplicity of infection of approximately ten organisms to one peritoneal cell when 1 ml had been added to the washed peritoneal cell culture. After incubation at 37 C in a moist atmosphere of 5% carbon dioxide in air for 25 min the medium was removed, and the cultures were vigorously washed in warm phosphate-buffered serum to remove as many extracellular organisms as possible and then re-incubated in medium-serum containing $1 \mu\text{g}$ of penicillin per ml.

Bactericidal assay. Taking time zero as the start of the *Listeria* phagocytic phase (i.e., time of inoculation of peritoneal cell cultures) cover slips were removed at 2-h intervals for 8 h, then at 12, 18, 24, 48, and 72 h. Triplicate cover slips were processed for: (i) direct visual counts of bacteria after methanol fixation, May-Grünwald-Giemsa staining, mounting on microscope slides, and randomization; (ii) quantitative tryptose soya agar pour plate dilution assay read at 36 h, after washing the cultures thoroughly to remove antibiotic, exposing the culture to distilled water for 15 min, and vigorously scraping it with a rubber policeman followed by sonic treatment to ensure complete lysis of peritoneal cells and dispersion of bacteria.

Method of quantitation. (i) Examination of 100 high-power fields, with oil immersion, to count *L. monocytogenes* and peritoneal cells permitted calculation of: percent infection of peritoneal cells, mean bacterial count per infected peritoneal cell, and mean bacterial count per peritoneal cell (infected and noninfected). (ii) A count of the total number of peritoneal cells in 100 high-power fields in infected, as compared with noninfected, cultures permitted calculation of the mean survival of peritoneal cells per field. (iii) The quantitative pour plate dilution assay permitted calculation of the viable intracellular bacterial population which, in the absence of viable extracellular organisms, reflected true intracellular proliferation, survival, or killing.

Lymphocyte activation of peritoneal cell cultures. Mouse single-cell spleen suspensions were obtained by the method described below, but glass-adherent cells were removed from such suspensions and the remaining nonadherent cells were added to the peritoneal cell cultures with or without antigen in a ratio of 20 lymphocytes to 1 peritoneal cell. The cells were cultured together for 18 h prior to the *Listeria* infection phase, and the peritoneal cells were counted again immediately prior to this infection phase to achieve a base line for the method of quantitation of bacterial killing.

Generation of supernatant fluids. The method of Adler et al. (1) was modified for the culture of lymphocytes from murine spleens. Briefly, a single-cell suspension of spleen cells was made by pressing a whole spleen through a sterile stainless-steel mesh into a petri dish containing RPMI 1640 medium. The cell suspension was centrifuged, resuspended in a small volume of RPMI 1640, and counted. A 3-ml volume of cell suspension in RPMI 1640, containing 10% fresh heat-inactivated human serum, $1 \mu\text{g}$ of penicillin per ml, and 3×10^6 cells per ml was cultured in a Falcon plastic tissue culture tube at 37 C in a moist atmosphere of 5% carbon dioxide in air for 60 h. Lymphocytes were stimulated with antigen in vitro. The identical strain derivative of *Listeria* as employed for immunization was used as antigen in a ratio of one heat-killed *Listeria* cell to one lymphocyte. In the case of lymphocyte cultures from BCG-immunized mice, preservative-free purified protein derivative (Central Veterinary Laboratory, Weybridge, Surrey) was used at a concentration of $10 \mu\text{g}/\text{ml}$. After culturing, the lymphocytes were centrifuged and the supernatant fluid was removed, pooled and, in the case of *Listeria*-stimulated cultures, filtered through a $0.45\text{-}\mu\text{m}$ membrane filter (Millipore Corp.) to remove organisms. Supernatant fluids were supplemented with 20% heat-inactivated fetal calf serum and then added to the normal mouse peritoneal cell cultures for 18 h before infection of the latter with live *L. monocytogenes*. At the end of the infection phase, the cultures were again incubated in the supernatant fluids for the duration of the experiment.

Preparation of ATS. Thymus cell suspensions from BALB/c were used as antigen in the preparation of antithymocyte serum (ATS) by the method of Pearson and Osebold (9). Briefly, cells nonadherent to plastic were twice inoculated intravenously into rabbits, in doses of 1.5×10^8 to 2×10^8 , with a 2-week interval between inoculations. Sera were collected 1 week after the last inoculation, filtered through a $0.45\text{-}\mu\text{m}$ membrane filter (Millipore Corp.), heat-inactivated, absorbed with erythrocytes, and stored at -20 C . Twofold serial dilutions of sera were tested for cytotoxic activity against thymocytes and peritoneal cells. The ATS was active against thymocytes with a cytotoxicity ranging from 2^8 to 2^{10} (where the titer was designated as \log_2 of the reciprocal of the greatest dilution of serum that caused greater than 50% of the cells to stain with trypan blue when viewed at $\times 400$ magnification). It was also active against peritoneal cells but at a mean titer six twofold dilutions lower than the mean titer for thymocytes. This cytotoxicity

was completely absorbed out with washed CBA mouse brain cells (Table 1). Normal rabbit serum was not reactive with thymocytes or peritoneal cells.

Spleen cells were treated with ATS or normal rabbit serum for some experiments. For these, ATS and normal rabbit serum were diluted 1:40 with Hanks balanced salt solution, and 0.5 ml was added to each of two tubes containing 10^6 spleen cells in 1 ml of Hanks balanced salt solution. To each tube was added complement and the suspension was agitated at 37 C for 30 min. The treated cells were then washed three times with Hanks balanced salt solution and suspended in medium as used in the experiment to be performed.

RESULTS

Experiments were performed to measure the potential of lymphocytes and lymphocyte culture supernatant fluids to activate normal

TABLE 1. Cytotoxic assay of ATS

ATS	Absorption with washed CBA mouse brain cells			
	Pre-			Post-
	Thymo- cytes	Non- adherent PC* ^a	Adherent PC* ^a	Thymo- cytes
1	8.5 ^b	6.4	5.0	0.0
2	11.0	8.2	4.2	0.2
3	9.4	7.6	4.5	0.0

^a PC*, Peritoneal cells adherent or nonadherent to glass.

^b Titers expressed as \log_2 of the reciprocal of twofold serum dilutions.

mouse peritoneal cells to kill intracellular *L. monocytogenes* in vitro. Table 2 shows the experimental design and the controls provided. Each test and control experiment was duplicated—one series using lymphocytes to attempt activation of the normal peritoneal cells and the other using the supernatant fluids from such cultures.

The numbers of *L. monocytogenes* visible per infected cell increased or decreased in the different experimental groups (Fig. 1). There was proliferation of *Listeria* within peritoneal cells that had been exposed to control lymphocytes or supernatant fluids (as set out in Table 1), including those derived from ATS-pre-treated cultures. However, *Listeria* were reduced in number within peritoneal cells that had been activated by: (1) *Listeria*- and BCG-immune lymphocytes stimulated with relevant antigen (*Listeria* antigen being the more effective of the two); (ii) supernatant fluids from *Listeria*-immune lymphocytes cultured with relevant antigen (but they were less effective than the lymphocytes themselves). In contrast, supernatant fluids from BCG-immune lymphocytes cultured with relevant antigen did not activate peritoneal cells.

The same proliferation or degradation of *Listeria* within peritoneal cells is shown in Fig. 2, expressed as the percentage of visibly infected peritoneal cells during the time course of the experiment, and a similar pattern is observed.

The viable *Listeria* count in peritoneal cell cultures during the experiment is shown in Fig.

TABLE 2. Experimental design

Test or control	Immune status of donor of lymphocyte culture	Antigen used to stimulate lymphocyte culture ^a
Test	<i>Listeria</i> immune BCG immune	<i>Listeria</i> PPD
Nonimmune control	Nonimmune Nonimmune Nonimmune	<i>Listeria</i> PPD Nil
<i>Listeria</i> -immune control	<i>Listeria</i> immune <i>Listeria</i> immune	Nil PPD
BCG-immune control	BCG immune BCG immune	Nil <i>Listeria</i>
Test pretested with ATS	<i>Listeria</i> immune BCG immune	<i>Listeria</i> PPD
Test pretreated with normal rabbit serum	<i>Listeria</i> immune BCG immune	<i>Listeria</i> PPD

^a PPD, Purified protein derivative.

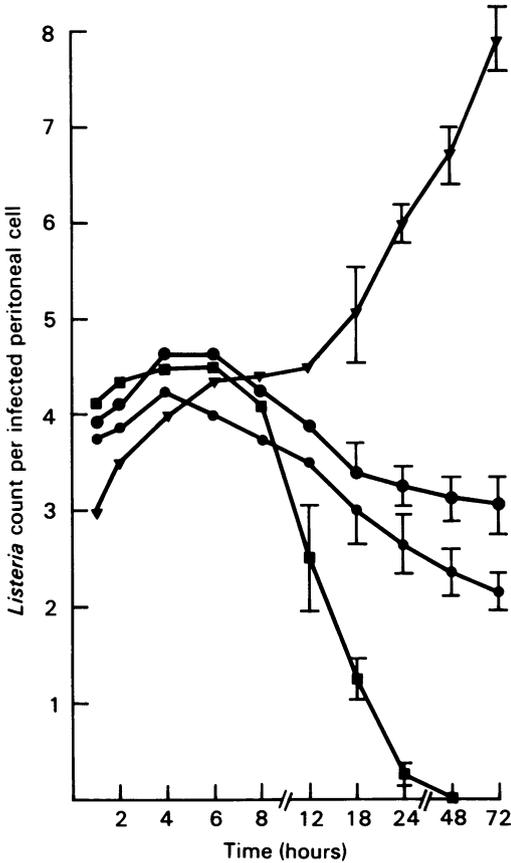


FIG. 1. Microscopically visible intracellular *Listeria* in peritoneal cell cultures activated by sensitized lymphocytes and their products. Symbols: ▼, peritoneal cell cultures treated with control lymphocytes or supernatant fluids derived from them, including those exposed to ATS-treated immune lymphocytes stimulated with relevant antigen (see Table 2); ●, peritoneal cell cultures treated with supernatant fluids from *Listeria*-immune lymphocyte cultures stimulated with *Listeria* antigen; ●, peritoneal cell cultures treated with BCG-immune lymphocytes stimulated with purified protein derivative antigen; ■, peritoneal cell cultures treated with *Listeria*-immune lymphocytes stimulated with *Listeria* antigen. Each point represents the mean \pm 1 standard deviation of triplicate cultures except in the case of ▼, where for clarity each point represents the mean - 1 standard deviation of triplicate cultures from all control situations including pretreatment with ATS.

3, and it is apparent that the apparent proliferation and killing observed microscopically (Fig. 1 and 2) were real.

The survival of peritoneal cells during the experiment is shown in Fig. 4. There was more than 10% cell death in cultures activated by antigen-stimulated *Listeria*-immune lympho-

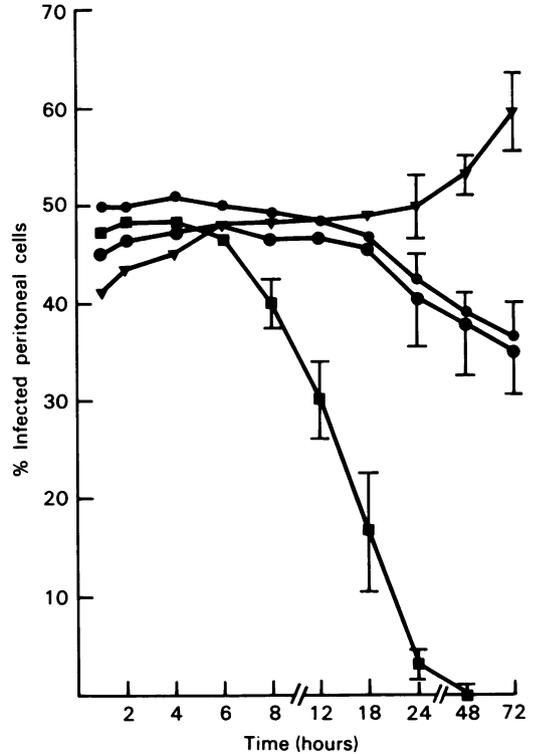


FIG. 2. Microscopically visible intracellular *Listeria* in peritoneal cell cultures activated by sensitized lymphocytes and their products. Symbols: ▼, peritoneal cell cultures treated with control lymphocytes or supernatant fluids derived from them, including those exposed to ATS-treated immune lymphocytes stimulated with relevant antigen (see Table 2); ●, peritoneal cell cultures treated with supernatant fluids from *Listeria*-immune lymphocyte cultures stimulated with *Listeria* antigen; ●, peritoneal cell cultures treated with BCG-immune lymphocytes stimulated with purified protein derivative antigen; ■, peritoneal cell cultures treated with *Listeria*-immune lymphocytes stimulated with *Listeria* antigen. Each point represents the mean \pm 1 standard deviation of triplicate cultures except in the case of ▼, where for clarity each point represents the mean \pm 1 standard deviation of triplicate cultures from all control situations including pretreatment with ATS.

cytes for 18 h prior to the *Listeria* infection, whereas there was approximately 35% cell death in cultures exposed to the supernatant fluid from such cultures for 18 h prior to, and throughout, the experiment.

DISCUSSION

The results of these experiments confirm the findings, both in vivo and in vitro, of several workers (2,5) that the lymphocytes responsible for activating peritoneal cell cultures to kill

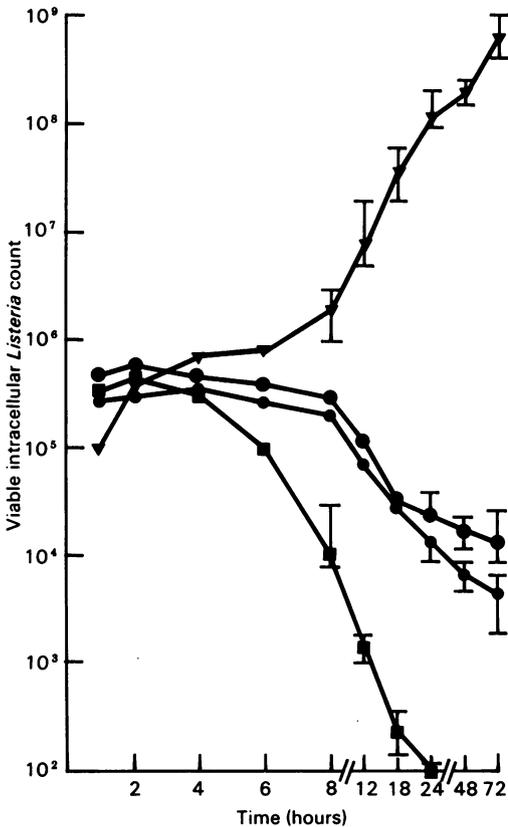


FIG. 3. Viable intracellular *Listeria* in peritoneal cell cultures activated by sensitized lymphocytes and their products. Symbols: ▼, peritoneal cell cultures treated with control lymphocytes or supernatant fluids derived from them, including those exposed to ATS-treated immune lymphocytes stimulated with relevant antigen (see Table 2); ●, peritoneal cell cultures treated with supernatant fluids from *Listeria*-immune lymphocyte cultures stimulated with *Listeria* antigen; ●, peritoneal cell cultures treated with BCG-immune lymphocytes stimulated with purified protein derivative antigen; ■, peritoneal cell cultures treated with *Listeria*-immune lymphocytes stimulated with *Listeria* antigen. Each point represents the mean \pm 1 standard deviation of triplicate cultures, triplicate pour plate dilutional assays being done on each, except in the case of ▼, where for clarity each point represents the mean \pm 1 standard deviation of triplicate cultures from all control situations including pretreatment with ATS.

intracellular *L. monocytogenes* are thymus derived.

The observation that supernatant fluids from antigen-stimulated immune lymphocyte cultures could also activate mouse peritoneal cells in vitro was made in a system in which the immunizing antigen was present on the target organism (6). The experiment carried out in the

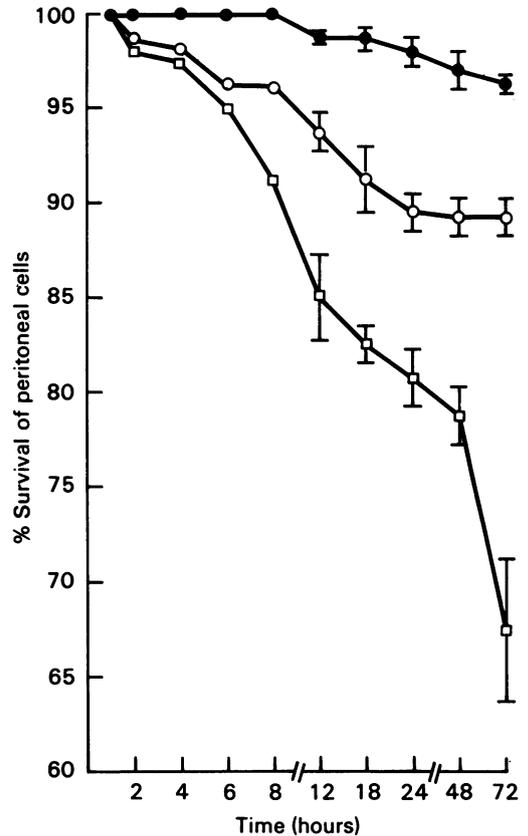


FIG. 4. Cell survival in peritoneal cell cultures activated by sensitized lymphocytes and their products. Symbols: ●, untreated uninfected peritoneal cell culture; ○, peritoneal cell culture treated with *Listeria*-immune lymphocytes in the presence of *Listeria* antigen for 18 h prior to infection with *Listeria*; □, peritoneal cell culture treated throughout the experiment with supernatant fluids from *Listeria*-immune lymphocyte cultures stimulated by *Listeria* antigen. Time zero was the start of the infection phase. Each point represents the mean \pm 1 standard deviation of triplicate cultures.

mouse system described in this paper provides evidence that activation can only be obtained by supernatant fluids when the immunizing antigen is also present on the target organism (in this case *Listeria*). More experiments need to be done with different target organisms to confirm this observation which is puzzling, since antigen-stimulated immune lymphocytes themselves, when allowed to come into contact with peritoneal cells, were successful in activating them despite the immunizing antigen (BCG) differing from those on the target organism (*Listeria*). However, there is no doubt that T-lymphocytes were concerned with the activating effects of supernatant fluids from anti-

gen-stimulated *Listeria*-immune lymphocyte cultures because pretreatment with ATS completely abrogated this activation.

The findings by Jones and Youmans (3) that supernatant fluid from nonimmune lymphocytes stimulated by antigen (*Listeria*) also showed a small, yet significant, degree of activating potential is also difficult to understand since *Listeria* are not known to produce endotoxin. It is possible, however, that this observation was in some way related to the use of high (40%) concentrations of horse serum in the medium, which after pinocytosis by the peritoneal cells might inhibit *Listeria* growth; it is not clear whether *Listeria* were killed in these experiments or merely inhibited from proliferating. In the experiments described in this paper no confirmation of this observation could be found since there was remarkably complete absence of activation in all the control situations explored.

It has been shown in the guinea pig that supernatant fluids from antigen-stimulated immune lymphocyte cultures can activate peritoneal cells to kill *Listeria* when an antigen unrelated to those on the target organism is used for the immunization (8). Therefore, it may be that there is a species difference in the mechanism of activation of peritoneal cells to kill intracellular organisms. It is interesting that with C₆₇B1 mice as donors of lymphocytes and peritoneal cells in the mouse system described in this paper prior treatment of lymphocytes with ATS did not completely abrogate the activation mediated by these cells (P.J. Cole, unpublished data). It is possible that there are not only species differences in the mechanism but also differences within a species. The influence of the H2 locus on this response is under investigation.

An alternative explanation for all of these unexpected findings is that *L. monocytogenes*, used in so many classic experiments, is in some way peculiar, and it is possible that further experiments with other target organisms will

show the mechanism required for killing this organism to be in some way atypical.

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