THE CYTOTOXIC PRINCIPLE OF THE
PHYTOFLAGELLATE PRYMNESIUM PARVUM

ZIPORA DAFNI and M. SHILO
From the Department of Microbiological Chemistry, the Hebrew University-Hadassah Medical School, Jerusalem, Israel

ABSTRACT
The cytotoxic events leading to lysis induced in Ehrlich ascites tumor (E.A.) cells by Prymnesium parvum cell extracts were followed microscopically and measured quantitatively as changes in E.A. cell volume, uptake of trypan blue, and release of macromolecular constituents from the cells. Cell swelling was the most immediate response to P. parvum cytotoxin, while cell death and lysis were later events distinguished by a decline in cell volume, uptake of dye, and appearance of cellular macromolecules free in the incubation medium. The pH and temperature were shown to affect the outcome of the lytic sequence. At either low pH or temperature, cells swelled but did not lyse until the pH or temperature was raised. On the other hand, cells swollen at the higher pH or temperature could be protected from lysis by lowering either the pH or the temperature.

INTRODUCTION
Prymnesium parvum, a phytoflagellate of the Chrysomonadinae, produces toxic principles which cause death of many gill-breathing animals and have often been the cause of mass fish mortalities (Otterström and Steemann-Nielsen, 1939; Reich and Aschner, 1947; Shilo and Aschner, 1953). Extensive studies have been carried out on the mode of action of the ichthyotoxin, and suitable assay systems for its quantitative determination have been developed (Yariv and Hestrin, 1961; Bergmann, Parnas, and Reich, 1963; Ulitzur and Shilo, 1964).

Cell extracts and culture supernatants of P. parvum exhibit a variety of biological activities which include hemolysis of mammalian erythrocytes (Yariv and Hestrin, 1961) and contraction of the isolated guinea pig ileum (Bergmann, Parnas, and Reich, 1964). In addition, various mammalian cells, including human amnion cells, Chang liver cells, and mouse peritoneal leukocytes, undergo morphological changes leading to lysis when brought in contact with P. parvum toxin (Shilo and Rosenberger, 1960). Ehrlich ascites tumor cells are extremely sensitive to the action of the toxin (Dafni, 1964). Since these cells have been widely used in the study of the mechanism of action of various injurious agents, they seemed a suitable model system for studying the action of the P. parvum cytotoxin. The nature of the cytotoxic event induced by the toxin in this model system and the effect of environmental conditions are described in this paper.

MATERIALS AND METHODS
Preparation of P. parvum Cytotoxin
Asexual cultures of Prymnesium parvum Carter (from a strain isolated from brackish water fishponds in Israel (Reich and Kahn, 1954)) were grown on a modified medium of Droop (with the omission of glucose) and under conditions described previously (Shilo and Shilo, 1962). Cells were harvested after 21 days of growth, and the toxic principles extracted in ethanol (Shilo and Rosenberger, 1960). The single extract which served in all the experiments described...
was prepared by extraction of 1 to 2 × 10⁸ *Prymnesium* cells per ml of ethanol. The dry weight of the toxic principles in each ml of ethanol was 4.5 mg. The extract was diluted in Krebs-Ringer phosphate buffer pH 7.4 just before addition to the incubation mixtures as described below.

**Propagation, Collection and Maintenance of Ehrlich Ascites Tumor Cells**

Ehrlich ascites tumor (E.A.) cells of the Landshütz strain (from the Department of Experimental Medicine, Hebrew University-Hadassah Medical School, Jerusalem) were grown intraperitoneally in albino mice of both sexes (12 to 15 g). Weekly i.p. passages were made using 0.2 ml of a freshly harvested peritoneal E.A. cell population. For the experiments, cells were harvested on the 6th day after inoculation. For collecting of the E.A. cells, mice were decapitated, drained of blood, and their peritoneal cavities rinsed repeatedly with physiological saline. The collected cells were washed twice (200 g for 3 min) with saline, and then resuspended and maintained in Krebs-Ringer phosphate buffer at different pH levels (as specified in the text). Cell suspensions containing 2.5 to 4 × 10⁶ cells/ml (optical density= 100 Klett units; Klett Summerson colorimeter, filter 42) served in all the experiments.

**Incubation of E.A. Cells with *P. parvum* Cytotoxin**

Incubation at 37 ° or 27 ° was carried out in 20-ml beakers containing 5 ml of the cell suspension and 5 × 10⁻⁴ ml of the ethanolic toxin extract.

**Assay of Cell Injury**

Three different parameters were used to measure cell injury.

1. **DYE UPTAKE:** Differentiation between normal and injured E.A. cells after different times of exposure to *P. parvum* cytotoxin was based on the uptake of trypan blue dye (Allied Chemical & Dye Corp., New York) by the injured cells. At different time intervals, aliquots of toxin-treated cell suspensions were taken and mixed with trypan blue solution in physiological saline to give a final concentration of 0.5% (w/v) trypan blue in the test mixture. After 1-min exposure to the dye, the total cell number and the per cent of stained cells were enumerated in a hemacytometer. 250 to 400 cells served for each estimation.

2. **SWELLING:** Measurements of the kinetics of swelling of toxin-treated E.A. cells were carried out in an electronic particle counter (Model B, Coulter Electronics, Hialeah, Florida). This instrument has been used widely in the estimation of cell numbers and volume distribution of different cell types (Mattern, Brackett, and Olson, 1956; Brecher, Schneiderman, and Williams, 1956; Brecher et al., 1962) and in following the kinetics of swelling of cell populations (Shilo and Shilo, 1962). Aliquots of the toxin-treated cell suspensions were taken after different incubation times and diluted in Krebs-Ringer phosphate buffer (pH 7.4) to give 25 ml of a cell suspension containing 4 × 10⁴ (±5%) cells/ml. The cell numbers and volume distribution of the E.A. cell population were then measured in the particle counter with an orifice of 100 μ (instrument setting: amplification = 1/2; aperture current setting = 1/2; gain trim = 10). The temperature of the cell suspension during these measurements was kept at 22-25°. The volume of 50% of toxin-treated cells after any given incubation time was expressed in relative units with the initial volume of 50% of the cells = 1.

3. **RELEASE OF INTRACELLULAR MACROMOLECULES:** The appearance of cellular macro-

---

Photomicrographs of typical toxin-treated Ehrlich ascites tumor cells (37°, pH 7.4) at different incubation times. Photographed under oil immersion with phase illumination (Zeptopan, Reichert). × 2000.

**Figure 1** Normal untreated Ehrlich ascites tumor cell.

**Figure 2** E.A. cell after 5-min incubation.

**Figure 3** After 10-min incubation.

**Figure 4** After 15-min incubation.

**Figure 5** After 30-min incubation.

**Figure 6** After 45-min incubation.

**Figures 7 and 8** After 50- to 60-min incubation.

**Figure 9** After 120-min incubation.

462 THE JOURNAL OF CELL BIOLOGY • VOLUME 28, 1966
molecular constituents in the suspension medium after incubation with *P. parvum* cytotoxin was measured spectrophotometrically and by chemical estimation. 5-ml aliquots of cell suspension taken after different incubation times and control cell suspensions were filtered through Whatman No. 1 filter paper, and the filtrate collected for assaying. In cases in which the filtrate remained turbid, it was centrifuged at 3,000 g for 8 min in the cold, and the cleared supernatant was employed in assays. Untreated cell suspensions (5 ml) lysed by addition of 0.05 ml of ethanolic digitonin (USP XVI, Fluka AG, Buchs SG, Switzerland) solution (1% w/v) served as the standard for total lysis in these assays.

**Spectrophotometry:** The absorption spectrum of the cell-free filtrate of toxin-treated cells in the range of 230 to 390 mÅ consistently showed the appearance of a single sharp peak at the 260-mÅ wave length. In our experiments, the optical density at 260 mÅ of the cell-free filtrate was measured in a spectrophotometer (Perkin-Elmer, Model 137 UV), with Krebs-Ringer phosphate buffer solution serving as blank.

**DNA Assay:** DNA in the cell-free filtrate was measured using a modification of Burton's (1956) method. Diphenylamine reagent (1 g of diphenylamine (A.R. Reidel-de-Haen AG, Seelze-Hannover) in 100 ml of glacial acetic acid containing 2.75 ml of concentrated H₂SO₄ without addition of acetylaldehyde) was prepared immediately before use. The reaction mixture was incubated at 30° for 16 to 18 hr, and the optical density determined using a Klett Summerson colorimeter (filter 60).

**RNA Assay:** RNA in the cell-free filtrate was determined using a modification of Drury's (1948) method. The orcinol reagent was prepared by dissolving 1 g of orcinol (British Drug Houses, Ltd., Poole, Dorset, England) in 100 ml of HCl (concentrated) containing 0.1 g of ferric ammonium sulfate·12 H₂O.

**Protein Assay:** Protein in the cell-free filtrate was measured by the method of Lowry et al. (1951).

**RESULTS**

**Kinetics of Swelling and Mortality of Ehrlich Ascites Tumor Cells Induced by *P. parvum* Cytotoxin**

Microscopic observations under phase contrast illumination (Figs. 1 to 9) show that cells exposed to the action of cytotoxin promptly underwent a series of morphological changes. Initially, cytoplasmic swelling and focal pouching with periph-

![Figure 10](image-url)

**Figure 10** Effect of *P. parvum* cytotoxin on E.A. cell volume and trypan blue uptake. Incubation mixtures (see Methods) were kept up to 120 min at pH 7.4, 37°. Estimations of cell volume and the trypan blue uptake are as described in Methods. Triangles, cell volume; circles with tails, trypan blue uptake; solid symbols represent toxin-treated cells; open symbols, untreated control cells.
eral extrusions were observed (Figs. 2 and 3). Later, the pseudopod-like extrusions grew (Fig. 4) and became confluent, with uniform involvement of a large part of the cell circumference (Figs. 5 to 7). The uniformly clear cytoplasmic space became progressively lighter during this stage of swelling (Fig. 5 in contrast to Fig. 6 and Fig. 7), while the nucleus was surrounded by clusters of cytoplasmic granules. Finally, the swollen cells burst, liberating cytoplasmic spheres (Fig. 8) and leaving naked nuclei and cellular debris (Fig. 9).

The kinetics of swelling (measured in the Coulter counter) and mortality (measured by the loss of ability to exclude dyes) of E.A. cells treated with *P. parvum* cytotoxin at pH 7.4, 37 °, are shown in Fig. 10. The increase in cell volume commenced immediately upon addition of the cytotoxin without any lag period, reaching a maximum value followed by a rapid decline. During this stage of cell volume increase, the E.A. cells underwent the morphological sequence shown in Figs. 2 to 6. No trypan blue uptake occurred during the period of swelling, but, with the onset of the decline in cell volume and the morphological appearance of lysis (Figs. 7 to 9), there was a concomitant increase in cell stainability.

Analyses of cell supernatants for intracellular macromolecular constituents after different times of incubation with toxin showed (Fig. 11) that 260-μm absorbing material, protein, and RNA were released from the injured cells but that there was no detectable release of DNA. Similar to dye uptake by injured cells, release of cytoplasmic macromolecules was low during the stage of cellular swelling but increased rapidly after the onset of the lytic phase and the decline in cell volume.
The Effect of Incubation Temperature and pH on the Different Parameters of the P. parvum Cytotoxic Activity

The results described in Figs. 10 and 11 clearly show that the swelling of the E.A. cells preceded all the other manifestations of the cytotoxic action measured. The effect of different environmental conditions on the swelling as compared to their effect on the other cytopathological phenomena in E.A. cells was, therefore, studied. At a pH of 6.4 (Fig. 12) or at a temperature of 27°C (Fig. 13), E.A. cells swelled but no significant leakage of macromolecules (as 260-mu absorbing materials) or uptake of trypan blue occurred. Cells already swollen in the presence of P. parvum cytotoxin under conditions of lowered pH (Fig. 12) or temperature (Fig. 13) were lysed (as expressed by dye uptake, release of intracellular macromolecules, and decline in cell volume) when the pH was returned to 7.4 or the temperature raised to 37°C.

Fig. 14 shows the swollen condition of an E.A. cell treated with P. parvum cytotoxin at pH 6.4, and Fig. 15 shows its subsequent lysis after the pH was raised to 7.4. Similar morphological changes were observed when the incubation temperature of toxin-treated E.A. cells was raised from 27°C to 37°C.

Cells incubated with toxin at pH 7.4 and at 37°C were transferred at various times to conditions of lower pH (Fig. 16) or lower temperature (Fig. 17). The volume decline and leakage of macromolecules inevitable at 37°C and pH 7.4 could be arrested at any stage of the lytic sequence by lowering the pH or the temperature. Even at a period when a large portion of the cells in the suspension were already lysed, the remaining highly swollen cells could be protected from lysis by lowering the pH or temperature of the incubation mixture.

DISCUSSION

Ehrlich ascites tumor cells have been used widely as a model system in studying the mechanisms underlying immune cytolysis (Green and Goldberg, 1960) as well as illuminating the nature of...
the damage inflicted upon cells by various injurious agents, including plant hormones (Schultz and Norman, 1965) and bacterial toxins (Ginsburg, 1959; Eaton, Scala, and Jewell, 1959). The morphological changes occurring in the cells (Easty and Ambrose, 1957; Flax, 1956; Goldberg and Green, 1959; Bickis, Quastel, and Vas, 1959; Ross, 1957; Bitensky, 1963), swelling of injured cells (Flax, 1956; Ellem, 1957; Green and Silverblatt, 1960; Ross, 1957), loss of the ability to exclude dyes (Pappenheimer, 1917; Gorer and O’Gorman, 1956; Eaton, Scala, and Jewell, 1959; Reif and Norris, 1960), and the leakage of cell constituents into the suspension medium (Ellem, 1957, 1958; Colter et al., 1957; Green, Barrow, and Goldberg, 1959; Goldberg and Green, 1960) are common effects of most of these cytotoxic agents. The cytotoxic activity of P. parum extracts on these cells showed many similarities to the effects of the other injury-inducing agents.

Different single criteria usually served, in the above-mentioned studies, as the measure of cell injury by cytotoxic agents, thus limiting the perspective of their interpretations of the mode of action of the cytotoxic agents. In our investigations, four criteria for following the cytotoxic action (dye exclusion, release of intracellular macromolecules, change in volume of the injured cells, and microscopically observed morphological changes) were compared. Different stages in the lytic sequence seem to be separable and are affected variously by different environmental conditions. Only by the simultaneous use of different parameters was it possible to assess the relationship of the stages to one another.

The detailed analysis of immune cytolysis of E.A. cells by Green and Goldberg (1960) has led these investigators to propose a mechanism in which primary irreversible damage is inflicted upon the cells by antibody in the presence of complement. They assumed the formation of functional “holes” in the cell membrane large enough to permit rapid exchange of inorganic cations and small molecules, but not of macromolecules. With disturbance in the K⁺ and Na⁺ equilibrium, the resulting osmotic imbalance
causes swelling of the cells and stretching of the damaged membrane, at which stage macromolecules leak out.

With *P. parvum* cytotoxin at pH 7.4 and at a temperature of 37°, the morphological sequence of lysis is similar to that observed in immune cytolysis. However, when incubation with *P. parvum* cytotoxin was carried out at pH 6.4 or at a temperature of 27°, the cells swelled considerably without any leakage of intracellular macromolecules. It thus appears that, in this case, even the highly stretched membrane did not become permeable to macromolecules. Moreover, these swollen cells did not lose their ability to exclude dye (trypan blue) and did not lyse. No irreversible damage seems to be inflicted upon the toxin-treated cells, even when markedly swollen, until a period very close to lysis. This is borne out by the fact that even when part of the toxin-treated cell population already is lysed, the highly swollen cells remaining intact can be protected from the inevitable lysis by changing the pH from 7.4 to 6.4 or by lowering the temperature from 37° to 27° (see Figs. 16 and 17). Thus, while in immune cytolysis the antibody and complement-dependent damage is irreversible and all later stages of the lytic sequence are predetermined, the lytic activity of *P. parvum* cytotoxin can easily be dis-
The earliest recognizable effect of *P. parvum* ichthyotoxin on the intact fish is the loss of the selective permeability of the gill tissue, allowing entrance of trypan blue and labeled I\(^{125}\) and macromolecules such as radioiodinated human serum albumin I\(^{131}\) into the gill tissue (Ulitzur and Shilo, 1966). This resemblance to the cytoxic effect in Ehrlich ascites tumor cells indicates that the principles involved may be closely related and that an elucidation of the mode of cytotoxic action could shed light on the mechanism of action of the toxin upon the intact fish.

The assistance of Mrs. B. Warshavsky in preparation of the manuscript is gratefully acknowledged.

Received for publication 11 October 1965.
FIGURE 17 Interruption of lytic sequence in incubation mixtures of E.A. cells by lowering of temperature. Incubation mixtures were initially incubated at 37°C, pH 7.4. Arrows (↓) indicate time of change of temperature to 27°C. Measurements of cell volume and OD at 260 m/μ given as in Fig. 11. Triangles, cell volume; circles, OD at 260 m/μ; hexagons, untreated control; solid symbols represent incubation at 37°C; open symbols represent incubation at 27°C.

REFERENCES

15. FLAX, M. H., Cancer Research, 1956, 16, 774.