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# Effect of Particulate Materials on Population Growth of the Free-Living Nematode *Caenorhabditis briggsae*[1,2] (40420)

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Recent investigations have shown that ingestion of particulate material has promoted population growth of amoebas (1) and ciliates (2). The authors have suggested that particulate material in nutrient media induces formation of food vacuoles and thus permits faster uptake of nutrients. Previous work with an axenic culture of free-living nematodes demonstrated that particulate protein (3–5) stimulates earlier maturation of *Caenorhabditis briggsae*. However, when polystyrene latex beads (250 nm) were added to the chemically defined medium in place of the particulate protein, no stimulation was observed (4). Later, Vanfleteren (6–8) observed larger population increases with acid-precipitated heme and also proposed the idea that acid-precipitated heme (9) can replace the particulate protein.

This study of the effects of particulate material on population growth of *C. briggsae* was prompted by the observation that population growth was greater when the organism was cultured in a turbid medium prepared by the addition of a separately heat-sterilized salt mixture which had become turbid on autoclaving, than when they were cultured in a clear medium prepared by the addition of a separately sterile-filtered salt mixture which remained clear after Millipore filtering. This led to the hypothesis that the particles themselves could stimulate population growth and that the particulate material does not necessarily have to be a protein moiety. Experiments were conducted to examine the effect of various inert particles on population growth, and to determine whether there is any particular size or size range that is most stimulatory. Finally, we wished to see if various particulates could replace casamino acids, which are commonly used in our laboratory as a source of proteinaceous growth factor(s) for the test organism.

*Materials and methods.* The chemically defined medium, *Caenorhabditis briggsae* Maintenance Medium (CbMM) (3), obtained from Grand Island Biological Company (Grand Island, NY) was used as the basal medium for the cultivation of the free-living nematode. The basal medium was supplemented with 50 μg/ml Millipore-filtered cytochrome c (Sigma Chemical Company, St. Louis, MO) and 50 μg/ml of β-sitosterol (Sigma Chemical Company, St. Louis, MO) emulsified in 0.13% Tween 80. The supplements were sterilized by Millipore filtration. In addition, some of the assay media were supplemented as indicated with 2.5–5.0 mg/ml of casamino acids (acid-hydrolyzed casein; Difco Laboratories, Detroit, MI) which remained a clear solution after sterilized by autoclaving.

The various particles used were Celite (diatomaceous earth from J. T. Baker Chemical Co., Philipsburg, NJ); glass powder (Corning Glass Works, Corning, NY); tin oxide (lapidary grade), acid-washed; diamond powder of various sizes (Diafin, La Jolla, CA); Microcrystalline Cellulose (Bio-Rad Lab., Richmond, CA); Solka Flok (Brown Company, Burlingame, New Hampshire); Cellophane Spangles (Vixkotyte Corp., Plainfield, NJ).

In each experiment, particulate stock solutions were made at the highest concentrations to be used. Serial dilutions were made for the lower concentrations. These stock solutions were then sterilized by autoclaving at 121° at 15 psi for 15 min and 0.5 ml of the sterilized particulate suspension was added to each culture tube containing the experimental medium.

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Diamond powder (50 mesh) and Cellophane Spangles were weighed directly into culture tubes, as they were too dense or too light to be properly dispersed for serial dilutions. Deionized water was then added to make up to 0.5 ml in volume. Culture tubes containing particulate suspensions were then sterilized by autoclaving at 121° at 15 psi for 15 min.

The nematode culture was maintained in 4% soypeptone, 1% yeast extract, and 10% heated liver extract (SP-YE-HLE) stock medium (10). It was washed two times in deionized water before being inoculated into a culture tube containing basal medium, 50 $\mu$g/ml of $\beta$-sitosterol and 50 $\mu$g/ml of cytochrome *c*. This is the preliminary culture.

The modified mass culture assay of Rothstein *et al.* (10) was employed. The free-living *C. briggsae* were used under axenic conditions in all assays; 500–700 nematodes/ml from the preliminary culture were inoculated into duplicate 18 × 150 mm culture tubes containing 5.0 ml of medium. The culture tubes were incubated at 20° on a tissue culture-type rotator at 1 rpm. The population was estimated after approximately 20–29 days by counting appropriate dilutions on a counting slide. Two counts were made for each tube as a cross check.

*Results.* Table I shows that population growth of *C. briggsae* doubled when the salt mixture was autoclaved before it was added to the salt-depleted CbMM as compared to the same salt mixture when Millipore-filtered. The autoclaved salt mixture contained a slight precipitate. This result suggested that particles might stimulate population growth in *C. briggsae*.

TABLE I. EFFECT OF DIFFERENT TREATMENTS OF SALT MIXTURE ON POPULATION GROWTH OF *C. briggsae*.

| Basal medium[a] + | Population (Number of worms × $10^3$/ml assay medium) 15 days | 28 days |
|---|---|---|
| Salt mixture[b] (Millipore-filtered) | 9 | 29 |
| Salt mixture[b] (Autoclaved) | 24 | 65 |

[a] Basal medium consisted of salt-depleted CbMM, supplemented with 50 $\mu$g/ml cytochrome c, 50 $\mu$g/ml $\beta$-sitosterol emulsified in 0.13% Tween 80, and 5.0 mg/ml casamino acids.

[b] Salt mixture contained (mg/ml): $KH_2PO_4$, 12.3; $K_3 \cdot$citrate$\cdot H_2O$, 4.86; $CaCl_2 \cdot 2H_2O$, 2.21; $CuCl_2 \cdot 2H_2O$, 0.065; $MgCl_2 \cdot 6H_2O$, 0.609.

We then used various particulate materials such as Celite (diatomaceous earth) and glass powder, which are chemically inert. A lapidary grade of acid-washed tin oxide was also used. Table II shows the results of various concentrations of these particles on population growth. As the concentration increased, population growth also increased. At a concentration of 2000 ppm, when the medium was supplemented with 5.0 mg/ml of casamino acids, the population increase was 4.4-fold for Celite, 3.9-fold for glass powder, and 3.7-fold for tin oxide; when casamino acids were omitted from the medium, the increase was 6.2-fold for Celite, 2.6-fold for glass powder, and 3.7-fold for tin oxide. The presence of casamino acids always resulted in increased population growth, irrespective of the concentrations of particle added to the me-

TABLE II. EFFECTS OF VARIOUS CONCENTRATIONS OF CELITE, GLASS POWDER, AND TIN OXIDE ON POPULATION GROWTH OF *C. briggsae* WITH AND WITHOUT CASAMINO ACIDS (CAA).

| Basal medium[a] + Particles | Population[b] (Number of worms × $10^3$/ml of assay medium) | | | | | |
|---|---|---|---|---|---|---|
| | Celite | | Glass powder | | Tin oxide | |
| | −CAA (21 days) | +CAA[c] | −CAA (29 days) | +CAA[c] | −CAA (25 days) | +CAA[c] |
| (ppm) | | | | | | |
| 0 | 7 | 19 | 7 | 15 | 9 | 25 |
| 16 | 14 | 24 | 7 | 17 | 14 | 31 |
| 80 | 17 | 35[d] | 8 | 18 | 20 | 64 |
| 400 | 19 | 38 | 7[d] | 29 | 17 | 81 |
| 2000 | 42 | 83 | 19 | 59 | 34 | 93 |

[a] The basal medium consisted of CbMM, 50 $\mu$g/ml cytochrome c, 50 $\mu$g/ml $\beta$-sitosterol emulsified in 0.13% Tween 80.

[b] Initial population: ~500 nematodes/ml.

[c] Casamino acids: 5.0 mg/ml.

[d] Difference between duplicate counts and average is greater than 10%.

dium, indicating that the particulate effect is in addition to the proteinaceous growth factor provided by casamino acids.

Table III shows the results of various sizes and concentrations of diamond powder on population growth of *C. briggsae*. For the 0.25 μm and 3 μm particles, population growth increased slightly as the concentration of particles increased and reached a maximum at 80 ppm. Higher concentrations were inhibitory. Observation under a microscope revealed the presence of black diamond particles in the intestines of most of the larger nematodes when 3 μm particles were fed; particles were seen in the intestines of much smaller nematodes when 0.25 μm particles were fed. The ingestion of such dense and hard particulate matter could possibly have adverse effects on the worms.

For the four other sizes (15 μm, 45 μm, 125 μm [150-mesh], 350 μm [50-mesh, 250 μm–500 μm]), population growth increased as the concentration of particles increased, and reached a maximum at the highest concentration. At 10,000 ppm, in the presence of casamino acids, population increases ranged from 2.3- to 2.8-fold. When casamino acids were omitted from the medium, increases ranged from 2.6- to 3.1-fold. The effect of particles at an intermediate concentration (400 ppm) on population growth was also similar in both media. Particles could not be seen in the guts of these nematodes. Such powders were thus probably too large for the stomata of the worms. For a large worm (~40 μm wide and 700 μm long), the estimated diameter of its stomata was between 2 and 3 μm.

Cellophane Spangles and Solka Flok are bulking agents commonly added to the diets of experimental vertebrates. Each of them and Microcrystalline Cellulose were tested to see if they had any effect on the nematodes (Table IV). Microcrystalline Cellulose was the most stimulating of the three; at a concentration of 10,000 ppm, it gave a 4.5-fold increase with casamino acids and an 8.1-fold increase without casamino acids. Solka Flok appeared to be slightly more effective than Cellophane Spangles in promoting population growth, but the high baselines of Cellophane Spangles' controls (with and without casamino acids) could very well mask the stimulation.

*Discussion.* The experiments described demonstrate the effectiveness of inert particulate materials in promoting population growth of *C. briggsae*. Increases in population growth produced when nematodes were cultured in a medium containing particulate material suggest that limiting factors besides inadequate nutrition are operating in the tube culture to prevent maximal population growth of this organism.

The fact that increases in population growth occurred when biologically inert particles (such as Celite, glass powder, and diamond powder) were added to the medium, and that the large-sized particles (diamond powder) were unable to enter the guts of the nematodes via the small stomata but still stimulate population growth, suggests that particulate matter outside the organism could

TABLE III. EFFECT OF VARIOUS CONCENTRATIONS AND SIZES OF DIAMOND POWDER ON THE POPULATION GROWTH OF *C. briggsae* WITH AND WITHOUT CASAMINO ACIDS (CAA).

| Basal medium[a] + Diamond powder (ppm) | Population[b] on 20th day (Number of worms × $10^3$/ml of assay medium) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.25 μm | | 3 μm | | 15 μm | | 45 μm | | 125 μm | | 350 μm | |
| | −CAA | +CAA[c] | −CAA | +CAA[c] | −CAA | +CAA[c] | −CAA | +CAA[c] | −CAA | +CAA[c] | −CAA | +CAA[c] |
| 0 | 8 | 18 | 8 | 18 | 8 | 18 | 8 | 18 | 8 | 18 | 8 | 18 |
| 16 | 12 | 23 | 9 | 22 | 10 | 25 | —[d] | — | — | — | — | — |
| 80 | 14 | 23 | 15 | 27 | 11 | 29 | 11 | 23 | 10 | 27 | — | — |
| 400 | 12 | 20 | 13 | 26 | 11 | 34 | 15 | 29 | 14 | 27 | 12 | 32 |
| 2000 | 12[e] | 18 | 8 | 15 | 17 | 39 | 17 | 29 | 15 | 36 | 18 | 38 |
| 10,000 | 7 | 13 | 6 | 13 | 25 | 50 | 24 | 42 | 21 | 43 | 23 | 44 |
| 20,000 | — | — | — | — | — | — | — | — | 26 | 45 | 30 | 50 |

[a] The basal medium consisted of CbMM, 50 μg/ml cytochrome c, and 50 μg/ml β-sitosterol, emulsified in 0.13% Tween 80.

[c] Casamino acids: 5.0 mg/ml.

[d] Not tested.

[e] Difference between average and duplicate counts is greater than 10%.

TABLE IV. EFFECT OF VARIOUS CONCENTRATIONS OF THREE CELLULOSE PRODUCTS ON POPULATION GROWTH OF *C. briggsae* WITH AND WITHOUT CASAMINO ACIDS (CAA).

| Basal medium[a] + Particles | Population[b] (Number of worms × $10^3$/ml of assay medium) | | | | | |
|---|---|---|---|---|---|---|
| | Microcrystalline cellulose | | Solka flok | | Cellophane spangles | |
| | −CAA | +CAA[c] | −CAA | +CAA[c] | −CAA | +CAA[c] |
| (ppm) | (21 days) | | (19 days) | | (25 days) | |
| 0 | 15 | 35 | 7 | 32 | 17 | 69 |
| 16 | 17 | 42 | 9 | 34 | —[d] | — |
| 80 | 18 | 42 | 9 | 46 | — | — |
| 400 | 26 | 48 | 13 | 63 | — | — |
| 2000 | 36 | 80 | 26 | 70 | 44 | 104 |
| 10,000 | 121 | 156 | 23 | 77 | 43 | 127 |

[a] The basal medium consisted of CbMM, 50 μg/ml cytochrome c, 50 μg/ml β-sitosterol emulsified in 0.13% Tween 80.

[b] Initial population: ~500 nematodes/ml for Microcrystalline Cellulose; ~700 nematodes/ml for Solka Flok; and ~600 nematodes/ml for Cellophane Spangles.

[c] Casamino acids: 2.5 mg/ml.

[d] Not tested.

act as a mechanical vector.

The population growth stimulation produced by four different sizes of diamond particles, ranging from 15 μm to 350 μm (~250 μm–500 μm) was about the same. This suggests that, within that range, size does not make much difference in stimulating population growth provided that the particles are not so small and hard (as the 0.25 and 3 μm sizes of diamond powder) that ingestion of a large quantity can be harmful.

The results of the experiments also show that a further increase in population growth occurred when casamino acids were added to the medium, irrespective of the type or concentration of particle being added. This indicates that particulate material cannot totally replace casamino acids.

From the observation reported above, it is possible to account for the increased population growth produced by particulate protein preparations and the lack of success of soluble proteins (4); these observations also explain the finding that particulate heme was found to be more effective than soluble heme for increasing population growth of *C. briggsae* (9). Increases in population growth attributable to particulate protein or precipitated heme are probably linked to the physical presence of particles in the medium and not necessarily due specifically to the precipitated forms of protein or heme.

We cannot dismiss the suggestion that particulate protein or particulate heme can stimulate population growth through phagocytosis (6) nor can we rule out the possibility that precipitates may adsorb nutrient molecules, thus making food available to the nematodes in a more concentrated form (4). However, the fact that biologically inert particles such as diamond powder, a poor adsorbent, could stimulate increases in population growth indicates that stimulation is not due to nutrient adsorption. Moreover, the fact that large-sized particles (diamond powder) although unable to enter the gut, therefore presumably unable to stimulate phagocytosis, are still able to stimulate population growth, suggested that particulate matter may simply act as a mechanical vector in the medium. Although the mechanism by which inert particles promote increase in population growth of soil-dwelling nematodes is not understood, it is possible that the presence of particles partially mimics the natural habitat of the nematode. The presence of particles might somehow modify the feeding behavior of the nematode.

*Summary.* Several particulate compounds such as Celite, glass powder, tin oxide, diamond powder, and three different cellulose products, Microcrystalline Cellulose, Solka Flok, and Cellophane Spangles, stimulated population growth in *C. briggsae.* Since inert diamond particles form a poor adsorbent, and since large particles did not enter the gut of the nematode, our results suggest that stimulation is not the result of nutrient adsorption or phagocytosis, but rather due to the physical presence of particles.

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