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Authors: Tokusumi, Yumiko, Nishi, Nozomu, and Takagi, Yoshiomi

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A Substance Secreted from *Tetrahymena* and Mammalian Sera Act as Mitogens on *Paramecium tetraurelia*

Yumiko Tokusumi[1], Nozomu Nishi[2] and Yoshiomi Takagi[1]

[1]*Department of Biology, Nara Women's University, Nara 630 and* [2]*Department of Endocrinology, Kagawa Medical School, Kagawa 761-07, Japan*

ABSTRACT—We previously isolated and purified Paramecium growth factor (ParGF) from a cell-free fluid of an early stationary mass culture of *Paramecium tetraurelia* (Tanabe *et al.*, 1990). The mitogenic activity of the purified ParGF and of the crude sample (ca. a 100-fold concentrate obtained by ultrafiltration of cell-free fluid) has been assessed based on restoration of the fission rate of the *jumyo* mutant of *P. tetraurelia* in daily reisolation cultures. With this assay system, we found that crude samples of *Tetrahymena pyriformis* and *T. thermophila* showed mitogenic activity. This suggests that *Tetrahymena* cells secrete a mitogenic factor(s) like ParGF. To some extent, fetal bovine serum (FBS) and calf serum (CS) also acted as mitogens on the *jumyo* mutant. Of nine mammalian growth factors assayed for their mitogenic effects on the *jumyo* mutant, epidermal growth factor (EGF) and transforming growth factor α (TGFα) were slightly and occasionally effective. These results support the idea of actual use of similar kind of growth factors to control cell divisions from protozoa to mammals.

INTRODUCTION

We isolated a *jumyo* mutant of *Paramecium tetraurelia* that has a short clonal life-span, about one-tenth that of the wild type stock (Takagi *et al.*, 1987b, 1989). This mutant divides slowly in daily reisolation cultures but as rapidly as wild type cells in mass cultures (Takagi *et al.*, 1989). This characteristic provided a clue that led to the finding of Paramecium growth factor (ParGF) and a method for its biological assay (Tanabe *et al.*, 1990). With this assay system (i.e., restoration of the fission rate of the *jumyo* mutant in daily reisolation cultures), ParGF or a functional homologue was found to be secreted from stocks of *P. tetraurelia* other than the *jumyo* mutant and from stocks of other *Paramecium* species (Takagi *et al.*, 1993). Although ParGF is the only growth factor identified so far, several other species of protozoa also may release a substance like this growth factor because unknown compounds produced during their culture promote cell proliferation of their own and other species (Christensen and Rasmussen, 1992; Lilly and Stillwell, 1965).

Use of our assay system to test for the secretion of growth factors from two species of *Tetrahymena* gave positive results. This led us to test mammal sera and growth factors for mitogenic activity on the *jumyo* mutant. Various serum-free culture mediums have been developed, first for immortal cell lines and later for mortal human diploid cells (Barnes and Sato, 1980; Phillips and Cristofalo, 1980; Walthall and Ham, 1980; Yamane *et al.*, 1981). Most cell cultures, however, require the addition of serum to synthetic medium for cell proliferation and maintenance. We first tested fetal bovine serum (FBS) and calf serum (CS) and found that these mammalian sera had some effect on the fission of the *jumyo* mutant. As there is some evidence that certain protozoa cells have a site to bind a mammalian growth factor (Andersen *et al.*, 1984; Christensen, 1993; Csaba and Kovács, 1990; Hide *et al.*, 1989), we assessed nine mammalian growth factors and found that EGF and TGFα acted as mitogens on the *jumyo* mutant although only slightly and occasionally.

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MATERIALS AND METHODS

Cells

The *jumyo* mutant (d4-SL4) of *Paramecium tetraurelia* (Takagi *et al.*, 1987b, 1989) was used. *Tetrahymena pyriformis* (wild type) and *Tetrahymena thermophila* (strain B1868III) were provided by Drs. T. Takemasa and Y. Watanabe, Tsukuba University.

Cultures

Tetrahymena cells were cultured in PPYD medium (Watanabe, 1963) or in the medium for *Paramecium* (Takagi *et al.*, 1987a). PPYD medium contained 1% proteose peptone (DIFCO), 0.5% yeast extract (DIFCO), and 0.87% dextrose (NAKARAI). The medium for *Paramecium* was 1% phosphate-buffered medium of 5 g/l Wheat Grass Powder (Pines International, Inc., USA) inoculated with a non-pathogenic strain of *Klebsiella pneumoniae* 2 days before use (here referred to as bacterized WGP medium).

For the mass culture, 1,000 ml flasks, each containing 400 ml of culture medium, were used. After the introduction of 1–10 cells/ml, the flasks were incubated at 26°C. The time at which the culture first became clear, indicative of the exhaustion of bacteria, was considered to be the early stationary phase.

For the daily reisolation culture, a single cell of the *jumyo* mutant was placed in 400 μl of culture medium in the well of a 3-well depression slide, housed in a moist chamber, and incubated at 26°C for a day. One of the cells produced was transferred daily to a new culture medium and the number of fissions was calculated from $\log_2 N$, where N is the number of cells produced. For the

microcapillary culture, a single cell was grown in a microcapillary (0.7 mm inside diameter, 126 mm long) containing 10 μl of culture medium that was kept in a moist chamber.

Preparation of the crude samples

Cells of *P. tetraurelia* in the early stationary phase culture were removed by filtration through two folds of two sheets of filter paper (TOYO). The resulting cell-free fluid was concentrated about 100-fold by ultrafiltration using a Diaflow cell (Amicon) and a Diaflow membrane YM2 (Amicon) with a nominal cut-off molecular weight of 1,000. This crude sample was designated "PBW" (mixture of substances derived from *Paramecium*, bacteria, and Wheat Grass Powder). In the same way, 100-fold concentrates were prepared from Wheat Grass Powder (WGP) medium that had not been inoculated with *Paramecium* or bacteria, and from bacterized WGP medium that had not been inoculated with *Paramecium* cells. The former was designated "W", the latter "BW".

Crude samples of *T. pyriformis* and *T. thermophila* were prepared from cell-free fluid in the same way. When *Tetrahymena* cells were cultured in PPYD medium, the crude PPYD used was a 100-fold concentrate that had not been inoculated with *Tetrahymena*.

All the crude samples obtained were stored in a freezer (–25°C) and thawed in a refrigerator before use.

Assay for mitogenic activity

The assessment of mitogenic activity was based on the promotion of cell proliferation of the *jumyo* mutant in daily reisolation and microcapillary cultures. The microcapillary assay was introduced first in this study; this differs from the former one in that only a small amount of the sample for assay was needed and in that the sample for assay was added not daily but one time on the first day.

For the daily reisolation cultures, cells of the *jumyo* mutant were placed in 9 wells of depression slides, each well containing 400 μl of culture medium, then cultured as daily reisolation lines for 5 days. A set of 9 lines was regarded as one batch, each set comprising the control and a single experiment. In each experiment, the substance whose effect on the growth of *jumyo* cells was to be examined was added daily. When the substance was the crude sample, the final concentration was adjusted to 1-fold that of the original; e. g., when the sample was a 100-fold concentrate, 4 μl was added to 400 μl of the culture medium. In some experiments, a 2- or 0.5-fold concentration of the original was used. The average fission rates of the control and experiment were compared 5 times for the daily cumulative number of cell divisions in the 9 lines. Lines discontinued due to death were included in the data up to the day of death. The substance added was considered effective as a mitogen when the differences in the average cumulative fissions on the 3rd, 4th, and 5th days were all statistically significant (U-test, $P<0.05$) because the mitogenic effect tended to appear first on the 2nd day.

For the microcapillary cultures, cells were cultured for 3 days in 10 microcapillaries each containing 10 μl of culture medium. A set of 10 microcapillaries was regarded as one batch, each set comprising the control and a single experiment. In each experiment, the substance whose effect on the growth of *jumyo* cells was to be examined was added on the 1st day. It was considered effective as a mitogen when the average number of cells in an experiment was significantly higher on the 3rd day (t-test, $P<0.05$).

Sera and growth factors

Fetal bovine serum (FBS) was obtained from ICN; calf serum (CS) from Gibco; bovine serum albumin (BSA) and insulin from Sigma Chemical Co.; mouse epidermal growth factor (mEGF) from Toyobo Co., Ltd.; human transforming growth factor α (TGFα) from Intergen Co.; human insulin-like growth factor I (IGFI) and II (IGFII) from Mallinckrodt Specialty Chemicals Co., Inc.; human platelet-

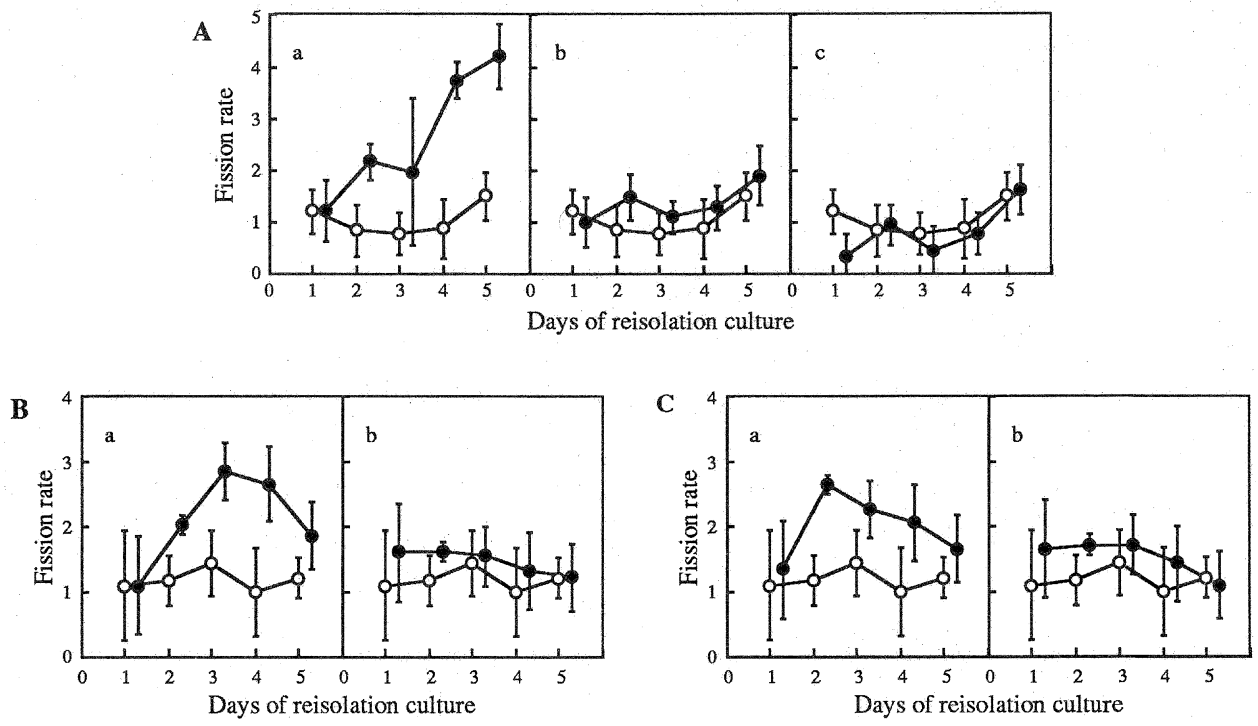

Fig. 1. Mitogenic activities of crude samples of *P. tetraurelia* (A), *T. pyriformis* (B), and *T. thermophila* (C) all grown in bacterized WGP medium. (A); PBW (a), BW (b), W (c). Controls (open circles) are for 9 daily reisolation lines of *jumyo* cells, and experiments (closed circles) for 9 daily reisolation lines supplemented daily with a crude sample 1-fold (Aabc, Ba, Ca) or 0.1-fold (Bb, Cb) the original. Daily reisolation cultures begun on day 0 were maintained for 5 days. The fission rate is given in $\log_2 N$, N being the number of cells on the day following reisolation. Bars indicate standard deviations.

derived growth factor (PDGF) from Upstate Biotechnology Inc.; acidic fibroblast growth factor (aFGF) from Wako Pure Chemical Industries Ltd.; and basic fibroblast growth factor (bFGF) from R&D Systems. Rat EGF (rEGF) was purified from the submaxillary gland of male Sprague-Dawley rats as described previously (Nishi *et al.*, 1988). mEGF and rEGF were dissolved in double distilled water; TGFα was dissolved in 10 mM acetic acid; insulin in 30 mM HEPES-HCl (pH 3.0) containing 100 mM NaCl; IGFII in 100 mM acetic acid; IGFI and PDGF were dissolved in 10 mM acetic acid containing 1 mg/ml BSA; and aFGF and bFGF in 10 mM Tris-HCl (pH 7.5) containing 10 mg/ml BSA and 0.03% CHAPS. When the growth factor solution was added to the culture medium, the pH was adjusted to 6.5–7.0.

Dialysis of FBS

For dialysis, we used molecularporous membrane tubing with a nominal cut-off molecular weight of 3,500 (Spectra/Por). This tubing containing 2 ml FBS was placed in 25 ml of double distilled water at 4°C overnight, then the water was exchanged for 250 ml phosphate-buffered saline (PBS), and the tubing kept for 8 hr at 4°C.

RESULTS

Mitogenic activity of the substance secreted from Tetrahymena

Before testing the effect of the substance secreted from *Tetrahymena*, we checked our assay system. As shown in Figure 1A, PBW effectively restored the fission rate of the *jumyo* mutant in the daily reisolation cultures (a), whereas BW (b) and W (c) were ineffective, evidence that the assay system functioned well.

We then examined the mitogenic activity of the crude sample prepared from *Tetrahymena* grown in bacterized WGP medium (Fig. 1B, C). The *jumyo* mutant supplemented with the crude sample prepared from *T. pyriformis* increased their fission rates (Ba). Diluted to one-tenth, the crude sample was still effective, but to a lesser degree (Bb). This also was true for the crude sample prepared from *T. thermophila* (C). These results suggest that ParGF, or a functional homologue, is secreted from *Tetrahymena*.

We next examined the mitogenic activity of the crude sample prepared from *Tetrahymena* grown in PPYD medium (Fig. 2). Because the saturation density in PPYD medium was higher than in bacterized WGP medium, crude samples 0.5-fold the original were used. Restoration of the fission rate of *jumyo* cells occurred when the crude sample prepared from *T. pyriformis* was added daily (A), as was true for the crude sample prepared from *T. thermophila* (B). The crude sample prepared from PPYD medium, however, was ineffective (C).

Mammalian serum acts as a mitogen on the jumyo mutant

We first examined the effect of FBS on *jumyo* cells in

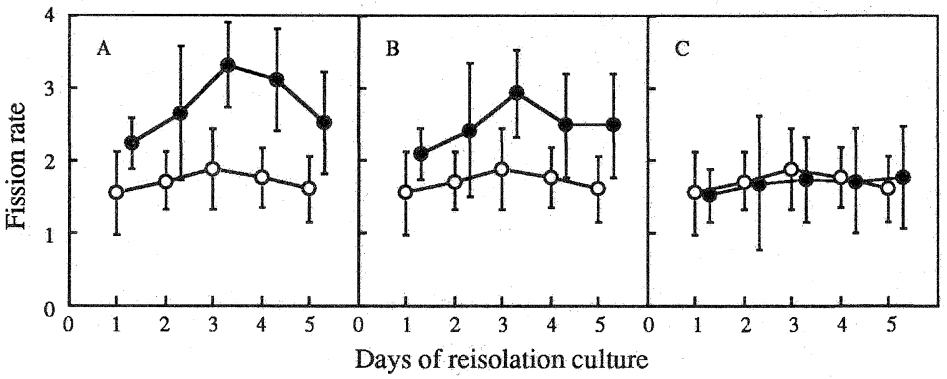

Fig. 2. Mitogenic activities of crude samples of *Tetrahymena* grown in PPYD medium. Crude samples 0.5-fold the original prepared from *T. pyriformis* (A), *T. thermophila* (B), and PPYD medium (C) were added daily. Circles and bars are the same as in Figure 1.

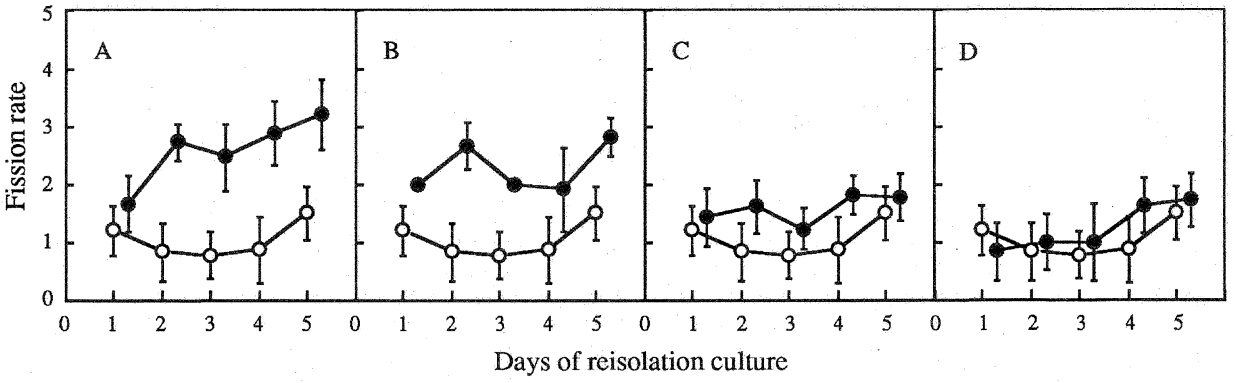

Fig. 3. Mitogenic activity of FBS on the *jumyo* mutant of *P. tetraurelia* in daily reisolation cultures. FBS was added daily at a final concentration of 6% (A), 2% (B), 0.6% (C), or 0.2% (D). Circles and bars are the same as in Figure 1.

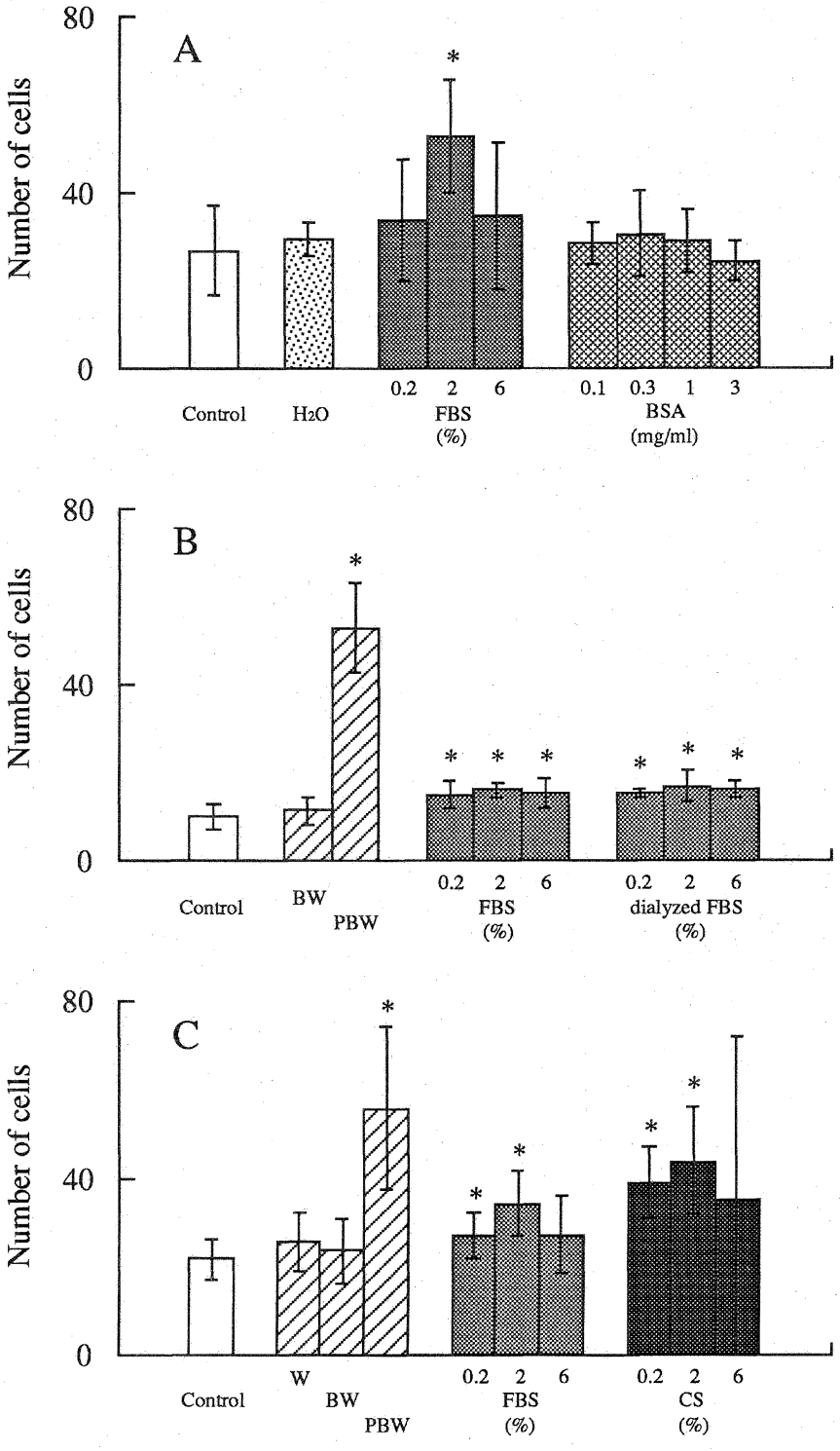

Fig. 4. Mitogenic activities of FBS, CS, and BSA on the *jumyo* mutant of *P. tetraurelia* in microcapillary cultures. Controls and experiments are for 10 lines of *jumyo* cells cultured in microcapillary tubes for 3 days. Double distilled water (H_2O) equivalent to 2% FBS was added. W, BW, and PBW were added to raise the concentration to 2-fold that of the original medium. FBS, dialyzed FBS, and CS were added and respectively raised the concentrations 0.2%, 2%, or 6%. BSA was added at a final concentration of 0.1, 0.3, 1, or 3 mg/ml. Bars indicate standard deviations. Asterisks indicate that the number of cells in the experiment was significantly higher than that in the control with nothing added (*t*-test, $P<0.05$).

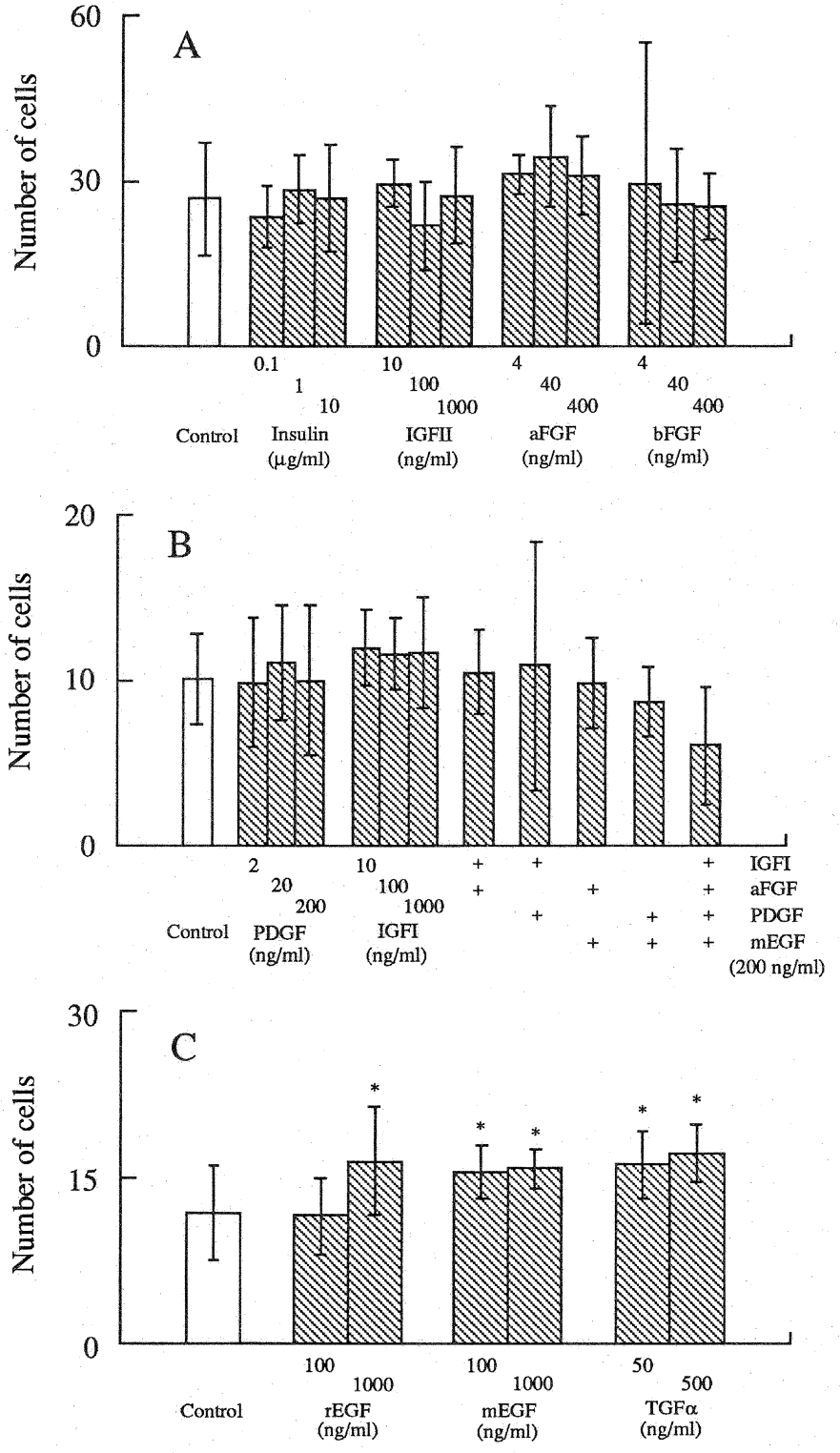

Fig. 5. Mitogenic activities of mammalian growth factors on the *jumyo* mutant of *P. tetraurelia* in microcapillary cultures. The growth factors were added at the final concentrations indicated. Bars and asterisks are the same as in Figure 4.

daily reisolation cultures (Fig. 3). The fission rate of the *jumyo* cells was restored by 6% (A), 2% (B), and 0.6% FBS (C), but not by 0.2% FBS (D). The mitogenic activity of 2% FBS on these cells was confirmed in more than 20 experiments (data not shown).

We next examined the effect of FBS on *jumyo* cells in microcapillary cultures (Fig. 4). Cell proliferation was not promoted by an addition of H_20 or 0.2% FBS, but it was significantly promoted by 2% FBS. No promotion occurred when 6% FBS was added. We examined the effect of BSA, a major component of FBS and found it ineffective at all the concentrations tested (Fig. 4A).

In the second test (Fig. 4B), BW was the negative control and PBW the positive control. The mitogenic activity of FBS in this test was not prominent; but, the difference compared with control was statistically significant (*t*-test, $P<$ 0.05). FBS was dialyzed to remove molecules of less than 3,500 daltons. The dialyzed FBS remained effective as a *jumyo* cell mitogen.

In the third test (Fig. 4C), we examined the effect of CS, as well as FBS, on *jumyo* cells. Both CS and FBS at concentrations of 0.2% and 2%, but not 6%, were effective. The mitogenic activity of 2% CS was somewhat similar to that of PBW.

Effect of mammalian growth factors

We examined the effect of nine mammalian growth factors on fission of the *jumyo* mutant (Fig. 5). Insulin at concentrations of 0.1, 1, and 10 μg/ml; IGFII at 10, 100, and 1,000 ng/ml; and aFGF and bFGF, both at 4, 40, and 400 ng/ml, were all ineffective (Fig. 5A).

PDGF at concentrations of 2, 20, and 200 ng/ml; IGFI at 10, 100, and 1,000 ng/ml; any two or four combinations of IGFI, aFGF, PDGF, and mEGF, each at 200 ng/ml, also were ineffective (Fig. 5B).

rEGF at 1,000 ng/ml, but not at 100 ng/ml; mEGF at 100 and 1,000 ng/ml; and TGFα at 50 and 500 ng/ml, however, were effective (Fig. 5C).

The mitogenic activities of mEGF and TGFα on *jumyo* cells also were examined in daily reisolation cultures. Of 37 tests of mEGF at concentrations ranging from 20 to 3,000 ng/ml, 19 were positive and 18 negative. There was no relation between concentration and effect. Of 12 tests of TGFα at concentrations from 50 to 1,280 ng/ml, 8 were positive and 4 negative. Again, there was no relation between concentration and effect.

DISCUSSION

Secretion of growth factor(s) from protozoa cells

We elsewhere reported that several species of *Paramecium* secrete ParGF or a functional homologue (Takagi *et al.*, 1993). We now have found that the crude samples prepared from two species of *Tetrahymena* acted in a similar way to ParGF. The mitogenic activities of the crude samples were assessed on the basis of the restoration of the fission rate of the *jumyo* mutant in daily reisolation cultures. The mechanism by which the *jumyo* mutant responds to ParGF remains unknown; therefore, we can not answer the question as to why the crude sample does not act on the 1st day of daily reisolation cultures in some experiments (Fig. 1). Although the fission rate of the *jumyo* mutant was unstable changing with every experiment, it was usually lower than that of the wild type (not shown in this study; usually 3-5 fissions per day) and was restored only by a fraction of the crude samples thought to contain a growth factor. The substances contained in the crude sample could be derived from protozoa cells (*Paramecium* or *Tetrahymena*), from bacteria, and from Wheat Grass Powder or PPYD, the last two of which were ineffective. Therefore we considered that the fission rate of the *jumyo* mutant was restored by the substance(s) derived from the *Paramecium* or *Tetrahymena* cells, probably ParGF or its homologue. Christensen and Rasmussen (1992) suggested that *T. thermophila* produces and releases growth stimulating factors into the surrounding medium. Lilly and Stillwell (1965) reported that during their logarithmic growth phases several species of protozoa produce substances effective for the promotion of growth in other species. Our results, which are consistent with these reports, suggest that many kinds of protozoa produce and release a growth factor(s), which might be the same or a similar substance.

Some lower eukaryotes such as *Saccharomyces cerevisiae* (Field and Shekman, 1980), *Dictyostelium discoideum* (Diamond *et al.*, 1981), *Leishmania donovani* (Gottlieb and Dweyer, 1981), and *Acanthamoeba castellanii* (Hohmann and Bowers, 1984) secrete lysosomal enzymes. *Tetrahymena* also secretes many lysosomal enzymes (Müller, 1972), some of which have been well characterized (Banno and Nozawa, 1984, 1985; Tiedtke, 1983). Most probably *Tetrahymena* cells secrete lysosomal enzymes and the growth factor simultaneously. If so, after secretion the growth factor must become the target of the lysosomal enzymes. Because crude samples of *Tetrahymena*, which should contain both, effectively promoted the proliferation of *jumyo* cells, there must be a mechanism that protects the growth factor from attack by the lysosomal enzymes.

ParGF is the only protozoa growth factor purified so far. It is now necessary to purify and determine the chemical characteristics of the growth factor(s) secreted from *Tetrahymena*.

Mammalian and protozoa growth factors

We found that FBS acted as a mitogen on the *jumyo* mutant. The mitogenic effect of 2% FBS was most stable. In Figure 3, however, 6% rather than 2% FBS appeared more effective; whereas, in Figure 4A, C, 2% rather than 6% FBS was more effective. This unsettled effect may be related to the unknown mechanism that drives the *jumyo* mutant to different fission rates every experiment. FBS contains various components and is especially rich in protein which averages 3.8 g/100 ml (Price and Gregory, 1982). Of these

proteins, BSA constitutes 2.3 g/100 ml (Price and Gregory, 1982); but, at the concentrations of 0.1, 0.3, 1, and 3 mg/ml it was ineffective for promoting cell proliferation (Fig. 3A). The nondialyzable portion of FBS is reported to stimulate the growth and development of the embryos and larvae of a parasite wasp (Greany *et al.*, 1983). In our study also, cell proliferation by the *jumyo* mutant was promoted by the nondialyzed portion of FBS. Recently, Hosoya *et al.* (1995) reported the mitogenic effect of 20% FBS on *T. pyriformis* and identified the effective component as α2-macroglobulin. Supposing that a substance was found to be a mitogen, the substance might be either a nutritional factor supplied from the outside or a hormonal growth factor produced by cells themselves. The mitogenic activity of FBS found in our study was at the concentration 1/10 that used by Hosoya *et al.* (1995) and was assayed by the *jumyo* mutant that responded only to a fraction including cell-derived factors as shown in Figure 1A. It appears, therefore, that a functional homologue of ParGF may be contained in FBS, most probably a growth factor. It remains to be investigated, however, whether α2-macroglobulin can act on the *jumyo* mutant.

We found that CS, a serum derived from cattle after birth, also acts as a mitogen on the *jumyo* mutant. Because the mitogenic activity of CS was higher than that of FBS (Fig. 4C) and mitogenic activity is known to be affected both by the kind of serum and the kind of cell, it should be interesting to examine the mitogenic activity of other sera on *jumyo* cells, such as pre-colostrum newborn calf serum derived from newborn calves within a few hours, newborn calf serum (NCS) derived from 7–10 day-old calves, and bovine serum (BS) derived from adult cows.

Trypanosoma brucei has a binding site to EGF (Hide *et al.*, 1989) and *Tetrahymena* to PDGF (Andersen *et al.*, 1984) and insulin (Christensen, 1993; Csaba and Kovács, 1990). We found that EGF and TGFα, i.e., an EGF-related protein that binds to the EGF receptor (Reynolds *et al.*, 1981), effectively promoted the proliferation of *jumyo* cells but that insulin, IGFI, IGFII, aFGF, bFGF, and PDGF were ineffective. Although this is the first report indicating that EGF and TGFα promoted cell divisions of *Paramecium* cells, it remains to be studied further that *Paramecium* cells, unlike *Tetrahymena* cells, did not respond to PDGF and insulin and that they responded to EGF and TGFα only slightly and occasionally. It is especially important to purify the mitogen derived from *Tetrahymena* and determine the molecular homology between it and ParGF and between ParGF and EGF.

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