MICROINJECTION OF IMMATUREN REJUVENATES 
SEXUAL ACTIVITY OF OLD PARAMECIUM

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SUMMARY
The sexual activity of aged Paramecium that underwent about 700 fissions after conjugation was remarkably decreased. Microinjection of a soluble cytoplasmic fraction prepared from young immature cells (about 20 fissions after conjugation) restored the sexual activity of the aged cells to that of presenescent mature cells. The factor responsible for the recovery of the sexual activity was shown to have the same characteristics as immaturin in terms of molecular size and net charge. Rejuvenation of sexual activity lasted up to 20 fissions after microinjection. However, cell division rate or fertility after self-conjugation was not increased by the microinjection. Our finding indicates that the decrease in sexual activity caused by aging was not due to defects in the genes for mating substances but to a decrease in the activity of a soluble protein responsible for the expression of mating activity. This is the first description of the restoration of a senescent defect by microinjection of young cytoplasmic components into Paramecium.

INTRODUCTION
The sexual maturation in early clonal development of Paramecium has been well defined as a function of the number of cell divisions (Sonneborn, 1957; Miwa & Hiwatashi, 1970; Bleyman, 1971). One of the unique advantages of this organism in studying clonal development and aging is that the life cycle can be reinitiated by conjugation at any time in the sexually mature period (Sonneborn, 1954). After conjugation, which resets the time of clonal life cycle at zero, sexual ability, which is tested by mating reaction with mating-reactive cells of complementary mating type, is repressed for 50–60 fissions in Paramecium caudatum (Miwa & Hiwatashi, 1970). Biochemical studies on the molecular nature of sexual activity in Paramecium revealed that membrane proteins, called the mating substances, play an important role in recognizing complementary mating type and in promoting cell contact at the beginning of fertilization (Takahashi et al. 1974). Haga & Hiwatashi (1981) reported that the repression of mating activity in the immature period was controlled by the soluble protein immaturin.

Clonal aging and senescence of Paramecium have been characterized by various changes in cellular functions (for a review, see Smith-Sonneborn, 1981). To analyse the intracellular events in senescent cells in Paramecium, cell fusion between young and old cells was induced by conjugation and old cytoplasm was proved to be

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responsible for the decrease in nuclear function in senescent cells (Sonneborn & Schneller, 1960).

An alternative approach to the study of clonal aging is microinjection. The microinjection technique has been successfully used in the analysis of the aging of germ nucleus (Karino & Hiwatashi, 1981) and the maturation of sexual activity in Paramecium (Haga & Hiwatashi, 1981; Miwa et al. 1975). Haga & Hiwatashi (1981) demonstrated that microinjection of immaturin isolated from immature cells results in the decrease or loss of mating activity in injected mature cells and thus rejuvenates the clonal age to that of the immature stage. In this study, we examined whether rejuvenation of sexual activity in senescent cells could be induced by microinjecting young cytoplasmic components. We will demonstrate the recovery of sexual activity in senescent cells by microinjecting of soluble proteins prepared from immature cells. The mechanism of rejuvenation will also be discussed.

MATERIALS AND METHODS

Culture conditions

Stock St29 of P. caudatum, syngen 3, mating type V, which has a high survival after self-conjugation, was used. Behavioural mutant, 13B (cnrB/cnrB, mating type VI), was used as a tester for mating reactivity (Takahashi, 1979). The culture medium was 1-25% (w/v) lettuce juice in Dryl's solution (Dryl, 1959) inoculated with Klebsiella pneumoniae 1 day before use (Hiwatashi, 1968).

The clone was maintained by the tube culture method throughout its lifespan (Karino & Hiwatashi, 1981).

Microinjection and quantitative assay of mating reactivity

Microinjection was performed by Koizumi's method (Koizumi, 1974). The volume of the injection was approximately 15 pl. Quantitative assay of mating reactivity was done by using the glass capillary method described by Haga & Hiwatashi (1981). The behavioural mutant CNR was used as a tester for mating reactivity to distinguish tested cells of the wild-type from tester in mating clumps. Because of the lack of a voltage-sensitive Ca²⁺-channel function, CNRs cannot swim backward when transferred into 20 mM-KCl in Dryl's solution. When mating clumps were transferred into 20 mM-KCl in Dryl's solution, tested cells showed long backward swimming, although testers did not (Takahashi, 1979). Mating reactivity of each clone was determined by counting wild-type cells in mating clumps. The wild-type cells were then isolated and incubated in a capillary with fresh culture medium.

Preparation of cell fraction and purification of immaturin

Cells of P. caudatum were homogenized and fractionated according to the methods described by Haga & Hiwatashi (1981). Immature cells, about 20 fissions after conjugation, were washed with solution A (10 mM-KCl, 5 mM-MgCl₂, 10 mM-Tris-HCl, pH 7.4) and then with solution A' (solution A + 0.25 M-sucrose) by slow centrifugation (100–200 g). The pellet was homogenized with an injection syringe (Sonneborn, 1954) and the homogenate was centrifuged at 105 000 g for 60 min at 2°C. The supernatant was filtered through a 0.45 µm Millipore filter. The filtrate was applied to a Sephadex G-50 column and each fraction was assayed by microinjecting a 15-pl sample into mature cells. The active fractions were combined and chromatographed on a DEAE-Sephadex A-25 column. The activity was eluted as a single peak at about 400 mM-NaCl.
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Fission rate and survival after self-conjugation

For counting fission rate, a single cell was isolated into a capillary with fresh culture medium and incubated at 25°C. The number of cells in each capillary was counted every day.

Survival after self-conjugation of injected cells was measured as follows. An injected cell was grown in a minitube that contained about 3 ml of fresh culture medium. About 10 fissions after microinjection, self-conjugation was induced by mixing with mating-reactive cells of a CNR mutant of mating type VI (Takahashi, 1979). Homotypic pairs of wild-type (injected clone) were isolated into K⁺-Dryfall's solution and incubated at 25°C (Mikami & Hiwatashi, 1975). After 2 days, exconjugants were isolated into tubes with fresh culture medium. Viable clones were defined as those that cleared the bacteria in the medium at stationary phase. Clones that could not clear the bacteria in 10–12 days of incubation were scored as non-viable.

RESULTS

Senescent decrease in sexual activity and its recovery by microinjection of an immature fraction

Sexual activity of presenescent and senescent cells in St29 were tested by mixing with mating-reactive cells of complementary mating type. This test was performed twice on the first and second days of stationary phase by counting the number of mating clumps that consisted of one cell to be tested and a group of reactive cells of the tester. As shown in Fig. 1, all clones of presenescent cells that underwent about

Fig. 1. Rejuvenation of mating reactivity in presenescent and senescent cells by microinjecting an immature fraction. A. Mating reactivity of the presenescent clones (150 fissions after conjugation; n = 53). B. Mating reactivity of the presenescent clones after microinjecting a soluble fraction prepared from immature cells (n = 21). C. Mating reactivity of senescent clones (700 fissions after conjugation; n = 31). D. Mating reactivity of the senescent clones after microinjecting the soluble fraction of immature cells (n = 17). Each measurement was made five to six fissions after microinjection in case of the presenescent clones and four to five in case of the senescent clones.
150 fissions after conjugation contained more than 80% of mating-reactive cells (A). After an additional 550 fissions, however, the percentage of mating-reactive cells in each clone greatly decreased (Fig. 1C). We tested if the decreased sexual activity in senescent cells could be rejuvenated by microinjecting young cytoplasmic components. A soluble fraction was prepared from immature cells of about 20 fissions after conjugation by ultracentrifugation. When presenescent mature cells were injected with the soluble fraction, the percentage of mating-reactive cells in each clone derived from injected cells was decreased. This was designated as the 'immaturin effect' (Fig. 1B) (Haga & Hiwatashi, 1981). In sharp contrast, when the senescent cells were injected with the same amount of the soluble fraction, the percentage of mating-reactive cells in each clone was increased to almost that of the presenescent cells (Fig. 1D).

Fractionation of the factor for rejuvenation of sexual activity in senescent cells

To determine whether immaturin is involved in the rejuvenation of sexual activity in senescent cells, the soluble fraction from immature cells was fractionated by the protocol established for immaturin purification (Haga & Hiwatashi, 1981). Because immaturin activity is usually eluted in fraction numbers 32–34 from a Sephadex G-50 column, these fractions were combined and injected into both presenescent and senescent cells in St29. When presenescent cells of age 150 fissions were injected with the fraction, the clones showed a decrease in mating reactivity when compared with uninjected controls (data not shown). In contrast, the clones of senescent cells showed an increase in mating reactivity after microinjection (Fig. 2B). Mating-reactive cells were then isolated from mating clumps and grown in new capillaries to test the mating activity after an additional three or four fissions. The retention of the rejuvenation effect in senescent cells was found until about 20 fissions after microinjection (Fig. 2C,D).

The fraction was further fractionated through a DEAE-Sephadex A-25 column. When presenescent cells were injected with a sample of fraction number 10, which had the highest activity of the immaturin effect, about 50% of clones showed a decrease in mating activity (data not shown). On the other hand, the mating activity of senescent clones was increased by microinjection of the same fraction to about threefold of that of uninjected clones (Fig. 3A,B). When specific activity was defined as the activity per protein injected, two purification steps increased the specific activity of the immaturin effect in presenescent cells about 600-fold and that of the rejuvenation effect in senescent cells about 350-fold of those of a crude soluble fraction (Table 1).

Effect of the soluble fraction on the fission rate and postconjugation survival of senescent cells

Since injection of immaturin set back the clock of aging in sexual activity not only in presenescent cells but also in senescent cells, it would be interesting to know if the injection could rejuvenate other cellular functions of senescent cells. To test this, we investigated the effects of the soluble fraction prepared from immature cells on
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Fig. 2. Retention of the rejuvenation effect of mating activity in injected senescent clones. The soluble fraction was concentrated by ultrafiltration and was applied to a Sephadex G-50 column and chromatographed at 5°C. Each fraction was assayed for immaturin activity by microinjection of 15-μl samples into presenescent cells. The active fractions of immaturin were combined and injected into senescent cells. Four to five fissions after injection, the mating reactivity of each clone was tested and mating reactive cells were isolated into new capillaries with fresh culture medium. A. The mating reactivity of uninjected senescent clones (fissions = 700, n = 48). B. Four to five fissions after injection (n = 25). C. 11–14 fissions after injection (n = 14). D. 17–22 fissions after injection (n = 12).

Fission rate and survival after self-conjugation in senescent cells. First, senescent cells of St29 at age 700 fissions were injected with the soluble fraction of immature cells and then incubated in capillaries with fresh culture medium. The number of cells in each capillary was counted after 5 days. As shown in Fig. 4, neither growth rate nor cell density in the stationary phase was increased by the microinjection.

In the second experiment, cells of about 650 fissions after conjugation were injected with the soluble fraction and grown in small tubes. About 10 fissions after injection, each clone was induced to conjugate by mixing with behavioural mutants, which were mating-reactive for complementary mating type. Homotypic pairs of senescent cells (wild-type) were isolated by the behavioural test in 20 mM-KCl in Dryl's solution and incubated in K⁺–Dryl's solution for 2 days (Mikami & Hiwatahi, 1975). Exconjugants were then isolated into fresh culture medium in test tubes. Ten to twelve days later, survival of each progeny was scored. There was no significant difference between injected and uninjected groups (Table 2).
Fig. 3. Rejuvenation of mating activity in senescent clones by injecting purified immaturin. Active fractions for immaturin after gel filtration on Sephadex G-50 were combined and applied to a DEAE-Sephadex A-25 column. The elution was done with a linear gradient of from 0 to 500 mM-NaCl in the buffer. The active fraction of immaturin (no. 10) was injected into senescent cells and the mating reactivity of each clone was tested after four to five fissions. A. The mating reactivity of uninjected senescent clones (fissions (/) = 700, \( w = 21 \)). B. The mating reactivity of injected senescent clones (fissions (/) = 700, \( w = 11 \)).

### Table 1. Purification of the rejuvenation factor

<table>
<thead>
<tr>
<th>Steps</th>
<th>Proteins injected (pg)</th>
<th>Senescent Specific activity (n)</th>
<th>Presenesthetic Specific activity (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>15-0</td>
<td>4·4 (17)</td>
<td>4·2 (21)</td>
</tr>
<tr>
<td>Sephadex G-50 (no. 32)</td>
<td>1·2</td>
<td>50·6 (25)</td>
<td>68·8 (23)</td>
</tr>
<tr>
<td>DEAE-Sephadex (no. 10)</td>
<td>0·02</td>
<td>1535·0 (11)</td>
<td>2500·0 (16)</td>
</tr>
</tbody>
</table>

Specific activity is defined as activity per injected protein (pg), while activity is the % of clones that contain less than 70% of mating-reactive cells in case of presenesthetic cells and more than 70% in case of senescent cells.

**DISCUSSION**

The expression of sexual activity in clonal development of *Paramecium* has been found to be a genetically programmed process (Myohara & Hiwatashi, 1978; Cohen & Siegel, 1963). The process is assumed to be controlled by the genes necessary for the synthesis of immaturin, which in turn control the genes for mating substances (Miwa et al. 1975; Tsukii & Hiwatashi, 1983). An important result of this study in the regulation of sexual activity is the recovery of mating activity in senescent cells by microinjection of a soluble factor isolated from young immature cells. Results described here suggest that the decrease in sexual activity in senescent cells is not due
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to the defects in genes for mating substances but to the decrease or loss of a soluble
factor, which is co-purified with immaturin activity. Aging must therefore affect
either immaturin itself or some other novel regulatory factors.

Four lines of evidence obtained here strongly suggest that the rejuvenating factor
for senescent cells is associated with immaturin. (1) The activity of rejuvenating
factor was co-purified with immaturin activity through two purification steps. These
purification steps increased the specific activity of rejuvenation more than 350-fold
and the immaturin activity about 600-fold. (2) Retention of the rejuvenation effect in
injected senescent cells was similar to that of immaturin effect. In the case of

![Comparison of growth rate between clones with and without microinjection of a
soluble fraction of immature cells. O---O Uninjected clones; (••••) injected
clones. A. Presenescent clones of 150 fissions after conjugation. B. Senescent clones of
700 fissions after conjugation.](image)

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Pairs isolated</th>
<th>Death or MR*</th>
<th>Surviving true progeny (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old (650) clone no. 1</td>
<td>47</td>
<td>44</td>
<td>3 (6)</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>42</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>44</td>
<td>1 (2)</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>39</td>
<td>39</td>
<td>0 (0)</td>
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<tr>
<td>Experiment</td>
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<tr>
<td>Old (650) clone no. 1</td>
<td>45</td>
<td>44</td>
<td>1 (2)</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>29</td>
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</tr>
<tr>
<td>3</td>
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<tr>
<td>5</td>
<td>31</td>
<td>30</td>
<td>1 (3)</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>28</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

All clones were derived from sister cells of a clone of St29 at 650 fissions.
*MR, macronuclear regeneration.
restoration of genetic defects in Ca-channel function by microinjecting a soluble factor, the restoration disappeared within a couple of cell divisions after microinjection (Haga et al. 1984). In contrast, the retention of the immaturin effect in injected presenescence cells continued for more than 10 fissions after microinjection (Miwa et al. 1975). The rejuvenation of sexual activity in injected senescent cells also continued up to 20 fissions after injection. (3) The ability to grow was not rejuvenated in senescent cells injected with the soluble fraction. Therefore, it is hard to assume that the increase in mating activity brought about by injection resulted from the activation of the total metabolic activity in senescent cells. Rejuvenation seems to be specific to the expression of mating activity. (4) The viability of progeny after self-conjugation in senescent cells was not increased by microinjection of immaturin. Micronuclear transplantation experiments revealed that the decrease in progeny survival at about 650 fissions after conjugation was not due to the defects produced in the micronucleus but to accumulating damage in macronuclear or cytoplasmic function (Karino & Hiwatashi, 1981). Rejuvenation by microinjection may be induced mainly in cell surface function rather than in intracellular events involved in the production of progeny. The recovery of mating activity might be brought about by a factor specific for the expression of mating substances. There may be a regulatory protein other than immaturin that is required for the expression of mating substances and is affected by aging. While there is no biochemical or molecular biological evidence to answer this question, however, all the lines of evidence discussed here suggest that immaturin is a good candidate for the rejuvenating factor in the recovery of sexual activity in senescent cells.

After injection of immaturin, presenescence and senescence cells showed quite opposite responses, in the former disappearance of mating activity and in the latter an increase. To explain the divergent responses, in cells of different age, to microinjection, we assume the presence of a factor that interacts with immaturin. This factor can be designated as anti-immaturin. Anti-immaturin is assumed to bind to immaturin and thus to inactivate it. When immaturin is predominant relative to anti-immaturin in a cell, mating activity is not expressed. When immaturin and anti-immaturin are well balanced to form a complex, the expression of mating activity is kept stable. The central idea of this model, therefore, is that one type of regulatory molecule can reversibly change its effect by interacting with complementary molecules. If this model is correct, some mutant that has a shorter immaturity period may make it possible to detect a factor neutralizing immaturin activity. In fact, Miwa suggested the presence of a factor that could inhibit immaturin activity in the cytoplasm of early mature mutations immediately before maturation (Miwa, 1984).

An alternative interpretation is also possible. For instance, a change in signal transduction caused by aging may result in different responses to the injected molecules.

The fact that the early mature mutants that had shorter immaturity periods were induced by mutagenesis suggests that the mechanism of gene expression coupled with cell division might be involved in this system. At present, we have limited information about the function of the biological clock in development. The study of
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the mechanism of immaturin action in Paramecium using the microinjection technique seems a promising way of understanding the molecular mechanism involved in the timing of gene expression in development.

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REFERENCES


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