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FACILITATION OF ELECTROFUSION OF MOUSE LYMPHOMA CELLS BY THE PROTEOLYTIC ACTION OF PROTEASES

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SUMMARY: Cell fusion of mouse lymphoma (L5178Y) was achieved by applying electrical pulses under dielectrophoresis. The presence of dispase, pronase or trypsin facilitated the electric pulse-induced cell fusion. Heat-inactivated pronase was no longer effective. Protease inhibitors (aprotinin and p-tosyl-L-lysine chloromethylketone) suppressed the effect of trypsin. Even in the absence of proteases, the cells pretreated with dispase or pronase underwent fusion with high probabilities, as far as free calcium ions were present in the external solution. It is concluded that facilitation of electrofusion by proteases is due to their proteolytic activities.

A novel cell fusion technique, electrofusion, has recently been established (1, 2) by a combination of dielectrophoresis (3) and controlled electrical breakdown of the membrane. It is known that electrofusion of animal cells is markedly facilitated by the presence of pronase or dispase during the application of electrical fields (4 - 8). However, pretreatment of leukemia cells or sea urchin eggs with the enzymes was ineffective, when the electrofusion was performed in a nominally Ca^{2+}-free medium. In addition, a protease inhibitor, PMSF, failed to abolish the pronase effect on the electrofusion of myeloma cells (8). Thus, Zimmermann and his colleague concluded that the enzyme effect on electrofusion is not directly linked with the proteolytic activities (2, 8). On the other hand, proteolytic treatment of erythrocytes is known to bring about fusion by itself (9) or enhance polyethylene glycol-induced fusion (10). Because of these discrepancies, we have re-examined whether the proteolytic activity is primarily involved in facilitating effects of proteases on electrofusion of lymphoma cells. The results show that facilitation of electrofusion by protease is due to the proteolytic function, though free calcium ions contaminated in the enzyme

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compounds have a share in an apparent effect of protease, if the external Ca^{2+} concentration is very low.

MATERIALS AND METHODS: Mouse Leukemic lymphoblasts, L5178Y, were cultured in Fischer medium supplemented with 10 % bovine serum. The cells were centrifuged, and the pellet was resuspended in the fusion medium composed of 0.3 M mannitol, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 5 mM Tris-HCl (pH 7.0 - 7.4) or in that devoid of $CaCl_2$. The cells were then washed with the fusion medium. In some cases, the cells were pretreated with 10 μg/ml dispase or 50 μg/ml pronase E in the fusion medium for 20 or 5 min, respectively, and then washed with the enzyme-free fusion medium.

The experimental set-up for electrofusion was similar to that of Zimmermann & Scheurich (11). The fusion chamber consisted of a slideglass with two parallel platinum wires (200 μm in diameter) placed at a distance of 200 μm. Dielectrophoresis of the cells were achieved by applying alternating fields with a function generator (Kikusui 455). Fusion was induced by a train of field pulses generated by a pulse generator (Nihon Kohden SEN-3201). To reinforce the output voltage and to reduce the output impedance of a pulse generator, a booster amplifier(built in collaboration with Mr. T. Mikami, Nihon Kohden, Kyoto) was employed. During the pulse application, alternating fields were automatically disconnected, using a switch device installed in the booster amplifier.

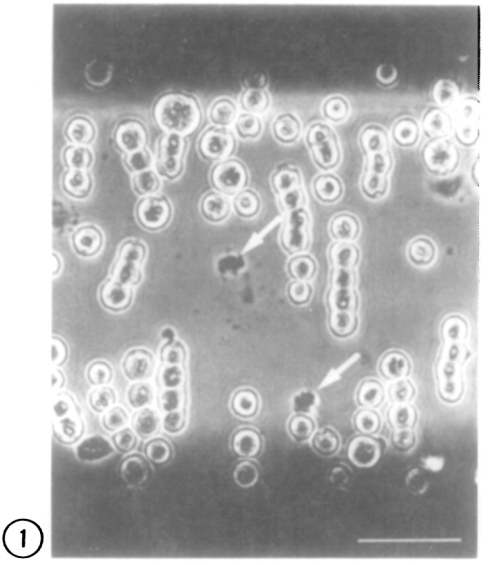
The fusion experiments were carried out at room temperature (20 - 25 °C) under a phase-contrast microscope (Nikon MTD) and monitored on a television screen (Tokyo Densi 9M 20A). The yield of fusion was determined by counting the percent of cells participating in cell fusion among viable cells of more than 100. Viability of the cells was identified by erythrosin B exclusion or by characteristic phase-contrast images of damaged cells; that is, loss of halo around the cells and their dark cytoplasmic feature (Fig. 1, arrows).

The concentrations of free calcium ions in the fusion media containing proteases were measured with Ca^{2+}-selective electrodes, which were made with Simon's neutral ligand sensor (12), as reported previously (13).

Dispase, pronase E and trypsin were from Godo Shusei, Kaken Chem., and E. Merck AG, respectively. Other chemicals employed in the present experiments were as follows: phenylmethylsulfonyl fluoride (PMSF, Sigma), p-tosyl-L-lysine chloromethylketone hydrochloride (TLCK, Nakarai), aprotinin (Sigma), ethylene glycol bis(β-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA, Nakarai), and erythrosin B (Sigma).

RESULTS AND DISCUSSION: Under an alternative electric field of 0.8 kV/cm and 100 kHz, the L5178Y cells adhered to each other in parallel with the field lines and formed so-called pearl chains. Cell fusion was initiated by four pulses, the intensity of which was progressively increased from 5 to 8 kV/cm, with a duration of 20 μs. Fusion observable under a microscope took place within several minutes (Fig. 1). Some of the fused cells started to become rounded immediately after switching off the dielectrophoretic field.

As shown in Fig. 2(a, b), the addition of dispase to the fusion medium devoid of Ca^{2+} prominently facilitated cell fusion, confirming previous observations (5-7). Viability of cells exposed to electrical fields was also



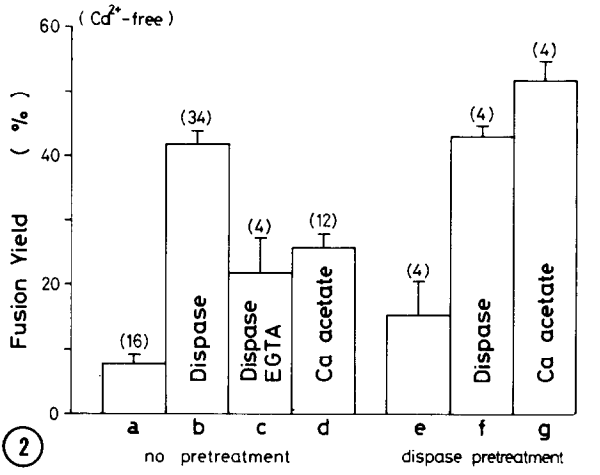
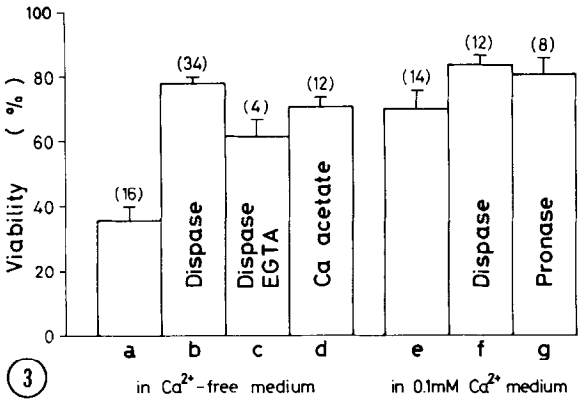


Fig. 1 Phase-contrast micrograph of lymphoma cells 5 min after the application of a train of pulses under dielectrophoresis with an alternative electric field in the presence of 10 μg/ml dispase. Arrows, irreversibly damaged cells. Bar, 50 μm. Shadows on top and bottom of the micrograph, a pair of platinum electrodes.

Fig. 2 Effect of dispase on electrofusion of lymphoma cells in a Ca^{2+}-free medium. a-d) cells without enzyme pretreatment, e-g) cells after pretreatment with dispase. In the fusion medium, no enzyme (a, e), 10 μg/ml dispase (b, f), 54 μM EGTA and 10 μg/ml dispase (c), or 5 μg/ml calcium acetate were added. Numbers in parentheses and vertical bars represent the number of observations and the standard error of the mean, respectively. The differences between the data of a) and b), c) or d), of b) and c) or d), as well as of e) and f) or g) are statistically significant ($P < 0.05$).

remarkably improved by the addition of dispase to a nominally Ca^{2+}-free solution (Fig. 3a, b). Such a stabilizing effect of protease has already been reported, though only qualitatively (1, 2, 4, 7). Dispase employed herein is known to contain 50 % (w/w) of calcium acetate, and, in fact, the free Ca^{2+} concentration of 10 μg/ml dispase was found to be about 30 μM using a Ca^{2+}-selective electrode. The addition of 54 μM EGTA, by which the free Ca^{2+} concentration was decreased down to 7 μM, partially suppressed effects of dispase on the fusion yield (Fig. 2c) and cell viability (Fig. 3c). The application of 5 μg/ml calcium acetate in a Ca^{2+}-free medium enhanced the fusion yield (Fig. 2d) as well as viability in the absence of dispase (Fig. 3d). However, these effects were significantly less than those of dispase which contained the same amount of calcium (Fig. 2b, Fig. 3b). These results indi-



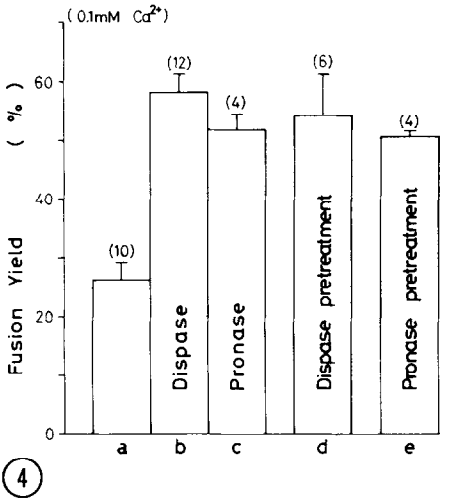


Fig. 3 Effects of dispase and pronase on viability of lymphoma cells after the application of electric pulses in the fusion medium devoid of Ca^{2+} (a-d) or containing 0.1 mM Ca^{2+} (e-g). No enzyme (a, e), 10 μg/ml dispase (b, f), 54 μM EGTA and 10 μg/ml calcium acetate (d) or 50 μg/ml pronase E (g) were added to the fusion medium. Numbers in parentheses and vertical bars are the same as in Fig. 2. The differences between the data of a) and b), c) or d), of b) and c) or d) as well as of e) and f) are statistically significant ($P < 0.05$).

Fig. 4 Effects of dispase and pronase on electrofusion of lymphoma cells in the fusion medium containing 0.1 mM Ca^{2+}. a-c) cells without enzyme pretreatment, d) cells after pretreatment with dispase, e) cells after pretreatment with pronase E. In the fusion medium, no enzyme (a, d, e), 10 μg/ml dispase or 50 μg/ml pronase E was added. Numbers in parentheses and vertical bars are the same as in Fig. 2. The differences between the data of a) and b), c), d) or e) are statistically significant ($P < 0.05$).

cate that effects of dispase cannot be explained solely by contaminated calcium ions. Supporting this inference, even in the presence of 0.1 mM Ca^{2+}, dispase still reduced the irreversible cell damage (Fig. 3e, f) and facilitated electrofusion (Fig. 4a, b). Pronase E also stabilized the cells (Fig. 3g) and facilitated cell fusion (Fig. 4c) in the 0.1 mM Ca^{2+} medium. Since the calcium contamination in 50 μg/ml pronase E was found to be only about 5 μM, effects of pronase E in the presence of 0.1 mM Ca^{2+} would mainly reflect the action of pronase itself. However, the effect of calcium contamination may not be ruled out, when higher concentrations of pronase are used (for example 1 - 3 mg/ml; 4 - 8).

The pretreatment of cells with dispase before the application of electrical fields only slightly increased the fusion yield under a Ca^{2+}-free condition (Fig. 2e). However, the addition of calcium (Fig. 2g) during the

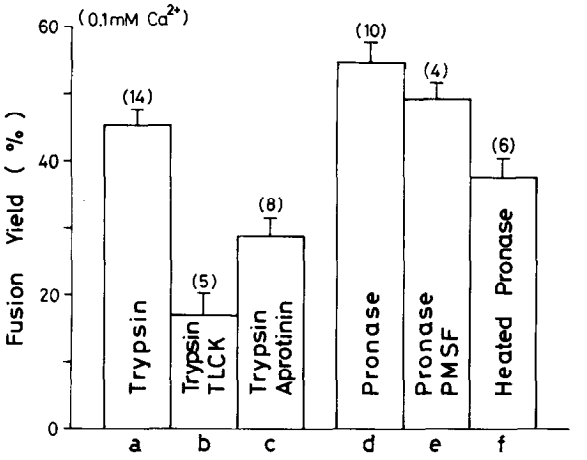


Fig. 5 Effects of protease inhibitors and heat-inactivated pronase on electrofusion of lymphoma cells in the fusion medium, to which 0.1 mM Ca^{2+} and 0.5 - 1 mg/ml trypsin (a, b, c), 50 - 500 μg/ml pronase E (d, e), 50 μg/ml heat-inactivated pronase E (f), 5 mM TLCK (b), 125 μg/ml aprotinin (c) or 2 mM PMSF (e) were added. Numbers in parentheses and vertical bars are the same as in Fig. 2. The differences between the data of a) and b) or c) as well as of d) and f) are statistically significant ($P < 0.05$).

field application resulted in fusion of the pretreated cells to the same extent as the addition of dispase (Fig. 2f). In the presence of 0.1 mM Ca^{2+}, the pretreatment of cells with dispase (Fig. 4d) or pronase E (Fig. 4e) facilitated cell fusion in a comparable degree to the effect of adding dispase (Fig. 4b) or pronase E (Fig. 4c). These results strongly suggest that proteolytic action is a primary factor for the effects of these enzymes on electrofusion.

Electrofusion performed in a 0.1 mM Ca^{2+} medium was facilitated by the addition of 0.5 - 1 mg/ml trypsin, which contained about 5 - 10 μM free Ca^{2+} (Fig. 5a). Protease inhibitors, TLCK (5 mM) and aprotinin (125 μg/ml), remarkably suppressed the facilitation by trypsin (Fig. 5b, c). However, another protease inhibitor PMSF (2 mM) failed to abolish the pronase effect (Fig. 5d, e). This agrees with the results reported by Vienken et al. (8). Since pronase is a mixture of several proteolytic enzymes (14), it is possible that PMSF may not inhibit all of them. In fact, heat treatment of pronase E (90°C, 3 min) markedly suppressed its effect on electrofusion (Fig. 5f).

Taken together, it is concluded that effects of proteases on electrofusion of lymphoblasts are due to their proteolytic activities, although the mechanism, by which cell fusion is facilitated, remains unknown. Previously, it was suggested that the protease effect is mainly due to general properties of proteases, which are common to other enzymes or proteins, including charges and hydrophobic properties (2, 8). This suggestion is not consistent with the present results. In fact, several other enzymes such as phospholipases and neuraminidase did not facilitate electrofusion of lymphoma cells (T. Ohno-Shosaku & Y. Okada, unpublished observations).

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