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# Activation of Human C3 by Living and Heat-killed Human Cells after Treatment with Trypsin.

**Ayako UEKI*, Yoshio MOCHIZUKI* and Tetsuo KIMOTO****

**Department of Hygiene and **Department of Pathology, Kawasaki Medical School, Kurashiki 701-01, Japan*
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**ABSTRACT.** Living and heat-killed, cultured human fibroblasts treated with 0.1% trypsin were found to activate the alternative complement pathway in human serum. The extent of C3 activation measured by conversion of C3 was dependent on the concentration of cells and associated with loss of hemolytic activity of complement.

Complement activation by heat-killed cells was significantly enhanced compared with that observed in viable cells. C3 conversion was detected in normal serum chelated with EGTA-$Mg^{++}$, but not in serum heated at 50°C for 30 min to inactivate factor B or in EDTA-chelated serum.

These data suggest that fibroblasts with modified membranes or killed by injury could induce antibody-independent activation of the complement system through the alternative pathway.

Whether a cell is recognized by the alternative complement pathway is determined by the capacity of its surface to regulate deposition and function of bound C3bBb sites.[1,2] Fearon et al.[3] described a glycoprotein of 205,000 mol wt from human erythrocyte membrane which can impair directly the formation and function of human C3bBb sites and serves as a cofactor for C3bINA-dependent cleavage of C3b. These surface constituents could represent a molecular basis for preventing inappropriate self-recognition.

The authors chose to look at fibroblasts as a major component of connective tissue which receives a large volume of tissue fluid and is exposed to toxic and cell modifying agents at the site of inflammation. In the case of some collagen disease such as systemic lupus erythematosus (SLE) complement is consumed, thus inciting an inflammatory reaction by the release of mediators.

In this paper living and heat-killed fibroblasts after treatment with trypsin were studied for their capacity to initiate complement activation.

## MATERIALS AND METHODS

*Human fibroblasts* : Diploid cell strain, Detroit 550 derived from human embryonal skin, was purchased from Flow Laboratory, USA. Another fibroblasts cell strain HEF-2 was established from human embryonal lung in our laboratory. MEM tissue culture medium was supplemented with 10% heat-inactivated fetal

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calf serum (GIBCO, USA), 2.5 μg/ml fungisone (Sankyo, Japan), 80 μg/ml gentamycin sulfate (Shionogi, Japan) and 1 mM HEPES buffer. A fibroblast monolayer was washed with phosphate buffered saline (PBS), and incubated with 0.1% trypsin at room temperature for 10 min. Cells were removed by gentle pipetting, washed 5 times in PBS, and a cell count was made using a hemocytometer. To prepare heat-killed cells, the cell suspension in PBS was incubated in a 60°C water bath for 1 hr. The cells retained the same morphological appearance under a light microscope, but less than 1% were able to exclude trypan blue dye.

*Normal human serum* : Blood group AB serum was used as a source of normal human complement in these experiments. The blood was clotted at room temperature for 30 min and then at 4°C for 1 hr, and the serum was stored at −70°C until use. To block classical pathway activity, serum was chelated with 10 mM EGTA and supplemented with 1 mM $MgCl_2$ (serum-EGTA-$Mg^{++}$). To block classical and alternative pathway activity, serum was chelated with 20mM EDTA (serum-EDTA).

*Incubation of serum with suspended fibroblasts* : Human fibroblasts were incubated with human blood group AB serum with frequent shaking at 37°C for 1 hr. As a control, AB serum without cells was also incubated at 37°C for 1 hr. The cells were removed by centrifuging, and the serum was collected and stored at −70°C.

*Titration of hemolytic activities* : The hemolytic activity of complement (CH50) in sample serum was measured by lysis of antibody-sensitized sheep erythrocytes (ShEA) according to the method of Mayer[4]. Because of the small volume of serum used in these experiments, the quantities of all reagent were reduced to 1/2.5[5].

*Assay for C3 conversion* : The percentage of native C3 converted to its more anodally migrating form was determined by antigen-antibody crossed electrophoresis (Laurell's method)[6]. The agarose gel electrophoresis (1% agarose with barbital buffer, 0.075M, pH 8.6) is performed for 4.5 hrs with a potential drop of 6V/cm. Afterwards a longitudinal strip of 5 mm width with the separated fractions are cut out, placed on the agarose gel plate containing the antiserum specific for human C3 (β1C/β1A) (MBL, Nagoya, Japan), and reelectrophoresed for 4.5 hrs. The specimens were left overnight at 4°C, washed and stained with Amido black B 12. The percentage C3 conversion was calculated from the areas under the peaks.

## RESULTS

*Crossed immunoelectrophoresis* : Evidence for the activation of alternative complement pathway was obtained by crossed immmunoelectrophoretic studies, which demonstrated the breakdown products of C3 after incubation of fibroblasts with blood group type AB serum. This finding was confirmed in the presence of EGTA (10 mM), which chelates the $Ca^{++}$ ions but not $Mg^{++}$ ions which are

necessary for the activation of the alternative pathway. However EDTA (20mM), which chelates both $Ca^{++}$ and $Mg^{++}$ ions, completely inhibited the appearance of the breakdown products of C3. C3 conversion was not observed in the serum before incubation, and only trace conversion was seen in serum incubated without cells. In addition, no C3 activation was detected in the serum pretreated at 50°C for 30 min to inactivate factor B (Table 1 & 4). Both living and heat-killed cells generated C3 cleavage, and increases in the concentration of cells resulted in increased C3 conversion to a maximum of 50.6% conversion occuring with $1\times10^7$ cells/50$\mu$l of serum (Fig. 1). Although there was some variation in the extent of conversion between several passages of cells, fibroblasts obtained from 2 different cell strains initiated C3 conversion in AB serum.

TABLE 1. C3 conversion and reduction of CH50 activity in serum after the incubation with fibroblasts.

| serum was incubated with | C3 conversion | Reduction of CH50 activity |
|---|---|---|
| none | 24.7±4.7 | — |
| $1\times10^6$ cells | 25.1±5.2 | 0 |
| $5\times10^6$ cells | N. T. | 30.0 |
| $1\times10^7$ cells | 44.4±1.3 | 52.7 |

Fibroblasts were treated with 1% trypsin for 10 min at room temperature, and washed throughly. Incubation of suspended cells with serum was performed at 37°C for 1 hr. C3 conversion was calculated after the crossed immunoelectrophoresis, and CH50 was measured according to Mayer.

TABLE 2. Loss of hemolytic activity in serum after the incubation with fibroblasts.

| cells/50$\mu$l serum | condition of cells | % reduction of CH50 |
|---|---|---|
| $1\times10^7$ | living | 55.0(%) |
| | heat-killed | 63.8 |
| $5\times10^6$ | living | 34.1 |
| | heat-killed | 52.4 |
| $1\times10^6$ | living | 26.2 |
| | heat-killed | 43.2 |
| $5\times10^5$ | living | 26.2 |
| | heat-killed | 36.7 |

Hemolytic activity of test sera was measured according to Mayer. Heat-killed cells were prepared by heating cell suspension at 60°C for 1 hr.

*Hemolytic activity of serum* : The effect of incubation of the serum with fibroblasts on the hemolytic activity of complement (CH50) was tested, and the serum showed a reduction in CH50 titer as shown in Tables 1 and 2. Increasing the concentration of cells resulted in reduction of CH50 activity, and this reduction coincides with the results of the crossed immunoelectrophoresis.

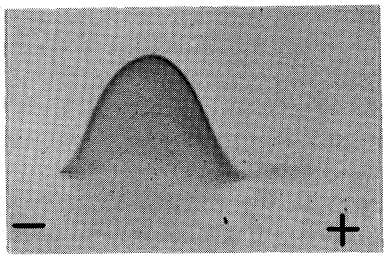

Fig 1.

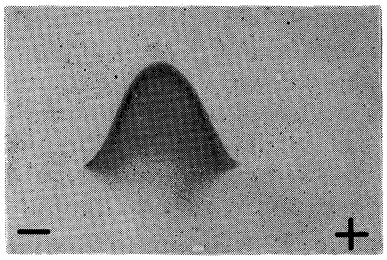

Fig 2.

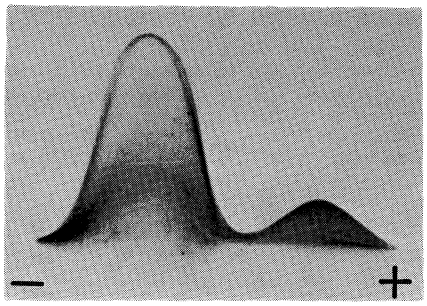

Fig 3,

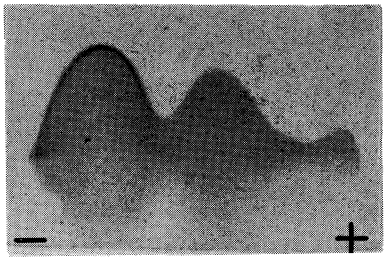

Fig 4.

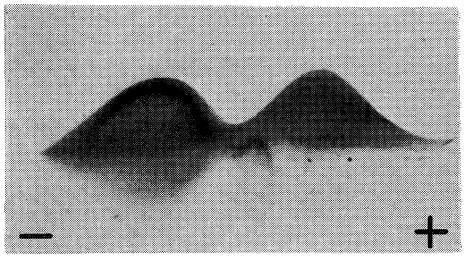

Fig 5,

Fig. 1. Crossed immunoelectrophoresis of fresh human serum to rabbit anti-human C3 ($\beta$1c/$\beta$1a) serum.
Fig. 2. EDTA-serum after the incubation with cells, $1\times10^7/50\mu$l, 37°C, 1 hr.
Fig. 3. Human serum (cell free) incubated at 37°C, 1 hr.
Fig. 4. Human serum incubated with cells, $5\times10^6/50\mu$l, 37°C, 1 hr.
Fig. 5. Human serum incubated with cells, $1\times10^7/50\mu$l, 37°C, 1 hr.

## DISCUSSION

Götze and Müller-Eberhard demonstrated that complement could be activated through the alternative pathway in the absence of antibodies[7]. Recently several authors reported the activation of the alternative pathway by human kidney cells[8], human liver cells[9], and erythrocytes stromata[10]. Such alternative

TABLE 3. Effect of EDTA, EGTA and heating of serum on C3 activation by human fibroblasts.

| serum absorption with | | human serum used for the incubation | C3 conversion $\beta$1A (%) |
|---|---|---|---|
| Cell free | | fresh human serum | 33.8±2.03 |
| Detroit 550 | $1\times10^6/50\mu l$ | fresh human serum | 36.8±2.37 |
| | $1\times10^7$ | fresh human serum | 48.3±3.73 |
| | $1\times10^7$ | pre-heated serum, 56°C, 30 min. | 0 |
| | $1\times10^7$ | fresh human serum + EGTA-$Mg^{++}$, 10 mM | 20 |
| | $1\times10^7$ | fresh human serum + EDTA, 20 mM | 0 |
| Zymosan | $50\mu g/50\mu l$ | fresh human serum | 58 |

Incubation of cells with EGTA-serum, blocking the classical pathway activity, demonstrated C3 conversion. EDTA-serum, where the classical and alternative pathway are blocked, C3 conversion could not be detected.

pathway activation might be a common property of human cell membrane moieties.

In this paper cultured, living and heat-killed human fibroblasts, treated with 0.1% trypsin, were found to be a potent activators of the complement system in human serum. When the cells were incubated with EGTA-serum, thus blocking classical pathway, C3 conversion could be observed. But with EDTA-serum in which both the classical and alternative pathways are blocked, C3 conversion was not seen. C3 conversion could not be detected when factor B was destroyed by pre-heating the serum at 50°C for 30 min.

The CH50 activities of the sample sera also suggest C3 conversion via the alternative pathway. After incubation of fibroblasts with EDTA-serum, the CH50 which was measured with sufficient $Mg^{++}$ and $Ca^{++}$ ions, remained high. On the other hand, when fibroblasts were incubated with EGTA-serum, the residual CH50 activity was considerably reduced, measurd with enough $Mg^{++}$ and $Ca^{++}$ ions.

This evidence strongly suggested that fibroblasts activated the complement system through the alternative pathway. Although the actual mechanism which is responsible for complement activation by fibroblasts treated with trypsin remains unknown, the treatment of cells with trypsin might alter cellular membrane constituents in such a way as to permit initiation of the alternative complement pathway. It is known that sheep erythrocytes that ordinarily can not activate the alternative complement pathway do so after treatment with neuraminidase to remove the membrane sialic acid residues[11,12]. Recently a glycoprotein of 205,000 mol wt, which was characterized as an inhibitor of the alternative complement pathway C3 convertase (C3bBb) and as a cofactor for the cleavage of the $\alpha$ chain of C3b in the fluid phase by C3b/C4b inactivator[3], was isolated from the membrane of human erythrocytes. Fearon identified the

glycoprotein as the C3b receptor (CR1). Iida et al.[13] also identified the glycoprotein from the membrane of human erythrocytes as a receptor for C3b (CR1) which promotes the dissociation of the alternative pathway C3 convertase C3bBb and the cleavage of C3b by C3b/C4b inactivator. CR1 also inactivates the C3 and C5 convertases of the classical pathway. Fearon proposed that the presence of CR1 on cell may prevent inappropriate self recognition by complement.

Although the mechanism by which trypsin treated fibroblasts activate complement is presently unknown, this antibody-independent complement activation provides evidence for an endogeneous system for recognizing altered or dameged cell membranes, and may play an important role in the pathogenesis of various human diseases or in the repair of some degenerated tissues.

## REFERENCES


1) Fearon, D.T., and Austen, K.F. : Activation of the alternative complement pathway with rabbit erythrocytes by circumvention of the regulatory action of endogeneous control proteins. J. Exp. Med. **146** : 22–23, 1977
2) Fearon, D.T., and Austen, K.F. : Activation of the alternative complement pathway due to the resistance of zymosan-bound amplification convertase to endogeneous regulatory mechanisms. Proc. Natl. Acad. Sci. USA. **74** : 1683–1687, 1977
3) Fearon, D.T. : Regulation of the amplification of C3 convertase of human complement by an inhibitory protein isolated from human erythrocytes membrane. Proc. Natl. Acad. Sci. USA. **76** : 5867–5871, 1979
4) Mayer, M.M. : Complement and complement fixation. In : Experimental Immunochemistry. Edited by E.A. Kabat and M.M. Mayer. Charles C.Thomas, Springfield, III. p. 133, 1961
5) Nagaki, K., Iida. K., and Inai, S. : A new method for the preparation of EAC14 cell with human or guinea-pig serum. J. Immunol. Methods **5** : 307–317, 1974
6) Laurell, C.B. Antigen-antibody crossed electrophoresis. Analyt. Biochem. **10** : 358–361, 1965
7) Götze, O., and Müller-Eberhard, H.J. : The C3 activator system : an alternative pathway of complement activation. J. Exp. Med. **134** : 90s–108s, 1981
8) Baker, P.J., and Osofsky, S.G. : Activation of human complement by heat-killed, human kidney cells grown in cell culture. J. Immunology **124** : 81–86, 1980
9) Corrocher, R., Falezza, G.C., Casaril, M. Aprili, G. and De Sandre, G. : Alternative pathway for complement activation triggered by human liver plasma membranes. Hepato-Gastroenterol. **28** : 195–198, 1981
10) Poskitt, T.R., Fortwengler, H.P. jr., Lunsijjs, B.J. : Activation of the alternative complement pathway by autologous red cell stromata. J. Exp. Med. **138** : 715–722, 1973
11) Pangburn, M.K., and Müller-Eberhard, H.J. : Complement C3 convertase : cell surface restriction of $\beta$1H control and generation of restriction on neuraminidase-treated cells. Proc. Natl. Acad. Sci. **75** : 2416–2420, 1978
12) Fearon, D. T. : Regulation by membrane sialic acid of $\beta$1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. Proc. Natl. Acad. Sci. **75** : 1971–1975, 1978
13) Iida, K., and Nussenzweig, V. : Complement receptor is an inhibitor of the complement cascade. J. Exp. Med. **153** : 1138–1150, 1981