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Relationship Between Bactericidal Action of Complement and Fluidity of Cellular Membranes

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The mode of complement-mediated killing of *Escherichia coli* B cells grown at 25 and 42°C and of *E. coli* K-12 UFA^{ts} cells grown in the presence of oleic and elaidic acids was examined in relation to their membrane lipid compositions and the thermodynamic properties of membrane phospholipids. Phospholipids isolated from 25°C-grown cells of strain B and from oleic acid-incorporated cells of strain K-12 UFA^{ts} had lower phase transition temperatures than did phospholipids from *E. coli* strain B grown at 42°C or those from strain K-12 UFA^{ts} grown on elaidic acid. The rate of cellular susceptibility to complement action closely correlated with the liquid crystalline phase of phospholipids of cell membranes. These findings suggest that membrane fluidity is obligatory for the final expression of complement action.

The mode of complement-mediated killing of bacterial cells has been studied by various approaches. Inoue and Kinsky (10) and Kinsky (11) produced a "lesion of membranes" by treating liposomes prepared with phospholipid and a marker with complement complexes. They verified that a target site at which complement operates is the phospholipid moiety of the membrane. Starkey and Wedgwood (19) observed that the susceptibility of bacterial cells to complement action varied with the growth temperature of the cell and with the temperature at which cells were treated with complement.

It is known (3, 4, 14, 15) that phospholipids undergo a temperature-dependent phase transition at a certain temperature. Below this temperature, the phospholipids are in a state of gel packing, with their fatty acyl chains in a restricted and ordered state; above this temperature, they are in a liquid crystal state in which the fatty acyl chains exhibit a high degree of molecular motion. This temperature is called the phase transition temperature, and it varies depending upon a variety of factors. Among them, the degree of unsaturation of the fatty acyl chains and the configuration of unsaturated fatty acids expressed in terms of *cis* or *trans* were reported to be major factors (4, 5). Increasing the degree of unsaturation results in a lowering of the transition temperature. An X-ray diffraction study reported (7, 20) that the membrane behaves in a fashion similar to that of phospholipid from the membrane with respect to phase transition temperature.

Thus, we approached the mechanism of complement-mediated killing of bacteria by correlating the complement effect with physicochemical properties of membrane phospholipids. The present experiments on complement-mediated killing of bacteria at various temperatures showed that the transition temperature with regard to the susceptibility of *Escherichia coli* to the bactericidal action of complement varied, depending on the condition of bacterial growth, and was near the phase transition temperature measured by differential scanning calorimetry (DSC), suggesting a correlation between membrane fluidity and susceptibility of bacterial cells to complement action.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains B and K-12 UFA^{ts} were used. The latter was originally isolated from strain K-12 3110 by Silbert and Vagelos (18), and was kindly given to us by Y. Akamatsu, National Institute of Health, Tokyo. This strain thermotropically requires unsaturated fatty acids for growth. A colony effectively incorporating elaidic acid was isolated from a culture of this strain by the method of Schairer and Overath (16).

Growth conditions. Strain B cells were grown at different temperatures to stationary phase in a medium composed of 2.5 g of NaCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $(NH_4)H_2PO_4$, 0.5 g of K_2HPO_4, 5 g of Casamino Acids, and 500 ml of distilled water. Strain K-12 UFA^{ts} cells were grown at 42°C to stationary phase in a medium composed of 7 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5 g of Na_3 citrate, 1 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1 mg of thiamine, 10 g of Casamino Acids, 0.5% Triton X-100, and 1,000 ml of distilled water supplemented with 0.005% oleic or elaidic acid.

Extraction of phospholipids. Cultured bacterial cells were harvested by centrifugation for 10 min at 6,000 × *g*, washed with 0.67 M phosphate buffer, pH

7.2, and suspended at a density of 100 mg/ml. The bacterial cells were disrupted through a French pressure cell at 70 kg/cm^2. The cell homogenates were centrifugated for 30 min at 29,000 × g, and the sediments were washed with 0.67 M phosphate buffer, pH 7.2, to obtain membrane fractions. Phospholipids were extracted from the membrane fractions according to the method of Bligh and Byer (2), condensed in nitrogen gas, and stored at −20°C.

Thin-layer chromatography. Thin-layer chromatography for detection and isolation of phospholipids was carried out by the method of Shibuya et al. (17). Briefly, silica gel thin-layer plates were developed with chloroform-methanol-water (65:25:4), and the spots corresponding to unsaturated fatty acids, phospholipids, amine (phosphatidylethanolamine), and glycerol (phosphatidylglycerol) were detected by exposure to iodine vapor, Zinzada reagent (molybdenum blue), ninhydrine, and periodate-Schiff reagent, respectively. Each phospholipid was recovered from the plates by extracting silica gel powder with chloroform-methanol solvent.

Gas-liquid chromatography. Gas-liquid chromatography for analysis of fatty acids was performed principally according to the method of Horning et al. (9). The chromatography was carried out at 180°C by using a Yanagimoto model GCG-550 FD gas-liquid chromatography equipped with a flame ionization detector. Stainless-steel columns, 1.8 meters long, were packed with 15% diethylene glycol succinate on Neopack AS, 60 to 80 mesh.

DSC. The phospholipid was thinly coated on the inner surface of the tube by rotary evaporation of a chloroform solution under vacuum. After the addition of glass beads (0.17 to 0.32 mm in diameter) and 0.15 M KCl, the tube was agitated by a mixer until phospholipid could no longer be seen adhering to its wall. Dispersions obtained were centrifuged for 60 min at 105,000 × g. DSC measurements were performed by using a heat leakage scanning calorimeter, SSC-540 (Daini Seikosha Co. Ltd., Tokyo), with 70-μl-volume volatile aluminum sample holders. About 20 mg (in a 20-μl volume) of the resulting sediment was placed in the sample holder, and 20 μl of water was placed in the reference holder. The holders were then hermetically sealed with a sample sealer. DSC thermograms of the lipid phase transition were observed at a heating rate of 400 s/degree Centigrade.

Complement. A pool of fresh sera isolated from 20 guinea pigs was used as a source of complement. Lysozyme was removed by absorption with 1% bentonite for 10 min at 4°C. Serum heated for 30 min at 56°C was added to the control tubes.

Antisera. Rabbits were immunized three times each with 5 × 10^8 cells of *E. coli* strain B or K-12 UFA^{ts} at 1-week intervals. Blood was isolated 1 week after the last immunization; the serum was decomplemented by heating for 30 min at 56°C.

Treatment of cells with antiserum and complement. The grown cells were washed with 0.01 M phosphate-buffered saline, pH 7.2, supplemented with 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+} (buffer A). Buffer A was used for dilution of antiserum and complement. The reaction mixture consisted of 3 × 10^7 cells, complement diluted 33-fold, and antiserum diluted 50-fold in a total volume of 0.5 ml. The mixture was incubated at various temperatures for 30 min for strain B or for 45 min for strain K-12 UFA^{ts}. After incubation, it was subjected to centrifugation at 4,000 × g for 20 min; the cells were washed three times with buffer A.

Assay for bactericidal activity. The bactericidal activity was determined by irreversible loss of β-galactosidase (β-gal)-synthetic activity. For β-gal induction, B cells treated with complement were incubated in a reaction mixture consisting of 0.67 M phosphate buffer, pH 7.0, 1% Casamino Acids, and 0.5 mM isopropyl-β-D-thiogalactopyranoside for 60 min at 37°C. K-12 UFA^{ts} cells were induced for β-gal synthesis in the same medium supplemented with 0.005% oleic acid for 60 min at 40°C. After induction, the reaction mixture was supplemented with 0.9 ml of 0.67 M phosphate buffer and 1 drop of toluene, shaken vigorously for 10 s, and incubated for 30 min at 34°C for breakdown of permeation barrier. After evaporation of toluene, cellular β-gal activity was assayed by the method of Hestrin et al. (8). The difference between β-gal units in induced and uninduced cells was taken as units of β-gal synthesized. Percent killing was calculated by the following equation.

$$\% \text{ killing} = \left(1 - \frac{\beta\text{-gal units synthesized by cells treated with fresh serum}}{\beta\text{-gal units synthesized by cells treated with heated serum}}\right) \times 100$$

RESULTS

Lipid composition of cell membranes. First, the fatty acid compositions of membrane phospholipids isolated from *E. coli* strain B cells grown in a semisynthetic medium at 25 and 42°C to stationary phase were studied. The fatty acid compositions of individual phospholipids are shown in Table 1. Thin-layer chromatography of extracted lipids confirmed the existence of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. The fatty acids given in Table 1 were detected by gas chromatographic analysis of each extracted phospholipid. The percentages of total unsaturated fatty acids detected in the membrane phospholipids of cells grown at 25 and 42°C were 57 and 35, respectively. Oleic and elaidic acid-incorporated cells were obtained by growing an unsaturated fatty acid auxotroph, strain K-12 UFA^{ts}, in a semisynthetic medium supplemented with oleic and elaidic acid, respectively, at 42°C to stationary phase. The fatty acid compositions of membrane phospholipids were examined in the same way. The total unsaturated fatty acids of oleic and elaidic acid-enriched cells were calculated to be 41 and 56%, respectively (Table 2).

Gel-liquid crystal transition temperatures of membrane phospholipids. The phospholipid was made into a paste from the

TABLE 1. *Fatty acid compositions of membrane phospholipids isolated from E. coli B cells grown at 25 and 42°C to stationary phase*

Fatty acid	Fatty acid composition (%)							
	25°C-grown cells[a]				42°C-grown cells			
	Total phospholipids	CL	PE	PG	Total phospholipids	CL	PE	PG
14:0	1	1			2	3	2	
16:0	30	21	28	30	44	46	44	43
16:1	11	8	14	11	12	14	11	11
17:Δ	10	3	11	7	15	9	16	12
18:1	46	64	41	46	23	24	22	29
19:Δ	2	3	2	2	4	4	5	4
TU[b]	57	72	55	57	35	38	33	40

[a] Abbreviation: CL, Cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.
[b] Total unsaturated fatty acids.

TABLE 2. *Fatty acid compositions of membrane phospholipids isolated from E. coli K-12 UFA^{ts} cells grown in the presence of oleic and elaidic acids to stationary phase*

Fatty acid	Fatty acid composition (%)	
	Oleic acid-grown cells	Elaidic acid-grown cells
14:0	8	10
16:0	47	33
16:1	9	10
17:Δ	2	1
18:1	32	46
19:Δ		
TU[a]	41	56

[a] Total unsaturated fatty acids.

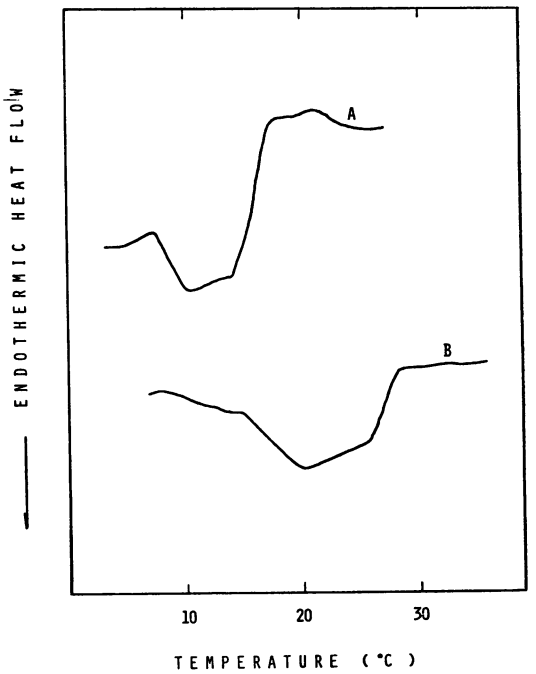

FIG. 1. *DSC heating curves of membrane phospholipids isolated from E. coli B cells grown at 25 and 42°C to stationary phase. DSC measurements were performed by using a heat leakage scanning calorimeter, SSC-540 (Daini Seikosha Co. Ltd., Tokyo). The heating rate was 400 s/degree centigrade. (A) Heating curve of membrane phospholipid isolated from E. coli B cells grown at 25°C. (B) Heating curve of membrane phospholipid isolated from E. coli B cells grown at 42°C.*

membranes of *E. coli* B cells grown at 25°C to stationary phase by the method described in Materials and Methods and was then transferred into a volatile DSC sample holder, and the lid was hermetically sealed. DSC analysis of the phospholipid obtained was carried out at a heating rate of 400 s/degree Centrigrade (Fig. 1). A broad peak of endothermic change was observed at a temperature range from 10 to 14°C. This indicates that the transition from a gel packing state to a liquid crystal state occurred in this temperature range. The curve returned to the baseline at about 17°C. This finding suggests strongly that the transition to the liquid crystal state ended at or just below 17°C. On the other hand, the phospholipid extracted from cells grown to stationary phase at 42°C exhibited the transition at temperatures ranging from 19 to 26°C. The peak was broader than that observed for the phospholipid from 25°C-grown cells. It appears that the transition ended at or just below 28°C. It is notable that the phase transition occurred at a lower temperature region in the phospholipid from 25°C-grown cells than in that from 42°C-grown cells. Phospholipids isolated from oleic and elaidic acid-enriched cells of strain K-12 UFA^{ts} showed relatively sharp DSC thermograms with apparent endothermic peaks at 19.5 and 33.2°C, respectively (Fig. 2). The endothermic figures suggest strongly that the transition was completed at about 27 and

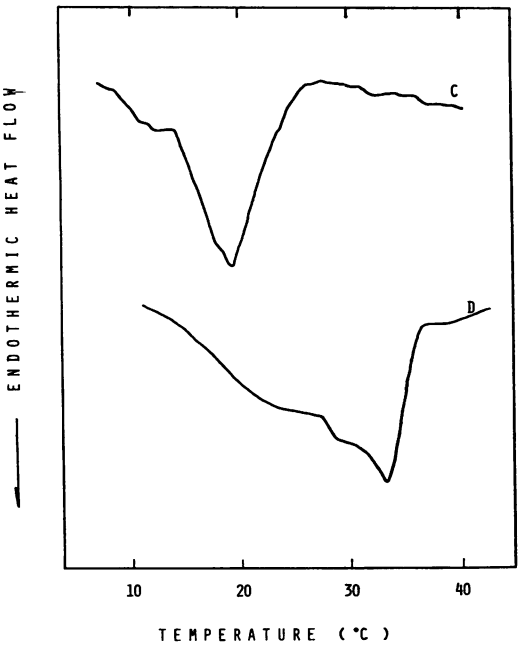

FIG. 2. *DSC heating curves of membrane phospholipids isolated from E. coli K-12 UFA^{ts} cells grown in the presence of oleic and elaidic acids to stationary phase. The conditions under which DSC was performed were as in Fig. 1. (C) Heating curve of membrane phospholipid isolated from E. coli K-12 UFA^{ts} cells grown in the presence of oleic acid. (D) Heating curve of membrane phospholipid isolated from E. coli K-12 UFA^{ts} cells grown in the presence of elaidic acid.*

37°C, respectively, in the phospholipids from oleic and elaidic acid-incorporated cells.

Complement-mediated bacterial killing as a function of temperature. Cells of *E. coli* B grown at 25°C to stationary phase were treated with rabbit antiserum to *E. coli* B cells and fresh guinea pig serum for 30 min at increasing temperatures. After the cells were washed, β-gal was induced by incubating them in a medium containing an inducer; the rate of bacterial killing was estimated from the irreversible loss of β-gal-synthetic activity. The level of bactericidal reaction was very low in the vicinity of 22°C (Fig. 3). The rate of bacterial killing increased rapidly with the increase in temperature at which the cells were treated with complement. The rapid increase in the rate of cellular killing suddenly shifted to a gradual increase at about 24°C. The increase in bactericidal rate above this temperature was gradual. *E. coli* B cells grown at 42°C to stationary phase were treated with the antiserum and complement at increasing temperatures, and the rate of cellular killing was measured in the same way. The susceptibility of the cells to complement action at various temperatures was lower than that of the 25°C-grown cells. The steep curve of cellular killing shifted to a gentle slope at around 27°C. Cells of *E. coli* K-12 UFA^{ts} grown in the presence of oleic acid were interacted with complement at increasing temperatures to determine the bacterial killing rate. A biphasic bactericidal curve having a sharp discontinuity at about 28°C was observed (Fig. 4). In contrast, the rapid increase in the killing rate of eladic acid-incorporated cells changed to a gradual increase at about 34°C. Some experiments demonstrated a shift of the rate in the range of 36 to 37°C.

DISCUSSION

The membrane lipid compositions of *E. coli* B cells grown at 25 and 42°C to stationary phase were examined. The membrane phospholipid of 25°C-grown cells was higher in unsaturated fatty acid content than was that of 42°C-grown cells. It is well known (4) that unsaturated fatty acids

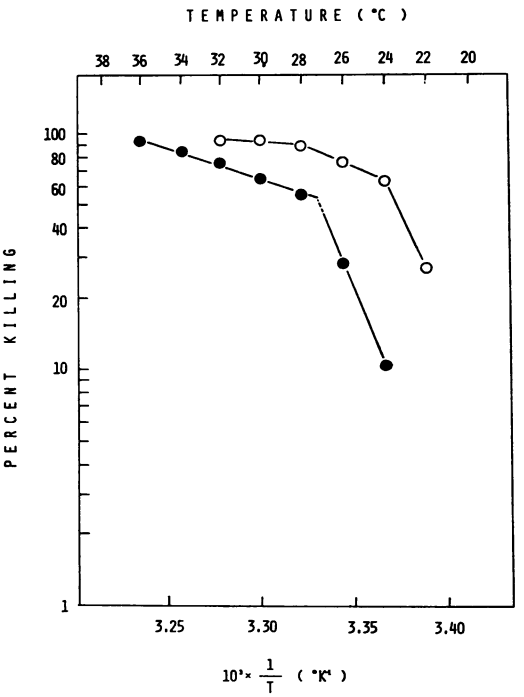

FIG. 3. *Complement-mediated killing rates of E. coli B cells grown at 25 and 42°C to stationary phase. Bacterial cells were treated with antiserum to E. coli B cells and quinea pig complement for 30 min at increasing temperatures. Percent killing (A) was calculated from irreversible loss of β-gal-synthetic activity by the equation given in the text. Symbols:* (○) *Cells grown at 25°C;* (●) *cells grown at 42°C.*

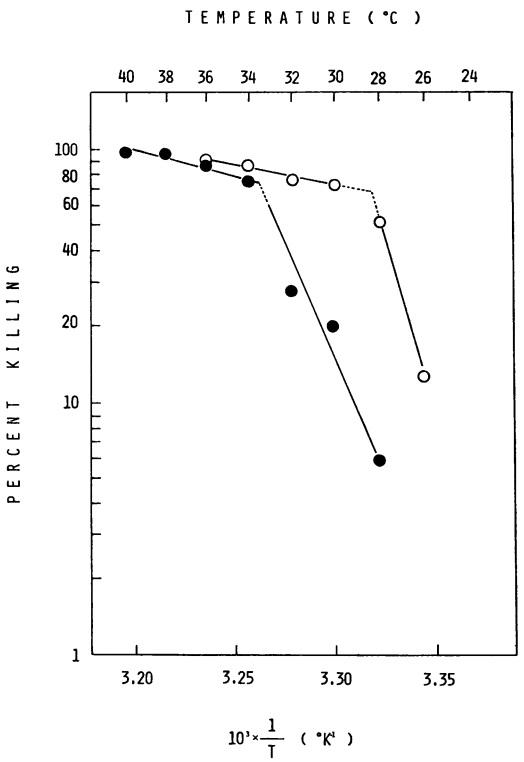

FIG. 4. *Complement-mediated killing rates of E. coli K-12 UFA^{ts} cells grown in the presence of oleic and elaidic acids to stationary phase. Experimental conditions were as in Fig. 3. Symbols:* (○) *Cells grown in the presence of oleic acid;* (●) *cells grown in the presence of elaidic acid.*

are lower in phase transition temperature than are saturated fatty acids. Accordingly, it was postulated that phospholipid extracted from 25°C-grown cells would exhibit a lower transition temperature than that extracted from 42°C-grown cells. Such was the case; the present DSC of membrane phospholipid from 25°C-grown cells demonstrated a broad peak of endotherm at temperatures ranging from 10 to 14°C, and the curve returned to the baseline at around 17°C; but phospholipid from 42°C-grown cells exhibited a broader transition at 19 to 26°C than did that from 25°C-grown cells, and the curve returned to the baseline at about 28°C, suggesting strongly that endothermic transition had finished at or just below 28°C. The complement-mediated killing experiments with 42°C-grown cells showed that the susceptibility of the cells to complement increased rapidly with the increase in temperature until about 27°C. The rapid increase in susceptibility appears to occur in the temperature range at which there is vigorous endothermic change. The abrupt reduction in the increasing rate of complement susceptibility occurred at a temperature (about 27°C) close to that (about 28°C) at which the transition to liquid crystal is considered finished. Above this temperature, further temperature increases did not cause an increase in the bactericidal rate at as high a level as that observed when the phase transition from gel to liquid crystal proceeded effectively. Thus, a close correlation between the effective action of complement and membrane fluidity was observed. It is known (5) that the phase transition temperature of oleic acid, which is a *cis*-configurated unsaturated fatty acid, lies in the range of 10 to 20°C and that of elaidic acid, which is a *trans*-configurated unsaturated fatty acid, lies in the range of 36 to 37°C. Thus, DSC on phospholipids extracted from oleic and elaidic acid-enriched cells of strain K-12 UFA^{ts} and complement-induced killing experiments were carried out to further examine the relationship between membrane fluidity and cellular susceptibility to complement action. The gel-liquid crystal transition of phospholipid from oleic acid-enriched cells occurred most actively at 19.5°C and is considered to have ended at or just below 27°C. The susceptibility to complement of oleic acid-enriched cells increased rapidly until about 28°C, and the greatly increasing rate of susceptibility shifted to the slightly increasing rate at that temperature. These results suggest strongly the existence of a close correlation between the liquid crystalline phase of the membrane and the efficient action of complement. The results obtained from physicochemical experiments on the membrane phospholipid of elaidic acid-enriched cells and from functional experiments on the cells showed the same correlation. Thus, the present series of experimental results is quite consistent with the view that the membrane fluidity permitting a high degree of molecular motion is obligatory to the efficient bactericidal action of complement.

Phospholipid extracted from 25°C-grown cells of strain B, which are relatively rich in unsaturated fatty acids, exhibited a lower transition temperature than that extracted from 42°C-grown cells, which are relatively depleted. The temperature during the shift from rapid to slow increase in complement susceptibility also was lower in the former than in the latter. Such a situation is consistent with the view mentioned above. However, there is considerable difference between (i) the temperature ranges of the main gel-liquid crystal transition and of the rapid increase in complement susceptibility and between (ii) the temperature ranges of the completion of phase transition and of the shift from rapid to slow increase in complement susceptibility. This

phenomenon is inexplicable. One possibility is that the phase transition temperature of the membrane is different from that of the phospholipid extracted from the membrane, since it is known (6) that the interaction of phospholipid with basic proteins affects the phase transition temperature of the phospholipid.

Kolb and Müller-Eberhard (13) interpreted the biological events underlying complement-induced hemolysis in the following way. The binding of C9 to to EAC1-8 complex is temperature insensitive and nonenzymatic; in contrast, C9-mediated hemolysis is indeed strongly temperature dependent. On the basis of these experimental data, they postulated that the temperature dependency of hemolysis is intimately related to the temperature-dependent fluidity of the biological membranes. Kitagawa and Inoue (12) proposed from their findings of temperature dependence of glucose release from complement-treated liposomes that liposome damage induced by complement requires some membrane fluidity.

Having defined the term bacterial killing in a strict sense, we took irreversible loss of protein synthesis instead of lack of colony development as an index of bacterial killing, since the latter may include such physiological impairment that cells are active in both respiration and macromolecule synthesis but inactive in cell division, as found in penicillin-treated bacterial cells, which do not result in cellular lysis (unpublished data).

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