DIMETHYLAMINOCHALCONE, AN INDICATOR OF STRUCTURAL CHANGES IN THE *E. coli* CELL MEMBRANE PRODUCED BY Ca^{++} CATIONS AND TRIS BUFFER

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An attempt was made to find hypothetical structural changes in the cell membrane of *Escherichia coli* under the influence of Ca^{++} cations with the aid of the uncharged fluorescent probe 4-dimethylaminochalcone (DMC). Effects of Tris buffer (0.01 M) at 0°C and of other agents, namely Mg^{++} cations and EDTA, also were tested for comparison. Treatment of the *E. coli* cells with Ca^{++} cations was shown to cause structural changes in the surface of the cell membrane which differed from the changes produced by treatment with Tris buffer at 0°C, Mg^{++} cations, and EDTA. DMC can be used with success as an indicator of structural changes in biomembranes.

KEY WORDS: Ca^{++} cations; Tris buffer; *Escherichia coli*; fluorescent probe.

Treatment of intact *Escherichia coli* cells with Ca^{++} cations in the cold leads to a marked increase in the ability of the cells to adsorb exogenous DNA and also enables this DNA to penetrate inside the cells [6, 7]. According to other workers' [5] and our own observations [3], interaction of Ca^{++} cations with intact cells of *E. coli* and other gram-negative organisms probably leads to structural changes in the surface of the cell walls and to liberation of products of lipopolysaccharide nature into the medium.

In this investigation, in order to record the changes mentioned above in the cell membranes of bacteria, an attempt was made to use the uncharged hydrophobic fluorescent probe 4-dimethylaminochalcone (DMC), suggested by Sorokov et al. [4]. This probe, which gives weak fluorescence in aqueous solutions, fluoresces brightly in suspensions of artificial biomembranes, and is nowadays being increasingly used to study membranous structures [1, 2]. For comparison with the effect of Ca^{++} cations, effects of the action of Tris buffer at 0-4°C, Mg^{++} cations, and EDTA on the membrane of *E. coli* cells also were investigated.

EXPERIMENTAL METHOD

Bacterial cells of *E. coli* K12JC7623 were grown to a concentration of $5 \cdot 10^8$ cells/ml in medium P [6] with aeration, then washed in 0.01 M $CaCl_2$ or 0.01 M Tris buffer (pH 7.2, 25°C) and resuspended in one of the following solutions: 0.05 M $CaCl_2$, 0.01 M Tris buffer (pH 7.2), 0.05 M $MgCl_2$, $2 \cdot 10^{-4}$ M EDTA (pH 7.2), and incubated at 0-4°C or 25°C for 20 min. This cell suspension will subsequently be called CS. Part of the CS was sedimented by centrifugation and the cells were resuspended in the same solutions. This suspension of washed cells will subsequently be called WCS. Solutions of DMC with a concentration of the latter of about 6-7 μM, in 0.01 M Tris buffer, pH 7.2, or 0.05 M $CaCl_2$, pH 6.0, were titrated with cells from CS and WCS to a concentration of not more than $1.7 \cdot 10^8$ cells/ml at 25°C. The intensity of fluorescence was measured on a Hitachi (Japan) model 204 spectrofluorometer in a 1-cm cuvette. Fluorescence was excited by light at λ = 420 nm and measured at λ = 495-506 nm.

EXPERIMENTAL RESULTS

Structural changes in the surface of the cell membrane would be expected to be reflected in the ability of the cells to interact with molecules of the probe. This can be judged from changes in fluorescence of the probe. Analysis of graphs of intensity of fluorescence of the

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TABLE 1. Values of 1/log I_{lim} and Corresponding Intensities of Fluorescence I_{lim} (in arbitrary units) of DMC in Cells Treated with Various Solutions

Index	CS				WCS			
	Tris buffer at 0°C	Ca^{2+}	Mg^{2+}	EDTA	Tris buffer at 0°C	Ca^{2+}	Mg^{2+}	EDTA
1/lg I_{lim}	0,52±0,03 (8)	0,50±0,03 (6)	0,51	0,67	0,41±0,02 (6)	0,40±0,02 (6)	0,41	0,31
($M \pm m$) I_{lim}	84	100	91	31	274	316	274	1700

Legend: Here and in Table 2, numbers in parentheses denote number of determinations; where no value of m is shown the result is the mean of two independent measurements.

TABLE 2. Number of Occupied Combining Sites with DMC in *E. coli* Cells After Various Treatments as a Ratio of CS in Tris Buffer at 0°C (100%)

Cells	Tris buffer	Ca^{2+}	Mg^{2+} or EDTA
CS	100	55±13 (4)	78 92*
WCS	59^{25} 31±6 (5)	42^{25} 56±14 (4)	70^{25} 30 3*

Legend. The index 25 gives the temperature at which the cells were treated.

probe as a function of cell concentration, plotted between reciprocal coordinates, and simple calculation [3] give quantitative estimates for the relative change in the total number of occupied combining sites for the probe on the surface of the cells and quantum yields of fluorescence. In the present investigation, because of the considerable gradient of the change in intensity of fluorescence (I), instead of values of 1/I, values of 1/log I were plotted along the axis. The molar ratio DMC/lipid was about 2 in all the experiments, and for that reason the number of occupied combining sites corresponded to the number of all accessible areas for binding with DMC. To minimize error during extrapolation of the titration curves, all the curves were plotted by the method of least squares. Despite this, the results of individual experiments varied considerably.

Values of 1/log I_{lim} and the corresponding values for the intensity of fluorescence when all molecules of the probe were bound, found by extrapolation of the titration curves in reciprocal coordinates, are shown in Table 1. Since the concentration of dye in all the experiments was constant, the relative increase or decrease in quantum yield can be judged relative to the values of I_{lim}. Relative changes in the number of combining sites (n) with DMC after "washing" of CS, and also of CS in different solutions (for example, n_{CS}/n_{WCS} in Tris buffer), are shown in Table 2. As regards their effect on binding of DMC by the cells, Ca^{++} cations were found to differ considerably from Tris buffer at 0°C, Mg^{++} cations, and EDTA.

According to the results, the number of combining sites with the probe and the quantum yield of fluorescence of the probe in CS treated with Ca^{++} in the cold and with Tris buffer at 25°C were virtually indistinguishable. After treatment of the cells with 0.01 M Tris buffer at 0°C, the number of sites was doubled although the quantum yields were unchanged. In WCS treated with Ca^{++} cations the number of combining sites remained the same, whereas in cells treated with Tris buffer it was reduced by two-thirds. The same decrease in the number of combining sites was observed also in WCS treated with Mg^{++} cations. An even more striking difference was observed in WCS treated with EDTA. The number of combining sites in this case was reduced to 5% of the initial value (in CS). The quantum yields of fluorescence of the probe changed considerably in all cases. Treatment of intact *E. coli* cells with EDTA and Tris buffer at 0-4°C is known to lead to marked structural changes in the cell wall, accompanied by loss of considerable amounts of lipopolysaccharide (LPS, to 50%), phospholipid (to

20%), and protein (to 2%) [4]. These structures probably are components of the micelle-like particles found on the surface of the external membrane (EM) on electron microscopy of preparations obtained by the freeze-etching method [9]. Micelle-like particles on the surface of EM may probably act as combining sites with DMC. Heterogeneity of fluorescence of DMC in preparations of artificial phospholipid membranes, due to differences in combining of the probe with the surface of the membrane depending on the DMC/phospholipid ratio, has been described by Dobretsov et al. [2]. The postulated the existence of three types of combining sites with DMC, differing in their degree of affinity for the probe. The number of types of combination of natural, heterogenous membranes may probably be greater still. In the present case, because of the excess of DMC, it might be supposed that all potential combining sites with the probe were occupied. The suggestion was confirmed in titration experiments in which the intensity of fluorescence was measured at 540 and 495 nm. The ratio between the intensities of fluorescence at these wavelengths, characterizing the relative redistribution of probe molecules from the "strong," more hydrophobic regions (I_{max} at 495 nm) to "weak," less hydrophobic (I_{max} at 540 nm) regions remained unchanged. The decrease in the number of combining sites after washing cells treated with Tris buffer at 0°C with Mg^{++} cations and EDTA, and increase in the quantum yields of fluorescence indicate that the weak combining regions, probably consisting of micelle-like particles, are "washed off" [9]. In the outer layer of EM, it is suggested, there is only a small fraction of phospholipids. These phospholipids are probably inaccessible for exogenous agents such as phospholipases [8], on account of screening by the b and d proteins of EM and the heptose-bound glucose of LPS. LPS on the surface of the cells is very labile, and under the influence of cations may condense, thus leaving the surface of EM "bare" [5]. Possibly in CS treated with Ca^{++} cations more of the phospholipids of the latent fraction are accessible for DMC molecules than in CS treated with Tris buffer at 0°C, Mg^{++}, and EDTA. It is also possible that Ca^{++} combines with both or one of the b and d proteins, leading to perturbations of the surface and ultimately to the effect mentioned above. The phospholipid regions are evidently characterized by a high quantum yield of fluorescence of the probe.

It is interesting to note that, according to the results of determination of rhamnose in cells supernatants, the content of carbohydrates (hypothetically, part of LPS) in cells treated with Ca^{++} was greater in nearly every case than in cells treated with Tris buffer at 0°C. Furthermore, in cell supernatants treated with Ca^{++}, more protein was found. Possibly, therefore, the increase in the quantum yield of fluorescence on the probe in WCS treated with Ca^{++} was due to liberation of the carbohydrate moiety of LPS and protein into the medium, thus "improving" access for DMC to phospholipids. Treatment of the cells with Ca^{++} cations at 25°C does not lead to any changes in the number of combining sites or in the quantum yield of fluorescence of the probe compared with treatment at 0°C, although the permeability of the cell membranes relative to molecules of exogenous DNA and to certain DNA-tropic antibiotics is substantially modified. In all probability these changes can be detected only by measuring fluorescence at temperatures close to 0°C [3]. The absence of differences at room temperature supports a transient effect of an increase in permeability at 0°C under the influence of Ca^{++}.

It can be concluded from the results that the use of DMC as fluorescent probe enables structural changes to be detected in biomembranes after treatment in various ways, although the precise molecular interpretation vary and are difficult. By means of this probe it was shown that interaction between Ca^{++} cations and *E. coli* cells leads to completely different structural changes in the surface of the cell membrane compared with the action of Tris buffer at 0°C, Mg^{++} cations, and EDTA. Possibly it is these differences that determine the unique feature of Ca^{++} cations that makes them irreplaceable for the induction of changes in permeability of the cell membranes for DNA molecules.

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COMPARATIVE STUDY OF ROSETTE- AND PLAQUE-FORMATION IN RATS INFECTED WITH *Mycoplasma arthritidis* AND *Acholeplasma laidlawii*

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Mycoplasma arthritidis was shown to inhibit rosette and plaque formation in rats infected with this species of mycoplasma. By the 15th day the immune response in the control and experimental groups was equal again. At later stages strong stimulation of populations of rosette-forming (RFC) plaque-forming (PFC) cells was observed, subsiding toward the 150th day. Conversely, *Acholeplasma laidlawii* stimulates RFC and PFC at all periods of infection. The relatinship between these phenomena and the pathogenic properties of mycoplasmas is discussed.

KEY WORDS: mycoplasma; rosette formation; plaque formation.

There is evidence that cellular immunity plays an essential role in protection against infection caused by mycoplasmas (arthritis, acute respiratory diseases, etc.) [4, 11]. The study of cellular reactions is therefore important in connection with the study of mechanisms of development of the lesions.

The dynamics of the primary immune response to sheep's red blood cells was investigated in animals infected with *Mycoplasma arthritidis* and *A. laidlawii*.

These species of mycoplasmas were chosen because one of them (*M. arthritidis*) is a generally accepted pathogenic species, giving rise to polyarthritis in mice and rats, whereas the other (*A. laidlawii*) belongs to a genus whose pathogenicity for rats has not been proved.

EXPERIMENTAL METHOD

All the experiments were carried out on Wistar rats weighing 140-250 g. *M. arthritidis* (strain PG6) and *A. laidlawii* were grown at 37°C in broth prepared from a tryptic digest of bovine heart with the addition of 20% normal horse serum. The animals were infected intraperitoneally with undiluted 4-day cultures of *M. arthritidis* [dose 10^8 colony-forming units (cfu) in 1 ml] and 2-day cultures of *A. laidlawii* (dose 10^9 cfj/ml), and also with cultures diluted 10 and 100 times with broth. Control animals were given an injection of 1 ml broth.

Immunization with sheep's red blood cells was carried out 5 days before the immune response was tested. The population of rosette-forming cells (RFC) was studied by the method of Biozzi et al. [3] in the modification of Khorobrykh et al. [1], and the population of plaque-forming cells (PFC) by the method of Jerne and Nordin [6].

EXPERIMENTAL RESULTS

During the first week after infection with *M. arthritidis* the immune response to sheep's red cells was sharply inhibited in the experimental animals compared with that in the control (Fig. 1). The degree of inhibition was a linear function of the dose of the mycoplasmas. After infection with the undiluted culture the number of RFC and PFC was reduced by 83 and 90% respectively compared with the control. A dose of 10^7 cfu/ml inhibited rosette and plaque

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