Biochimica et Biophysica Acta, 508 (1978) 260—276

BBA 77977

THE BINDING OF LIPOPOLYSACCHARIDE FROM *ESCHERICHIA COLI* TO MAMMALIAN CELL MEMBRANES AND ITS EFFECT ON LIPOSOMES

M. DAVIES *, D.E.S. STEWART-TULL and D.M. JACKSON

Microbiology Department, University of Glasgow, Alexander Stone Building, Garscube Estate, Bearsden, Glasgow (U.K.)

(Received September 7th, 1977)

Summary

The kinetics of the absorption of ^{32}P- or ^{14}C-labelled lipopolysaccharide from *Escherichia coli* NCTC 8623, serotype 0 125, chemotype XII, to erythrocytes, leukocytes, peritoneal macrophages and peritoneal lymphocytes was examined. Under variable conditions maximal levels of binding were found due to saturation of receptor sites on the cell membrane or steric hindrance by bound lipopolysaccharide. During adsorption slight leakage of haemoglobin was found but complete lysis of erythrocytes was ruled out after noting the effect of lipopolysaccharide on artificial lipid bilayers.

The affinity of lipopolysaccharide to cell membranes revealed a consistent pattern of cyclic fluctuation between adsorption and desorption. A model was proposed to explain this cyclic fluctuation in binding based on membrane reorganization. It was significant that the cycle of lipopolysaccharide adsorption-desorption proceeded to completion even if the process was interrupted. The indication was that, once triggered, membrane reorganization occurred independently without influence from the test environment.

Introduction

The adherence of bacteria or their antigens to mammalian erythrocytes is a recognized phenomenon [1—7]. This adherence caused an alteration in the serological specificity of the erythrocytes [8] and it was found that the modification was due to polysaccharide determinants. The bacterial antigens which adhered to the erythrocytes were referred to by different workers as "erythrocyte-modifying antigens" [9], "haemosensitins" [10], or "erythrocyte-coating

* Present address: Department of Immunology, University of Manitoba, 795 McDermot Avenue, Winnipeg, R3E 0W3, Canada.

antigens" [11]. They included the O-somatic antigens of the gram-negative bacteria [6,12]. The lipopolysaccharide complex of polysaccharide, lipid and protein [13,14], morphologically resembled membrane fragments of varying shapes [15—18]. The membranous fragments were visualized as a bilayer, each half of which was composed of polysaccharide co-valently linked with lipid A. In aqueous solution the two halves bond together with the non-polar hydrophobic lipid buried inside the structure and the polysaccharide moiety exposed to the environment [19]. These lipopolysaccharide membranous structures possessed an affinity for mammalian cell membranes [4,20].

The present investigation was carried out as part of a study on the relationship between the affinity of bacterial substances for mammalian cell surfaces and their activity as immunological adjuvants. Since Ginsberg et al. [21] showed that lipopolysaccharide caused cellular modifications and Johnson et al. [22] showed that lipopolysaccharide preparations stimulated an adjuvant effect in mice it was decided to measure the extent and behaviour of the binding of lipopolysaccharide to mammalian cell membranes.

Materials and Methods

Culture

Escherichia coli NCTC 8623 was obtained from the National Collection of Type Cultures, Colindale, London, U.K. The serotype of this strain is O 125 : K70 (B15) : H19 and the chemotype is XII. The basal core of the lipopolysaccharide contains heptose, 2-keto-3-deoxy-octonate, glucosamine, galactose and glucose. The specific side-chain polysaccharide is composed of galactosamine, mannose and fucose [14].

Media

(i) Starter cultures were grown in a medium containing 30 g casamino acids (Difco Laboratories Ltd.), 10 g yeast extract (Oxoid Ltd.) and 10 g glucose (British Drug Houses, Poole, Dorset) per litre. The casamino acids and yeast extract base were sterilised at 121°C for 15 min, the glucose was filtered and added aseptically to the sterile base medium.

(ii) The minimal medium of Davis and Minglioli [23] was used to grow large quantities of organisms for lipopolysaccharide production. This medium was modified for use in the production of ^{32}P-labelled lipopolysaccharide. The medium contained 0.1 g $MgSO_4 \cdot 7H_2O$/1.0 g $(NH_4)_2SO_4$/0.5 g $Na_3C_6H_5O_7 \cdot 2H_2O$/5.0 ml trace element solution/20.0 ml sterile 10% (w/v) glucose solution/975 ml distilled water. Since the medium was phosphate free, 10 g/l casamino acids were added [24].

(iii) Mammalian cell maintenance medium was prepared by aseptically adding 5.0 ml sterile normal rabbit serum, 0.5 g lactalbumin hydrolysate, 30 units heparin (100 000 I.U./ampoule: Koch Light Laboratories, London) and 1.0 ml Cristamycin (10 000 units penicillin and 10 mg streptomycin/ml: Glaxo Laboratories Ltd., U.K.) to 96.0 ml Hanks balanced salt solution.

Growth of E. coli NCTC 8623

Starter cultures were prepared in liquid medium incubated at 37°C for 24 h.

The resultant growth, 1% (v/v), was used to inoculate the Davis and Minglioli minimal medium. Cultures were incubated at 37°C for 7 h in an orbital incubator shaking at 150 rev./min. Cells were harvested by centrifugation at 2000 rev./min (1300 × *g*) for 30 min in a 6 L Mistral centrifuge (Manufacturing and Scientific Equipment, Crawley, U.K.). The cell pellet was washed three times with sterile 0.85% (w/v) NaCl.

Extraction of lipopolysaccharide from E. coli NCTC 8623

The phenol/water method of Westphal et al. [25] was used. The aqueous extracts were concentrated using Carbowax 4000 (B.D.H. Ltd.) and centrifuged at 34 000 rev./min (100 000 × *g*) for 4 h in an MSE 65 centrifuge. The pellet was resuspended in 50 ml distilled water and added to a solution of ribonuclease (5 mg in 50 ml 0.05 M Tris · HCl buffer at pH 7.7). After incubation at 37°C for 6 h the mixture was recentrifuged and the pellet was washed until nucleic acid contamination, as shown by ultraviolet spectrophotometry, was removed. The lipopolysaccharide was freeze-dried and stored in a refrigerator.

Lipopolysaccharide preparations

Commercial preparations of lipopolysaccharide from *Salmonella typhi* 0901, *Shigella flexneri* and *E. coli* 055:B5 were obtained from Difco Laboratories Ltd., Mich., U.S.A. Samples of lipopolysaccharide from *Bordetella pertussis* X mode and *B. pertussis* C mode originally derived from a culture of *B. pertussis* 18344 (Connaught Medical Laboratories, Toronto) were kindly provided by Professor A.C. Wardlaw, and samples of lipopolysaccharide from *Bordetella bronchiseptica* and *Franciscella tularensis* were kindly provided by Dr. A. MacLennan, Microbiological Research Establishment, Porton.

Measurement of the uptake of carbohydrate by erythrocytes

The detailed procedure was described by Stewart-Tull et al. [26].

Preparation of radioactively-labelled lipopolysaccharide from E. coli NCTC 8623 using [^{14}C]glucose

(a) Diauxic growth. A starter culture was set up in Davis and Minglioli minimal medium containing glucose as the scole carbon source. This culture was inoculated (1%, v/v) into minimal medium containing lactose (200 mg/100 ml) and [^{14}C]glucose (23 μCi/100 ml). The culture was connected to a flask containing 1 M NaOH to absorb evolved $^{14}CO_2$ and was incubated at 37°C for 6 h in an orbital incubator. The cells were harvested and lipopolysaccharide was prepared as described above.

(b) Pulse-labelling. A starter culture was inoculated into minimal medium with glucose as sole carbon source and incubated at 37°C for 5 h in an orbital incubator. The cells were harvested by centrifugation at 2000 rev./min (1300 × *g*) for 15 min in an MSE 6L Mistral centrifuge; the pellet of cells was washed twice in 0.85% (w/v) NaCl. The cells were resuspended in 100 ml minimal medium without glucose and placed in a flask connected by a side-arm to a flask containing 1 M NaOH. Glucose containing 460 μCi (Amersham Radiochemicals Ltd., specific activity 230 Ci/mol glucose) was injected into the medium through a sterile port and the culture was incubated at 37°C for 63 min

(1.5 doubling times of starter culture). After incubation cells were harvested and the lipopolysaccharide was extracted.

Preparation of radioactively-labelled lipopolysaccharide from E. coli NCTC 8623 using [^{32}P]orthophosphate

A starter culture (1%, v/v) was used to inoculate the modified Davis and Minglioli minimal medium containing [^{32}P]orthophosphate (Amersham Radiochemicals Ltd., specific activity 1 mCi/ml) at a concentration of 2 mCi/l. The culture was incubated at 37°C for 7 h in an orbital incubator. The cells were harvested and the lipopolysaccharide was extracted.

Measurement of cell-associated and free radioactivity in reaction mixtures

The radioactivity of samples containing ^{14}C and ^{32}P was measured by a liquid scintillation method in an Automatic Beta-Gamma Spectrometer (Nuclear Enterprises, Edinburgh, U.K.). After incubation of cells with either ^{14}C- or ^{32}P-labelled lipopolysaccharide, cell-associated radioactivity was determined as follows. The cells were pelleted by centrifugation at 3500 rev./min (1750 $\times g$) for 5 min and the pellet of cells was washed twice with 0.85% (w/v) NaCl. The pellet was resuspended and subjected to wet oxidation [27] by mixing 0.2 ml with 0.2 ml 60% (w/v) $HClO_4$ (B.D.H., Analar) in a scintillation vial. After thoroughly mixing 0.4 ml 30% (w/v) H_2O_2 (B.D.H., Analar) was added and the vial was sealed and heated at 70—80°C for 1 h with intermittent shaking. After cooling 8 ml of a xylene-based multipurpose liquid scintillant (NE260, Nuclear Enterprises Ltd.) were added and the radioactivity was measured.

The radioactivity associated with the supernatant fluids obtained after centrifugation at 3500 rev./min was measured using 0.5-ml aliquots and 10 volumes of liquid scintillant.

Mammalian erythrocyte suspensions

The samples of blood from all species were collected into 3.8% (w/v) sodium citrate to prevent coagulation. Sheep and horse blood were collected aseptically from the jugular vein using the procedure described by Stewart-Tull and Rowe [28]. Blood was withdrawn from the ear of New Zealand White rabbits, from anaesthetized Ham 1/Cr mice by cardiac puncture and from an arm vein of human volunteers. Blood was centrifuged and the packed cells were washed with 0.85% (w/v) NaCl and diluted to provide appropriate concentrations [29].

Preparation of human leukocyte suspensions

Heparinized human venous blood (10.0 ml) was carefully layered on to 8.0 ml Ficoll/Hypaque (specific activity 1.076) in a 25-ml screw-capped container. The mixture was centrifuged at 400 $\times g$ for 20 min and the plasma decanted. The layer containing leukocytes and platelets was carefully pipetted off, resuspended in 5.0 ml Hanks balanced salt solution and centrifuged at 700 $\times g$ for 10 min. Platelets were removed by this procedure of washing and differential centrifugation. The pellet of leukocytes was resuspended in Hanks solution and washed twice with cell maintenance medium before use.

Mouse peritoneal macrophage and lymphocyte suspensions

Mice (Ham 1/Cr strain) were injected intraperitoneally with 10 ml cell maintenance medium. After 15 min the animals were killed, peritoneal washings were removed with a Pasteur pipette, placed in sterile glass petri-dishes and incubated at 37°C for 1 h to allow macrophage adherence. The supernatant was decanted into sterile petri-dishes and re-adsorbed at 37°C for 1 h. After the second adsorption to glass the supernatant fluid was examined for the presence of lymphocytes. The glass-adherent cells were suspended in cell maintenance medium and re-incubated at 37°C for 1 h; the supernatant was discarded. Glass-adherent cells were resuspended in cell maintenance medium and checked for the presence of macrophages.

Samples of both cell suspensions were stained with 0.003% (w/v) neutral red to determine viability; live cells stain with neutral red. Further samples of the cell preparations were treated with 1% (w/v) trypan blue. Although this stain is excluded by viable cells it is an intravital stain taken up by engulfment and seen as discrete granules in the cytoplasm of macrophages [30]. Hence the macrophage contamination of the lymphocyte preparation was determined and vice versa.

Preparation of artificial lipid bilayers-liposomes

Phosphatidylcholine was prepared according to the method of Pangborn [31] and liposomes as described by Stewart-Tull et al. [26]. The mixtures containing 100 μmol total lipid for negatively charged liposomes (70 μmol phosphatidylcholine/20 μmol cholesterol/10 μmol dicetyl-phosphate) or for positively charged liposomes (70 μmol phosphatidylcholine/20 μmol cholesterol/10 μmol octadeculamine) were dried down in vacuo and resuspended in 6.0 ml 0.145 M potassium chromate. The lipids were re-dispersed by ultrasonic treatment. The negatively or positively charged liposomes containing chromate were separated on a Sephadex G-50 column equilibrated with 0.145 M KCl/NaCl.

Weighed amounts of lipopolysaccharide were added to 1.0 ml liposomes in a dialysis sac. The mixtures were dialysed against 0.145 M KCl/NaCl solution at 37°C and the percentage release of chromate was monitored spectrophotometrically at 370 nm by comparison with the $E_{370,max}$ obtained after total disruption of 1.0-ml untreated liposomes with saponin.

Results

The effect of variable amounts of lipopolysaccharide on adsorption to a constant number of erythrocytes and vice versa

The amount of lipopolysaccharide adsorbed by rabbit or human erythrocytes was found to be dependent upon the amount of lipopolysaccharide in the reaction mixture when the total carbohydrate was measured. The maximum amount of lipopolysaccharide adsorbed by a single rabbit erythrocyte was 13.7 pg and human erythrocyte was 16.12 pg. A direct relationship between the amount of lipopolysaccharide added to the test and the amount adsorbed by the erythrocytes was not evident.

The next step was to examine the binding of ^{32}P-labelled *E. coli* NCTC 8623 lipopolysaccharide by rabbit erythrocytes using $8.12 \cdot 10^6$—$1.56 \cdot 10^9$ cells in

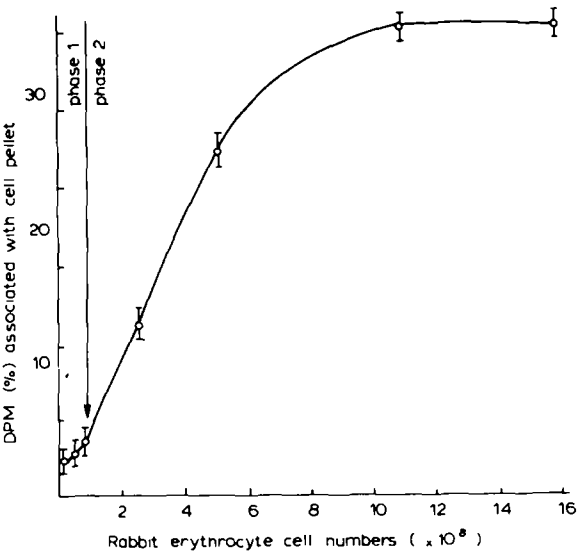

Fig. 1. The uptake of ^{32}P-labelled lipopolysaccharide from *E. coli* NCTC 8623 by varying numbers of rabbit erythrocytes. The amount of lipopolysaccharide adsorbed by the cells was calculated in terms of the radioactivity (dpm) associated with the cell pellet. The bar represents the standard error associated with each observation.

the reaction mixtures. The different concentrations of erythrocytes were incubated at 37°C for 1 h with a constant amount of lipopolysaccharide (total dpm ^{32}P-labelled lipopolysaccharide/test was 17 477) in a total volume of 1.1 ml. The erythrocytes were sedimented by centrifugation and the dpm associated with the cell pellet was determined. It was noticed that the binding of lipopolysaccharide as a function of erythrocyte concentration produced a sigmoid curve (Fig. 1). When the number of erythrocytes in the test exceeded $1 \cdot 10^9$ no further binding of the lipopolysaccharide occurred; the maximum amount adsorbed was 34.0%.

With ^{14}C-labelled lipopolysaccharide a similar effect was observed (Fig. 2). When the total dpm ^{14}C-labelled lipopolysaccharide/test was constant at 4803 there was a 33.6% uptake of lipopolysaccharide by the maximal level of $314 \cdot 10^6$ rabbit erythrocytes (A1). However, when the total dpm ^{14}C-labelled lipopolysaccharide/test was halved to 2104 there was a proportionate increase in the amount of lipopolysaccharide adsorbed to the erythrocytes, namely 59.5% (A2). When different concentrations of ^{14}C-labelled lipopolysaccharide were incubated at 37°C for 1 h with either $157 \cdot 10^6$ or $314 \cdot 10^6$ erythrocytes it was found that the amount adsorbed was proportional to the amount of lipopolysaccharide added. With $314 \cdot 10^6$ erythrocytes and ^{14}C-labelled lipopolysaccharide (4606 dpm/test) 35.4% of lipopolysaccharide was adsorbed (B1) and with $157 \cdot 10^6$ erythrocytes 27.35% of lipopolysaccharide was adsorbed (B3). The relationship between the binding of constant amounts of lipopolysaccharide to varying numbers of erythrocytes (A) and between constant numbers of erythrocytes and variable amounts of lipopolysaccharide (B) is shown clearly in Fig. 2. It was apparent that the experimental conditions reached similar proportions at points A_1 and B_1 with 33.6% and 35.04% adsorption of lipopolysaccha-

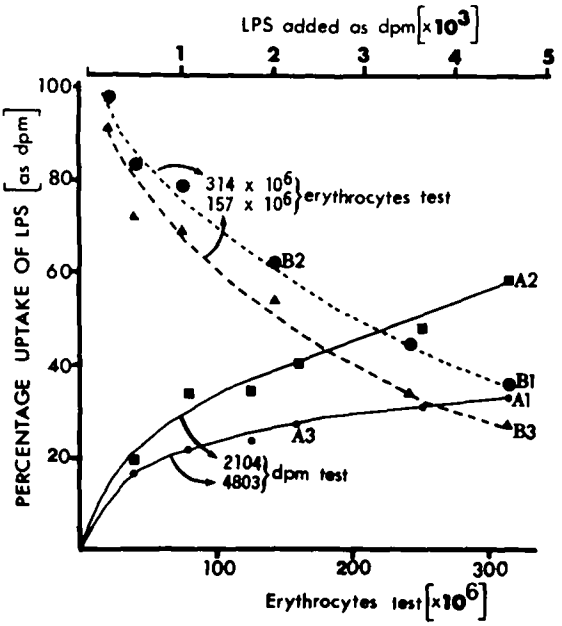

Fig. 2. The uptake of constant amounts of ^{14}C-labelled lipopolysaccharide (LPS) from *E. coli* NCTC 8623 by varying numbers of erythrocytes (lower abscissa, A curves) or the uptake of variable amounts of ^{14}C-labelled lipopolysaccharide by constant numbers of erythrocytes (upper abscissa, B curves).

TABLE I

THE EFFECT OF DIFFERENT AMOUNTS OF LIPOPOLYSACCHARIDE FROM *E. COLI* NCTC 8623 ON THE EFFICIENCY OF BINDING TO RABBIT AND HUMAN ERYTHROCYTES

Variable amounts of lipopolysaccharide were incubated with $4.8 \cdot 10^8$ human or rabbit erythrocytes at 37°C for 1 h.

Lipopolysaccharide added (mg)		Carbohydrate content of erythrocytes (mg/100 ml to Haldane standard) *		Increase in carbohydrate content				Lipopolysaccharide adsorbed **/pg	
				mg/100 ml (T—N)		mg 3 (T—N)/100			
		Rabbit	Human	Rabbit	Human	Rabbit	Human	Rabbit erythrocyte	Human erythrocyte
T	18.0	280.98	212.76	94.33	111.0	2.83	3.33	13.70	16.12
	9.0	279.84	209.42	93.19	107.66	2.79	3.23	13.50	15.64
	4.5	242.28	146.76	55.63	45.0	1.66	1.35	8.04	6.54
	2.25	212.00	130.42	25.35	28.66	0.76	0.86	3.66	4.16
	1.125	197.86	111.06	11.21	9.3	0.33	0.28	1.58	1.37
	0.56	192.72	104.42	6.07	2.66	0.18	0.08	0.85	0.37
N, untreated		186.65	101.76	—	—	—	—	—	—

* The carbohydrate content of cells was expressed as mg/100 ml hexose sugar to the Haldane haemoglobin standard of 14.6 g/100 ml using the formula: Hexose content of erythrocytes = $MCHC_A / MCHC_B \times$ hexose mg/100 $ml_B \times 14.6$/haemoglobin (g/100 ml)$_B$. A, untreated cells; B, treated cells.

** The amount of lipopolysaccharide adsorbed to the erythrocytes was determined form 3 (T—N)/H where the hexose content of *E. coli* 8623 (H) was 43.0% and the reaction mixture was 3.0 ml.

ride, respectively. By halving the amount of lipopolysaccharide at points A_2 and B_2 the uptake values were 59.5% and 61.5%, respectively. By halving the number of erythrocytes, at points A_3 and B_3 the uptake values were 27.7% and 27.35%, respectively.

Variation in the binding of lipopolysaccharide by mammalian cells in relation to the time of incubation of reaction mixtures

A constant amount of ^{32}P-labelled lipopolysaccharide from *E. coli* NCTC 8623 (total 4906 dpm/test) was added to $157 \cdot 10^6$ erythrocyte or leukocyte suspensions prepared from the blood of a human volunteer. The mixtures were incubated at 37°C for 10—140 min. The amount of lipopolysaccharide adsorbed in terms of the radioactivity associated with the cells was plotted against the time of incubation (Fig. 3). In each experiment it was noticed that there was a cyclic fluctuation in the adsorption of ^{32}P-labelled lipopolysaccharide. There were two peaks of maximum adsorption to erythrocytes at 60 and 114 min and to leukocytes at 50 and 110 min of incubation. Rabbit erythrocytes showed similar patterns of activity with peaks at 60 and 130 min. The cyclic fluctuation in adsorption of lipopolysaccharide to cells was unrelated to the type of radioactive label because the adsorption of ^{14}C-labelled lipopolysaccharide to human erythrocytes showed peaks at 60 and 130 min. The possibility existed that micelles of lipopolysaccharide might sediment with erythrocytes. However, in control experiments there was no evidence of sedimentation at 1750 $\times g$.

The adsorption of ^{32}P-labelled lipopolysaccharide (total 4906 dpm/test) to mouse peritoneal macrophages or lymphocytes (Fig. 4) shows that the uptake by peritoneal macrophages was greater than that of peritoneal lymphocytes. Nevertheless, the cyclic fluctuation in the adsorption pattern was still evident. The maximum adsorption to macrophages occurred at 54 and 89 min and to lymphocytes at 42 and 70 min.

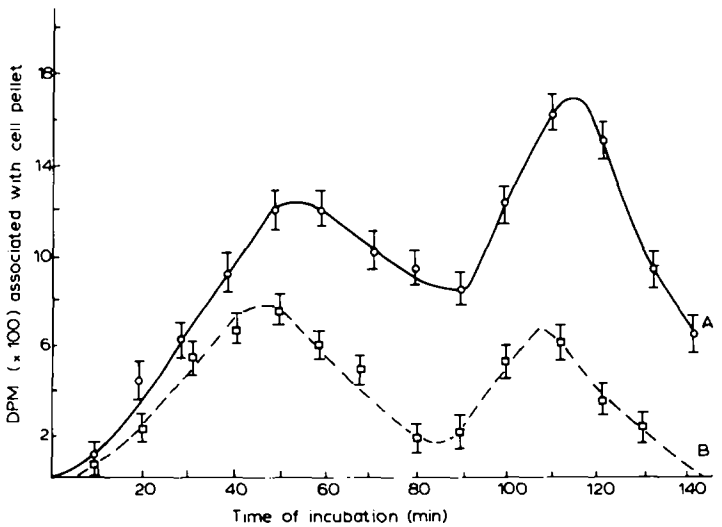

Fig. 3. The variation with time of incubation in the uptake of ^{32}P-labelled lipopolysaccharide from *E. coli* NCTC 8623 by A, human erythrocytes and B, human leukocytes.

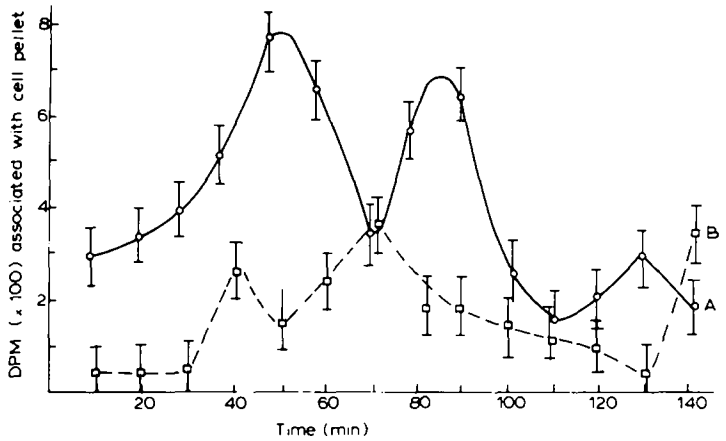

Fig. 4. The variation with time of incubation in the uptake of ^{32}P-labelled lipopolysaccharide from *E. coli* NCTC 8623 by A, mouse peritoneal macrophages and B, mouse peritoneal lymphocytes.

The effect of interrupting the incubation period on the adsorption of ^{32}P-labelled lipopolysaccharide to rabbit erythrocytes

Incubation of rabbit erythrocytes with ^{32}P-labelled lipopolysaccharide from 0 to 240 min at 37°C produced the pattern of adsorption shown in Fig. 5A,

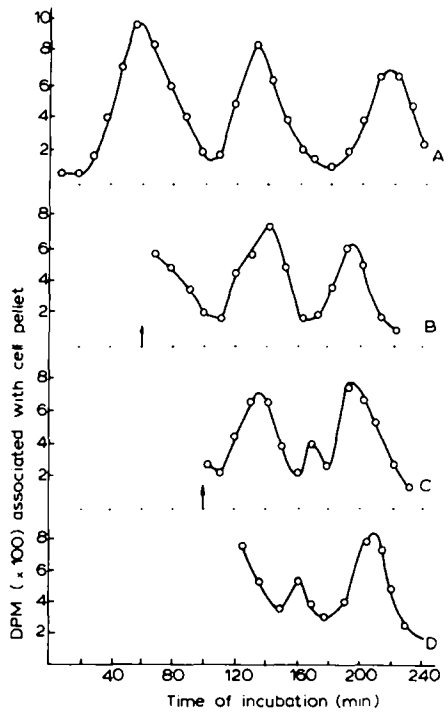

Fig. 5. The variation with time of incubation in the uptake of ^{32}P-labelled lipopolysaccharide by rabbit erythrocytes showing the effect of interrupting the binding cycle. A represents a complete uninterrupted test; B, C and D were interrupted at 60, 100 and 120 min, respectively, the erythrocytes were washed and re-incubated with fresh ^{32}P-labelled lipopolysaccharide.

where the amount of lipopolysaccharide adsorbed by the cells in terms of cell-associated radioactivity (dpm) was plotted against the time of incubation. Peaks of maximum adsorption occurred at 60, 130 and 200 min. An aliquot of erythrocytes incubated for 60 min (t_{60} cells) with ^{32}P-labelled lipopolysaccharide was washed in 0.85% (w/v) NaCl, a fresh sample of ^{32}P-labelled lipopolysaccharide was added and incubation was recontinued. It was found that peaks of maximum adsorption occurred after 80 and 140 min, (Fig. 5B) which corresponded to the peaks at 140 and 200 min in the uninterrupted test. A similar effect was observed with erythrocytes washed after 100 and 130 min (t_{100} and t_{130} cells, respectively) and subsequently re-incubated with fresh samples of ^{32}P-labelled lipopolysaccharide. The t_{60} cells were interrupted at the peak of maximum adsorption, whereas the t_{100} cells were interrupted at the point of maximum desorption. However, in both cases the adsorption of lipopolysaccharide to the erythrocytes revealed a continuation of the pattern of adsorption and cyclic fluctuation (Figs. 5C and 5D) which was observed in the uninterrupted test system.

The supernatant from the t_{60} test mixture was added to an untreated suspension of rabbit erythrocytes and incubated at 37°C. These erythrocytes showed peaks of uptake similar to those shown in Fig. 5A and confirmed that free lipopolysaccharide remained in a soluble state and was not sedimented with erythrocytes during low speed centrifugation.

Inhibition of the adsorption of radioactively-labelled lipopolysaccharide to erythrocytes by variable amounts of unlabelled homologous lipopolysaccharide

Human erythrocytes ($157 \cdot 10^6$/test) and ^{14}C-labelled lipopolysaccharide (987 dpm) from *E. coli* NCTC 8623 were incubated at 37°C for 1 h in the presence of variable amounts of unlabelled lipopolysaccharide (5—70 μg) from the same organism. It was found that as the amount of unlabelled lipopolysaccharide increased the dpm associated with the cell pellet decreased. This effect was

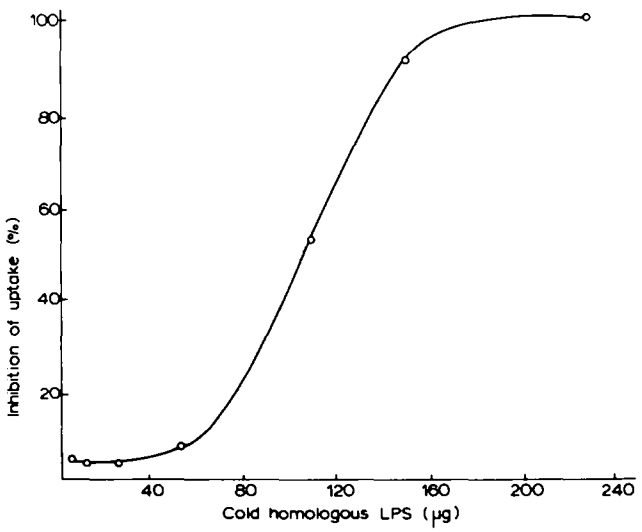

Fig. 6. Inhibition of uptake of ^{32}P-labelled lipopolysaccharide from *E. coli* NCTC 8623 to rabbit erythrocytes by the addition of unlabelled homologous lipopolysaccharide (LPS).

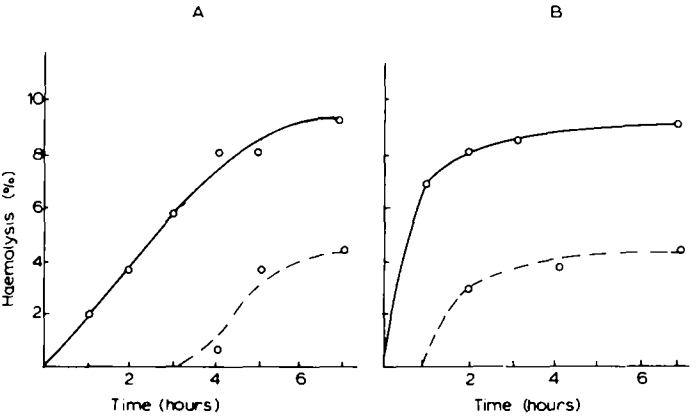

Fig. 7. Haemoglobin leakage from, A, sheep erythrocytes or B, horse erythrocytes on incubation with lipopolysaccharide from *E. coli* NCTC 8623. The solid line represents leakage with 2 mg/ml lipopolysaccharide and the interrupted line with 1 mg/ml.

linear in the presence of 15—50 μg unlabelled lipopolysaccharide in the mixture. Similarly, rabbit erythrocytes ($157 \cdot 10^6$/test) and ^{32}P-labelled lipopolysaccharide (15 076 dpm) from *E. coli* NCTC 8623 were incubated at 37°C for 1 h in the presence of unlabelled lipopolysaccharide (6—220 μg). It was found that unlabelled lipopolysaccharide inhibited the adsorption of ^{32}P-labelled lipopolysaccharide and this effect was linear in the presence of 60—140 μg unlabelled lipopolysaccharide (Fig. 6).

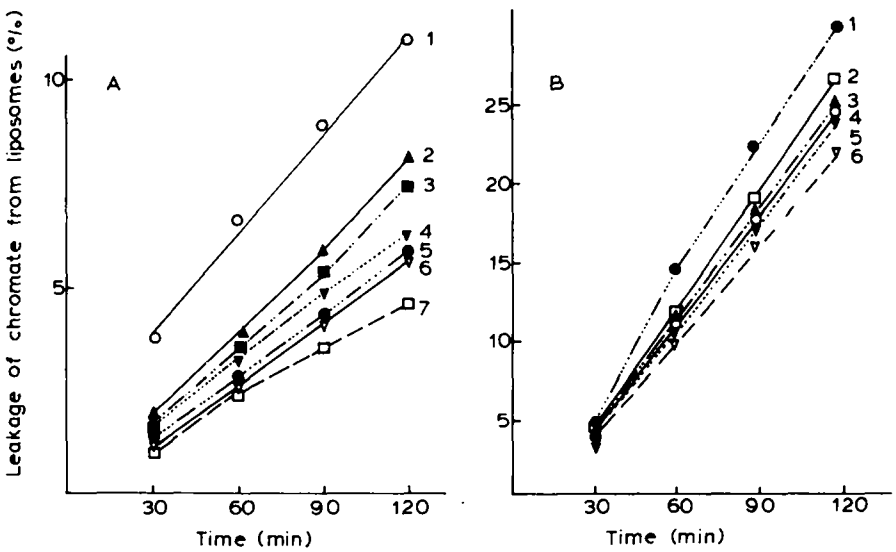

Fig. 8. (A) The effect of 1.0 mg lipopolysaccharide from different gram-negative organisms on negatively-charged liposomes. 1, control untreated liposomes; 2, liposomes treated with *B. bronchiseptica* lipopolysaccharide; 3, *Salm. typhi* lipopolysaccharide; 4, *E. coli* lipopolysaccharide; 5, *Fr. tularensis* lipopolysaccharide; 6, *Shig. flexneri* lipopolysaccharide; 7, *B. pertussis* lipopolysaccharide. (B) The effect of 1.0 mg lipopolysaccharide on positively-charged liposomes. 4, Control untreated liposomes; 1, liposomes treated with *Fr. tularensis* lipopolysaccharide; 2, *Shig. flexneri* lipopolysaccharide; 3, *B. bronchiseptica* lipopolysaccharide; 5, *E. coli* lipopolysaccharide; 6, *B. pertussis* lipopolysaccharide.

Leakage of haemoglobin from erythrocytes and divalent anions from liposomes in the presence of lipopolysaccharide

During this study it was noticed that the supernatants from the reaction mixtures were coloured red and showed E_{max} at 412 and 540 nm. This indicated that haemoglobin was released from the erythrocytes during incubation with lipopolysaccharide. There was a linear relationship between the concentration of lipopolysaccharide (0.3—20.0 mg) and the degree of leakage; 1.0 mg lipopolysaccharide caused 4.2% and 2.0 mg caused 9.0% leakage from $5 \cdot 10^9$ sheep erythrocytes (Fig. 7A). With horse erythrocytes 1.0 mg lipopolysaccharide caused 4.4% leakage and 2.0 mg 9.5% leakage (Fig. 7B). This effect was generally greatest with horse erythrocytes and least with human erythrocytes. The effect of free haemoglobin on the lipopolysaccharide was examined by sus-

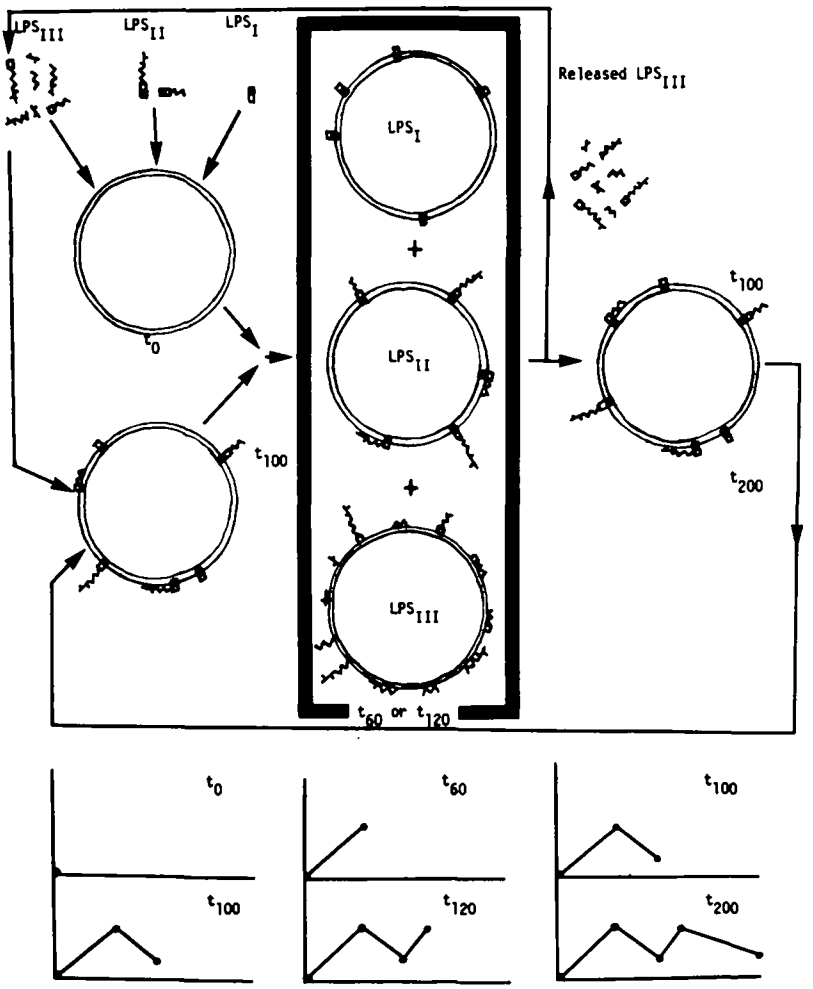

Fig. 9. Hypothetical model to explain the cyclic fluctuation in the pattern of binding of lipopolysaccharide (LPS) molecules to mammalian cell membranes. The small graphs represent the effect of time of incubation on the binding of lipopolysaccharide to membranes. ■, Lipid A; □, heptose, ⌒, basal core polysaccharide; ⌇, specific polysaccharide.

pending 10 mg lipopolysaccharide in 10.0 ml of a 10% haemoglobin solution. No evidence of aggregation, either visually or spectrophotometrically, was found and it was concluded that free haemoglobin did not cause cosedimentation of lipopolysaccharide with erythrocytes.

The release of chromate ion from phospholipid-cholesterol liposomes was expressed as the percentage of total CrO_4^{2-} trapped inside the liposomes. The total sequestered anion was determined by physical disruption of the liposomes using saponin. Various lipopolysaccharide preparations were added to samples of negatively-charged liposomes and the percentage release of chromate was determined. It was noticed that no lipopolysaccharide preparation interacted with the liposomes in such a way as to drastically increase the permeability to divalent anions. In all instances the release of chromate was less than the release from untreated negatively-charged liposomes (Fig. 8A). Similarly, with positively charged liposomes with the exceptions of lipopolysaccharide from *Fr. tularensis* and *Shig. flexneri* the release of chromate was not significantly different from that observed with untreated liposomes (Fig. 8B).

Discussion

Initially, the adsorption of lipopolysaccharide to membranes was found to be related to the number of cells until a maximum level was reached beyond which the addition of more erythrocytes to the system did not result in further adsorption of lipopolysaccharide. By varying the amount of lipopolysaccharide in contact with a constant number of erythrocytes it was found that the amount of lipopolysaccharide adsorbed was proportional to lipopolysaccharide concentration. A limit was reached, however, when further increases in the number of available lipopolysaccharide molecules did not result in a corresponding increase in bound lipopolysaccharide. It is apparent that the binding of lipopolysaccharide to membranes is controlled by both the availability of receptor sites on the membrane and differences in the polarity of the partial or complete lipopolysaccharide molecules. Consequently, saturation of receptor sites on the erythrocyte membrane and/or steric hindrance due to bound lipopolysaccharide could prevent the binding of additional lipopolysaccharide molecules.

The most interesting observations were those which involved time of exposure of erythrocytes to lipopolysaccharide. The binding of lipopolysaccharide to erythrocytes of all species tested showed a cyclic fluctuation between adsorption and desorption with even amplitude of the waves. Similar cycles of binding were observed with leukocytes, peritoneal macrophages or peritoneal lymphocytes. Since the binding of lipopolysaccharide to cell membranes is considered to be a passive process it appears that the sudden desorption of lipopolysaccharide is caused by changes at the cell surface. It was significant that some lipopolysaccharide always remained in a cell-associated state possibly due to penetration of the membrane. Vogel [32] and Hammerling and Westphal [33] suggested that stable lipopolysaccharide · cell membrane complexes were formed via lipid-lipid hydrophobic interactions. Lüderitz et al. [4], Springer et al. [34] and Adye et al. [35] confirmed that a relatively weak association existed between lipopolysaccharide molecules and the cell membrane. Gimber

and Rafter [36] also proposed a mechanism of charge-charge interactions between lipopolysaccharides and the leukocyte surface. Benedetto et al. [37] reported that the affinity of lipopolysaccharide for cell surfaces could be explained by the penetration of amphipathic lipopolysaccharide particles into the cell membrane. This interaction involved the lipid-lipid attraction between lipid A and membrane lipids [33] and a surface adsorptive effect due to charge-charge attraction. The results from our experiments involving constant lipopolysaccharide-variable numbers of cells and vice versa together with those showing cyclic binding of lipopolysaccharide suggest that both stable and labile interactions occur. It would seem possible that lipid-lipid hydrophobic interactions could account for Phase 1 binding, whereas weak charge effects between hydrophilic polysaccharide and the cell surface could account for Phase 2 binding.

Cižnar and Shands [20] stated that during tests on the binding of lipopolysaccharide to erythrocytes haemolysis occurred and this was attributed to membrane disorganization caused by lipopolysaccharide attachment. In this investigation there was no evidence of progressive haemolysis of erythrocytes but a short period of haemoglobin release after the addition of lipopolysaccharide to erythrocytes. This leakage of haemoglobin never exceeded 10% and appeared to be correlated with Phase 1 binding. It has been suggested that some of the erythrocytes haemolyse during the binding of lipopolysaccharide and during the ghosting step remove additional lipopolysaccharide from the medium by vesicularisation of the membrane. Although such a mechanism cannot be totally discounted it would seem to be highly unlikely since it would lead to a rapid plateau effect and no cyclic fluctuation of lipopolysaccharide adsorption would occur. For obvious reasons it would not explain the adsorption patterns observed with macrophages and lymphocytes. In addition, the release of CrO_4^{2-} from negatively- and positively-charged phospholipid-cholesterol liposomes did not indicate large-scale membrane disorganization caused by lipopolysaccharide. Bangham et al. [38,39] showed that the constituent bilayers of liposomes possessed physical properties which closely resembled those of cell membranes. Since lipopolysaccharide did not cause a significant release of divalent anions from liposomes it would seem unlikely that gross alterations in the permeability of the erythrocyte membrane would occur with the consequent release of larger molecules of haemoglobin.

It was concluded that lipopolysaccharide extracted from gram-negative organisms by the phenol/water method consisted of a heterogeneous mixture of lipopolysaccharide subunits, designated LPS_I, LPS_{II} and LPS_{III}. This proposal is further substantiated by the finding of T.H. Birkbeck (personal communication) that the lipopolysaccharide preparation from *B. pertussis* 18334 showed three discrete Schlieren peaks in the analytical ultracentrifuge which was indicative of a heterogeneous mixture. Two of these peaks contained material with high sedimentation coefficients and the third was composed of 13 S material. After alkali treatment of the lipopolysaccharide only the 13 S peak appeared. The LPS_I moieties might consist of amphipathic (lipid A - heptose) and have the greatest affinity for cell membranes binding through hydrophobic interactions involving the mixing and insertion of lipid A fatty acids into membrane lipid bilayers. This interaction might lead to altered permeability and the

release of haemoglobin during Phase 1 binding. LPS_{II} moieties might also consist of amphipathic molecules (lipid A-heptose-basal core polysaccharide) or (lipid A-heptose-basal core polysaccharide-*O*-specific polysaccharide) which bind onto the surface of cells in a similar fashion to LPS_I through hydrophobic interactions. In addition, the polysaccharide would be layered onto the surface through hydrophilic interactions or project into the environment. The LPS_{III} moieties might consist of (heptose-basal core polysaccharide), (heptose-basal core-*O*-specific polysaccharide), (basal core-*O*-specific polysaccharide) or (*O*-specific polysaccharide). Such moieties would be held at the surface possibly by Coulombic attraction between oppositely charged ionic groupings, i.e., hydrophilic interactions.

It would seem that the inherent differences in the binding characteristics of a heterogeneous population of lipopolysaccharide molecules could account for the cyclic fluctuations in binding. As shown in Fig. 9, at zero time (t_0) the cell is exposed to the mixture of fragments and molecules of lipopolysaccharide and the adsorption process commences. The lipophilic lipopolysaccharide moieties (I and II) bind strongly through lipid-lipid interactions and the hydrophilic LPS_{III} binds weakly to the membrane (t_{60}). At this stage it would appear that membrane reorganization occurs which leads to the release of LPS_{III} molecules (t_{100}) but not to the release of those molecules containing lipid A. The in vitro process continues to fluctuate with the adsorption of LPS_{III} (t_{120}) and subsequent release (t_{200}). Other explanations of the cyclic pattern of binding are possible. Spatial rearrangements in the membrane may cause altered surface charges with the breakdown of the charge-charge interaction with lipopolysaccharide. The hypothetical pattern of binding in Fig. 9 could also be due to the activation of membrane-bound hydrolytic enzymes initiated by the adsorption of lipopolysaccharide. These enzymes might destroy the lipopolysaccharide receptors and cause desorption. Subsequently, new receptors would be inserted into the membrane and the cycle could be repeated.

Singer [40] and Singer and Nicholson [41] presented a detailed analysis of the thermodynamics of membrane systems which led to the "fluid" mosaic model of membrane structure. In this respect it is interesting to note that when binding commenced it was found to proceed in a cyclic manner even if the process was interrupted (Fig. 5). Perret [42] showed that linear or branched systems of any degree of complexity tend towards a steady state in a constant environment if the velocity constants of intermediates stages assume unchanging values. If these constants have interdependent values the system permanently oscillates about a mean value, fails to attain a steady state and remains in a constant state of flux. The classical example in biology is provided by the Lotka-Volterra oscillation [43]. The adsorption of lipopolysaccharide would seem to initiate a reversible reorganization within the cell membrane and the ratio of bound to free lipopolysaccharide would remain in a state of flux. The binding of lipopolysaccharide molecules to the membrane and release into the chemical environment would therefore also remain in a state of flux, because in such a closed experimental system the chemical attraction of lipopolysaccharide for lipopolysaccharide might overcome the affinity for the cell membrane and lead to the formation of free micelles in the suspending fluid.

Information concerning the molecular interaction between lipopolysaccha-

ride and membrane might be obtained from the study of the effect of temperature on lipid mobility. However, this interaction involves the hybridisation of two membrane systems containing lipids with different melting points. Similarly, alteration of the ionic environment might allow an assessment of nonpolar or ionic binding. Penetration of the lipid A of lipopolysaccharide into the membrane would be controlled by the surface pressure. Native lipopolysaccharide was found by Benedetto et al. [37] to cause changes in the surface pressure of phospholipid monolayers ranging from 2 to 6 dynes/cm depending on the state of monolayer compression. However, in a solution of ionic strength sufficiently high to suppress ionic interaction, e.g. 1 M NaCl, the plasmolyzing effect may cause an increase in surface pressure due to compression forces associated with cellular shrinkage, this in turn would certainly influence the extent of lipopolysaccharide penetration. Further work is being carried out in an attempt to explain the binding phenomenon.

Acknowledgements

M.D. was in receipt of a research studentship from the Medical Research Council of Great Britain during the course of this investigation. One of us (D.E.S.S.-T.) is indebted to Dr. John H. Freer for valuable discussions during the preparation of this paper.

References

1 Boyden, S.V. (1951) J. Exp. Med. 93, 107—120
2 Keogh, E.V., North, E.A. and Warburton, M.F. (1948) Nature 161, 687—688
3 Lüderitz, O., Westphal, O., Eichenberger, E. and Neter, E. (1958) Biochem. Z. 330, 21—33
4 Lüderitz, O., Westphal, O., Sievers, K., Kröger, E., Neter, E. and Braun, O.H. (1958) Biochem. Z. 330, 34—46
5 Middlebrook, G. and Dubos, R.J. (1948) J. Exp. Med. 88, 521—528
6 Neter, E. (1956) Bact. Rev. 20, 166—188
7 Neter, E., Westphal, O., Lüderitz, O. and Grozynski, E.A. (1956) Ann. N.Y. Acad. Sci. 66, 141—156
8 Springer, G.F. and Horton, R.E. (1964) J. Gen. Physiol. 47, 1229—1250
9 Neter, E., Zalewski, N.J. and Zak, D.A. (1953) J. Immunol. 71, 145—151
10 Sorkin, E. and Boyden, S.V. (1955) J. Immunol. 75, 22—27
11 Roundtree, P.M. and Barbour, R.G.H. (1952) Austr. Ann. Med. 1, 80—83
12 Neter, E., Bertram, L.F., Zak, D.A., Murdock, M.R. and Arbesman, C.E. (1952) J. Exp. Med. 96, 1—15
13 De Petris, S. (1967) J. Ultrastruct. Res. 19, 45—83
14 Lüderitz, O., Staub, A.M. and Westphal, O. (1966) Bact. Rev. 30, 192—255
15 Beer, H., Braude, A.I. and Brinton, C.C. (1966) Ann. N.Y. Acad. Sci. 133, 450—475
16 Burge, R.E. and Draper, J.C. (1967) J. Mol. Biol. 28, 205—210
17 Shands, J.W., Graham, J.A. and Nath, K. (1967) J. Mol. Biol. 25, 15—21
18 Work, E., Knox, K.W. and Vesk, M. (1966) Ann. N.Y. Acad. Sci. 133, 438—449
19 Shands, J.W. (1973) J. Infect. Dis. 128, 5197—5201
20 Ciżnar, I. and Shands, J.W. (1971) Infect. Immun. 4, 362—367
21 Ginsberg, H.S., Goebel, W.F. and Horsfall, F.L. (1948) J. Exp. Med. 87, 411—424
22 Johnson, A.G., Gaines, S. and Landy, M. (1956) J. Exp. Med. 103, 225—246
23 Davis, B.D. and Minglioli, E.S. (1950) J. Bacteriol. 60, 17—28
24 Rowley, D., Howard, J.G. and Jenkin, C.R. (1956) Lancet 366—367
25 Westphal, O., Lüderitz, O. and Bister, F. (1952) Z. Naturforsch. 76, 148—155
26 Stewart-Tull, D.E.S., Davies, M. and Jackson, D.M. (1977) Immunology 33, in the press
27 Mahin, D.T. and Lofberg, R.T. (1970) in Current Status of Liquid Scintillation Counting (Bransome, E.D., ed.), pp. 212—221, Grune and Stratton
28 Stewart-Tull, D.E.S. and Rowe, R.E.C. (1975) J. Immunol. Methods 8, 37—46
29 Davies, M. and Stewart-Tull, D.E.S. (1973) Immunology 25, 1—9

30 Culling, C.F.A. (1957) Handbook of Histopathological Technique, Butterworth, London
31 Pangborn, M.C. (1951) J. Biol. Chem. 188, 471—476
32 Vogel, R.A. (1967) Proc. Soc. Exp. Biol. N.Y. 94, 279—283
33 Hammerling, V. and Westphal, O. (1967) Eur. J. Biochem. 1, 46—50
34 Springer, G.F., Adye, J.C., Bezkorovainy, A. and Murthy, J.R. (1973) J. Infect. Dis. 128, 5202—5212
35 Adye, J.C., Springer, G.F. and Murthy, J.R. (1973) Z. Immun. Forsch. 144, 491—496
36 Gimber, P.E. and Rafter, G.W. (1969) Arch. Biochem. Biophys. 135, 14—20
37 Benedetto, D.A., Shands, J.W. and Shah, D.O. (1973) Biochim. Biophys. Acta 298, 145—157
38 Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) J. Mol. Biol. 13, 238—252
39 Bangham, A.D., Standish, M.M. and Weissman, J. (1965) J. Mol. Biol. 13, 253—259
40 Singer, S.J. (1971) in Structure and Function of Biological Membranes (Rothfield, L.I., ed.), pp. 145—222, Academic Press, New York
41 Singer, S.J. and Nicholson, G.L. (1972) Science 175, 720—731
42 Perret, C.J. (1960) J. Gen. Microbiol. 22, 589—617
43 Volterra, V. (1931) Lecons sur la théorie mathématique de la lutte pour la vie, Gauthier-Villars, Paris