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THE AFFINITY OF BACTERIAL POLYSACCHARIDE-CONTAINING FRACTIONS FOR MAMMALIAN CELL MEMBRANES AND ITS RELATIONSHIP TO IMMUNOPOTENTIATING ACTIVITY

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Summary

The natural affinity of various bacterial glycopeptides and lipopolysaccharides for mammalian cell membranes was estimated quantitatively by comparison with the adsorption of lipopolysaccharide from *Escherichia coli* NCTC 8623 to erythrocytes, thymocytes, bone marrow cells, spleen cells, peritoneal lymphocytes and macrophages. Immunopotentiating activity was estimated by measuring the ability of the bacterial fractions to stimulate a humoral response to ovalbumin in HAM/1CR mice. When the affinity for mammalian cell membranes was compared with the stimulation of the antibody response, it was found that a negative correlation for peritoneal macrophages ($r_s = -0.94$, $P < 0.0005$) and a positive correlation for peritoneal lymphocytes ($r_s = +0.97$, $P < 0.0005$) and spleen cells ($r_s = +0.76$, $P < 0.005$) existed.

Introduction

These are numerous reports that bacteria or their antigens adhere to the membrane of mammalian erythrocytes [1—3]. In particular, the adherence of lipopolysaccharide from Gram-negative bacteria [3] and mycobacterial polysaccharide-containing fractions [4] to erythrocytes was observed. Lipopolysaccharide, mycobacterial peptidoglycolipids and glycopeptides could bind to various mammalian cells, including erythrocytes, macrophages and lymphocytes [5,6]. Since lipopolysaccharide [7,8] and fractions from mycobacteria

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[9—12] possessed immunopotentiating activity it was postulated [13,14] that these adjuvants could act as specific derepressors for immunoglobulin synthesis. This hypothesis required the binding of the immunopotentiating substance to both the cell membrane and to the immunoglobulin. In 1973, Davies and Stewart-Tull [15] showed that a glycopeptide from *Mycobacterium tuberculosis* could bind simultaneously to sheep erythrocytes and to guinea-pig IgG_2. In this study the ability of a particular polysaccharide-containing fraction to bind to mammalian cells was related to its ability to act as an immunoadjuvant.

Materials and Methods

Preparation of labelled lipopolysaccharide from Escherichia coli NCTC 8623. The detailed procedure was described by Dabies et al. [5]. Briefly, *E. coli* NCTC 8623 was grown in Davis and Mingioli minimal medium supplemented with ^{32}P-labelled glucose as the sole carbon source. The labelled lipopolysaccharide was extracted using the phenol-water method [16].

Lipipolysaccharide preparations. Commercial preparations of lipopolysaccharide from *Salmonella typhi* NCTC 8393, (0 901) *Salmonella typhimurium* NCTC 5710, *Shigella flexneri* and *E. coli* ATCC 12014 (0.55:B5) were obtained from Difco Laboratories Ltd., MI, U.S.A.

Preparation of lipopolysaccharide from E. coli B (NCIB 9484). Lipopolysaccharide from *E. coli* B was extracted by diethyl ether extraction or by ethylenediaminetretraacetic acid (EDTA) extraction. In the diethyl ether extraction process bacterial cells were homogenized in distilled water, followed by treatment with diethyl ether for 1 min. After centrifugation at 7500 rev./min ($6000 \times g$) for 15 min at 4°C, the aqueous and diethyl ether phases were decanted. Diethyl ether extraction was repeated and the pooled aqueous phases were dialysed against distilled water at 4°C for 1 week. The dialysed extract was centrifuged at 14 000 rev./min ($25\,000 \times g$) for 30 min. The sediment, resuspended in distilled water, was centrifuged at 36 000 rev./min ($100\,000 \times g$) for 4 h: this step was repeated four times and finally the sediment was freeze-dried [17].

The EDTA extraction was a modification of Lieve's method [18]. The bacterial cells were stirred in 0.1 M Tris-HCl buffer, pH 8.0. The suspension was centrifuged at 2000 rev./min ($1300 \times g$) for 15 min, the pellet was resuspended in 0.12 M Tris-HCl buffer, EDTA was added to a final concentration of $5 \cdot 10^{-4}$ M and the mixture was incubated at 37°C for 1 h. $MgCl_2$ was added to give a final concentration of 0.01 M, to neutralize excess EDTA. The mixture, cooled to 4°C, was centrifued at 6500 rev./min ($5000 \times g$) for 10 min. The supernate was decanted and centrifuged at 14 000 rev./min ($23\,500 \times g$) for 30 min. The pellet of lipopolysaccharide was washed twice with distilled water and finally centrifuged at 36 000 rev./min ($100\,000 \times g$) for 2 h. The pellet containing lipopolysaccharide was freeze-dried.

Preparation of mycobacterial glycopeptide fractions. Glycopeptides (ST208, ST210) were prepared from large volumes of culture filtrate of *M. tuberculosis* var. hominis strain DT, obtained from the Ministry of Agriculture, Central Veterinary Laboratories, Weybridge, Surrey, as described by Stewart-Tull et al. [10]. 30 g heat-killed *M. tuberculosis* strain C whole cells were extracted with

2% lauryl sulphate (50 : 50, v/v) solution for 48 h at 37°C. The cells were removed by centrifugation and the supernate was dialysed for several days against running tap-water to minimize the amount of residual lauryl sulphate and subsequently freeze-dried (ST 82). A similar aliquot of heat-killed cells was extracted by the phenol-water method [16], the aqueous phase was dialysed to remove phenol and freeze-dried (ST 211).

Composition of the lipopolysaccharides and glycopeptides. The mycobacterial glycopeptides (ST 208, ST 210) contained the basic structure of the cell wall monomer. The sugars found in this monomeric unit were glucosamine, muramic acid, muramic acid 6-phosphate, arabinose, galactose and mannose. The amino acids present were alanine, glutamic and meso-DAP typical components of the mycobacterial cell wall [10]. The glycopeptide was shown to have a molecular weight of 35 000 [14]. The glycopeptide obtained by lauryl sulphate extraction was composed of alanine, glutamic acid, glucosamine, galactose and mannose.

The chemical composition of the O-specific chains in the lipopolysaccharides has enabled them to be classified into chemotypes according to the scheme of Lüderitz et al. [19]. The standard *E. coli* NCTC 8623 lipopolysaccharide contains galactose, glucose, mannose and fucose; chemotype XII, serotype O 125:K70 (B15). *E. coli* ATCC 12014 containing glucose, galactose and colitose is in chemotype XI and serotype O 55:K59 (B5). The lipopolysaccharide of *Sh. flexneri* contains glucose, galactose and rhamnose; chemotype VII.

The lipopolysaccharides of *S. typhi* NCTC 8393 (chemotype XVI; serotype O 9, 12) and *S. typhimurium* NCTC 5710 (chemotype XIV; serotype O 4, 5, 12) both contained glucose, galactose, rhamnose and mannose together with tyvelose or abequose, respectively.

E. coli B was found to synthesise only R-polysaccharides with 4-amino-4,6-dideoxyhexose; preparations lacked the O-specific polysaccharide [20].

Preparation of mammalian erythrocyte suspensions. Blood samples from all species were collected in 3.8% (w/w) sodium citrate. Blood was withdrawn from the ear vein of New Zealand White rabbits, from the arm vein of human volunteers or was obtained by cardiac puncture from anaesthetized adult HAM/1CR mice. The blood was centrifuged and the packed cells were washed with 0.85% (w/v) NaCl and made up to the appropriate concentration.

Preparation of lymphoid cells from adult HAM/1CR mice. Cell suspensions of thymus, spleen, bone marrow, peritoneal lymphocytes and peritoneal macrophages were prepared from mice by standard techniques. All cell suspensions were prepared from exanguinated mice. Spleens and thymuses were disrupted to provide single-cell suspensions, first by mincing with a scalpel and then expelling the fragments through a narrow-bore syringe. Large fragments were allowed to sediment and the supernate was used as the cell suspension.

Bone-marrow cells were obtained from the femurs. After the heads of the bones were removed, the marrow cells were flushed out with Hanks medium to provide a suitable cell suspension.

Lymphocytes and macrophages were obtained from peritoneal washings incubated in glass dishes at 37°C for 1 h. The supernate containing non-adherent cells was decanted and reabsorbed on glass for 1 h. The adherent cells, removed from the glass with a rubber policeman were also reabsorbed on glass. The

twice absorbed non-adherent cells were considered to be a lymphocyte preparation and the adherent cells a macrophage preparation [5].

Measurement of the relative affinity of the polysaccharides for mammalian cell membranes. Details for measuring the uptake of labelled lipopolysaccharide from *E. coli* NCTC 8623 were described by Davies et al. [5]. The mammalian cells were mixed with the labelled lipopolysaccharide and incubated at 37°C for 1 h. The suspension was centrifuged at 3500 rev./min (1750 × *g*) for 5 min. The radioactivity associated with the supernate (free lipopolysaccharide) was measured directly by a liquid scintillation method, while the radioactivity associated with the pellet (bound lipopolysaccharide) was measured using a wet oxidation method [21]. The uptake of ^{32}P-labelled lipopolysaccharide by mammalian cells was inhibited by the addition of homologous unlabelled lipopolysaccharide [5] and this observation formed the basis of the method to determine the relative affinities of the bacterial polysaccharides. The degree of inhibition of uptake of ^{32}P-labelled lipopolysaccharide from *E. coli* NCTC 8623 caused by unlabelled homologous lipopolysaccharide was compared to the degree of inhibition caused by the heterologous lipopolysaccharide and mycobacterial fractions. The measurements were based on a four point bioassay system, with the reaction mixture consisting of $1.57 \cdot 10^8$ mammalian cells in 1.0 ml of medium and 0.1 ml of the radioactive lipopolysaccharide. To this mixture was added either 60 μg (low dose) or 120 μg (high dose) of the inhibiting polysaccharide. By comparing the uptake in uninhibited mixtures with that observed in mixtures containing the unlabelled polysaccharide, the degree of inhibition of uptake was calculated. This was plotted against the log value of the amount of unlabelled polysaccharide and the quantity required to cause 50% inhibition of uptake was calculated, this was termed the inhibition dose 50 (ID_{50}) of the polysaccharide. The relative affinity value was obtained by comparing the ID_{50} of the homologous lipopolysaccharide with the ID_{50} of heterologous lipopolysaccharide or mycobacterial fraction. The lipopolysaccharide from *E. coli* NCTC 8623 was given a relative affinity value of 1.0.

Immunization scheme for the production of anti-ovalbumin sera in mice. Adult HAM/1CR mice were immunized against ovalbumin by the following scheme. The injection mixture (0.1 ml) per mouse consisted of 20 μg ovalbumin (Grade V, Sigma Chemical Co., St. Louis, MO) together with either 30 or 60 μg of the bacterial polysaccharide in physiological saline. Groups of five mice were injected intravenously on days 1, 5 and 10 and were exsanguinated by cardiac puncture on day 21. Control groups consisted of animals given only 20 μg ovalbumin in saline.

Titration of anti-ovalbumin antibody in mouse sera by the passive haemagglutination technique. The indicator cells were formalinized group O human erythrocytes, coated with the antigen, ovalbumin, in the presence of tannic acid. Each mouse antiserum was absorbed twice with formalinized tanned human erythrocytes to remove heterophile antibody. Serial doubling dilutions of the absorbed antisera were made in 0.1 ml phosphate-buffered saline, supplemented with 1% (v/v) normal rabbit serum, also absorbed with formalinized tanned human erythrocytes in the wells of a haemagglutination plate. The coated indicator cells (0.1 ml) were added to each well. The plates were incubated in a moist chamber at room temperature for 18 h. The end-point of the

assay was taken as the highest dilution of the antiserum that caused complete haemagglutination: results were expressed as $\log_2$ titre.

Measurement of the relative immunopotentiating activity of the polysaccharide preparations. This activity was defined as the ability of the polysaccharide to stimulate increased levels of anti-ovalbumin antibody in HAM/1CR mice. Experiments were set up as four-point bioassays, the ability of a particular polysaccharide was compared to the ability of lipopolysaccharide from *E. coli* NCTC 8623 to stimulate production of anti-ovalbumin antibody. A linear relationship between $\log_{10}$ dose of the polysaccharide and $\log_2$ haemagglutination titre was observed earlier [22].

Statistical analysis. The data was analysed by standard procedures for a four-point assay using high and low doses of the standard *E. coli* lipopolysaccharide and the appropriate test fraction [23]. The results obtained were used to calculate the adjuvanticity values relative to the standard *E. coli* lipopolysaccharide which was assigned unit value.

The relative affinity values of each bacterial fraction for the cell membranes was compared to their relative immunopotentiating activity using the Spearman rank correlation coefficient (r_s). The r_s values and their significance were calculated by standard methods [24,25].

Results

Determination of the relative affinity values of the bacterial fractions for mammalian cells

Initially the raw data were processed so that the uptake of each bacterial fraction by each mammalian cell type was compared with the uptake of *E. coli* NCTC 8623 lipopolysaccharides by mouse erythrocytes. A distinct pat-

TABLE I

THE RELATIVE UPTAKE OF BACTERIAL POLYSACCHARIDE-CONTAINING FRACTIONS BY MOUSE CELLS

All figures relate to the uptake of lipopolysaccharide from *E. coli* NCTC 8623 by mouse erythrocytes, this being given the value of 1.0.

Bacterial polysaccharide-containing fractions	Relative affinity values					
	Erythrocytes	Peritoneal lymphocytes	Spleen cells	Bone-marrow cells	Thymus cells	Peritoneal macrophages
Lipopolysaccharide from:						
E. coli NCTC 8623	1.00	2.19	5.63	6.49	6.50	11.50
Sh. flexneri	0.58	1.29	4.05	5.25	5.46	18.20
S. typhi NCTC 8393	1.24	2.47	5.85	4.02	7.93	7.70
ST82 Mycobacterial glycopeptide	1.10	2.19	5.12	6.49	7.15	11.50
ST208 glycopeptide from *M. tuberculosis* strain DT	0.63	1.86	5.29	5.77	7.21	14.60

TABLE II

THE RELATIVE AFFINITIES OF BACTERIAL POLYSACCHARIDE-CONTAINING FRACTIONS FOR CELLS FROM HAM/1CR MICE

The relative affinity values relate to the uptake of lipopolysaccharide from *E. coli* NCTC 8623 for each particular cell type, these being given the value of 1.0. Unless otherwise stated the lipopolysaccharide preparations were extracted by the phenol-water method. n.t., not tested.

	Fraction (pg) bound to 1 μm^2 erythrocyte membrane	Relative affinity values					
		Erythrocytes	Thymus cells	Bone-marrow cells	Peritoneal lymphocytes	Peritoneal macrophages	Spleen cells
Lipopolysaccharides							
E. coli NCTC 8623 (O 125:B15)	0.12	1.00	1.00	1.00	1.00	1.00	1.00
S. flexneri	0.07	0.58	0.84	0.81	0.59	1.59	0.72
E. coli ATCC 12014 (O 55:B5)	—	n.t.	0.86	0.70	0.75	1.25	0.90
S. typhi NCTC 8393 (O 901)	0.15	1.24	1.22	0.62	1.13	0.67	1.04
S. typhimurium NCTC 5710	0.09	0.79	1.63	0.49	0.82	1.42	0.87
E. coli B (diethyl ether extraction)	0.06	0.54	0.74	0.74	0.82	1.48	0.88
E. coli B (EDTA extraction)	0.13	1.09	0.69	0.71	0.65	1.60	0.79
Mycobacterial fractions							
ST82 glycopeptide from *M. tuberculosis* strain C	0.13	1.10	1.10	1.00	1.00	1.01	0.91
ST208 glycopeptide from *M. tuberculosis* strain DT	0.08	0.63	1.11	0.89	0.85	1.27	0.94
ST210 glycopeptide from *M. tuberculosis* strain DT	0.14	1.20	1.50	0.40	0.98	1.03	1.05
ST211 water phase of phenol/H_2O extract of *M. tuberculosis* strain C	0.06	0.50	0.50	0.76	0.92	1.20	0.79
ST213 water-soluble PPD	0.08	0.67	0.81	0.91	0.93	1.13	0.86

tern emerged for each of the bacterial fractions tested (Table I). Erythrocytes appeared to bind the fractions to a lesser extent than macrophages. In the case of the latter, the results were probably higher than expected due to some engulfment of the polysaccharides. The other cell types showed intermediate binding capacity, with thymus cells > bone-marrow cells > spleen cells > peritoneal lymphocytes.

The uptake of both lipopolysaccharide and mycobacterial fractions by a variety of mammalian cells was determined and the relative affinity values calculated by comparison with the uptake of *E. coli* NCTC 8623 lipopolysaccharide for each particular cell type (Table II). The data indicated that the relative amount of each fraction adsorbed tended to vary with the cell type. For example, *S. typhimurium* NCTC 5710 lipopolysaccharide had relative affinity values which ranged from 0.49 for bone-marrow cells to 1.63 for thymus cells. This indicated that either the relative number or avidity of receptors for lipopolysaccharide varied considerably and was dependent on the cell type. The amount of lipopolysaccharide that one cell type could absorb varied depending on the source of the lipopolysaccharide. For example, the relative affinity values for thymus cells varied from 0.69 for *E. coli* B lipopolysaccharide to 1.63 for *S. typhimurium* NCTC 5710 lipopolysaccharide.

Determination of the relative adjuvant activity of the bacterial fractions

The adjuvant activity of the polysaccharide-containing fractions was deter-

TABLE III

THE DETERMINATION OF THE RELATIVE ADJUVANT ACTIVITY OF LIPOPOLYSACCHARIDES FROM GRAM-NEGATIVE BACTERIA

For the mean anti-ovalbumin antibody titre data analysis of variance of raw data revealed that there was a significant difference (a) between the titre obtained with the high dose and the low dose, (b) among the effects produced by the test polysaccharides compared to the standard *E. coli* lipopolysaccharide. There was no significant deviation from parallelism. The differences were significant at the less than 1.0% level. Data in parentheses are the standard deviations of the mean anti-ovalbumin antibody titres expressed as $\log_2$.

Expt.	Fractions	Mean anti-ovalbumin antibody titre		Relative adjuvant activity *	95% confidence limits	
		60 μg lipopolysaccharide (high dose)	30 μg lipopolysaccharide (low dose)		Lower limit	Upper limit
1	*E. coli* NCTC 8623	11.0 (0.84)	6.8 (0.70)	1.0	—	—
	Sh. flexneri	3.8 (1.09)	1.4 (0.57)	0.24	0.12	0.35
	E. coli ATCC 12014	6.4 (0.54)	2.6 (0.54)	0.46	0.39	0.54
	E. coli B (EDTA extraction)	4.0 (1.64)	1.4 (0.89)	0.18	0.06	0.30
2.	*E. coli* NCTC 8623	10.0 (0.81)	7.6 (1.34)	1.0	—	—
	E. coli B (ether extraction)	7.0 (0.81)	4.3 (2.30)	0.50 **	0.18	0.84
3.	*E. coli* NCTC 8623	8.2 (1.48)	6.6 (0.54)	1.0	—	—
	S. typhi NCTC 9393	10.0 (0.81)	8.2 (0.83)	1.99 **	1.34	2.65
4.	*E. coli* NCTC 8623	8.4 (0.89)	7.6 (0.89)	1.0	—	—
	S. typhimurium NCTC 5710	7.2 (1.14)	6.2 (1.22)	0.44 **	0.15	0.83

* Ratio of test polysaccharide/*E. coli* lipopolysaccharide (given the value of 1.0).

** Differences between high and low dose significant between 1 and 5% and difference in effect produced by the test polysaccharides compared to the standard *E. coli* lipopolysaccharides significant between 1 and 5%.

TABLE IV

THE DETERMINATION OF THE RELATIVE ADJUVANT ACTIVITY OF MYCOBACTERIAL POLYSACCHARIDES AND GLYCOPEPTIDES

For the mean anti-ovalbumin antibody titre data analysis of variance of raw data revealed that there was a significant difference (a) between the titre obtained with the high dose and the low dose, and (b) among the effects produced by the test polysaccharides compared to the standard *E. coli* lipopolysaccharide. There was no significant deviation from parallelism. The differences were significant at the less than 1.0% level. Data in parentheses are the standard deviations of the mean anti-ovalbumin antibody titres expressed as $\log_2$.

Expt. No.	Fractions	Mean anti-ovalbumin antibody titre		Relative adjuvant activity *	95% confidence limits	
		High dose (60 μg fraction)	Low dose (30 μg fraction)		Lower limit	Upper limit
1.	*E. coli* 8623 lipopolysaccharide	11.0 (0.81)	6.8 (0.70)	1.0	—	—
	ST82	9.8 (0.44)	6.0 (1.0)	0.90 **	0.84	0.95
	ST208	7.6 (1.0)	7.0 (1.4)	0.58	0.25	0.86
	ST211	8.0 (1.22)	4.4 (0.54)	0.62	0.50	0.72
2.	*E. coli* 8623	10.0 (0.81)	7.6 (1.34)	1.0	—	—
	ST210	9.6 (0.57)	7.3 (0.57)	0.95 ***	0.60	1.40
	ST213	9.0 (0.57)	6.2 (1.25)	0.79	0.62	0.83

* Ratio of test polysaccharide/*E. coli* lipopolysaccharide (given the value of 1.0).

** The difference in effect produced by the mycobacterial polysaccharide compared to the standard lipopolysaccharide was significant between 1.0 and 5.0%.

*** No significant difference between the effect produced by the mycobacterial polysaccharide and that produced by the standard *E. coli* lipopolysaccharide.

mined by measuring the levels of anti-ovalbumin antibodies in HAM/1CR mice. A value for the relative adjuvant activity of each fraction was obtained by comparison with the activity of *E. coli* NCTC 8623 lipopolysaccharide which was given the unit value for adjuvant activity [23]. The data are presented in Table III for lipopolysaccharide and Table IV for the mycobacterial fractions. The lipopolysaccharide fractions varied considerably in their activity. The poorest adjuvant, lipopolysaccharide from *E. coli* B (EDTA extraction) stimulated anti-ovalbumin antibodies to a level 82% lower than that obtained with the lipopolysaccharide from *E. coli* NCTC 8623 (phenol-water extraction). *S. typhi* NCTC 8393 lipopolysaccharide proved to be the best adjuvant, producing a level that was 99% greater than the level obtained with the standard *E. coli* NCTC 8623 lipopolysaccharide. The mycobacterial fractions showed a similar but narrower range of activity varying from 58% (ST 208) to 95% (ST 210) of the activity stimulated by the standard *E. coli* lipopolysaccharide.

Comparison of the relative affinity of the bacterial fraction for mammalian cells and their ability to stimulate anti-ovalbumin antibodies

The rank of the relative affinity values for each mammalian cell type was compared with the rank obtained for the relative adjuvant activity. A negative correlation ($r_s = -0.94$, $P < 0.0005$) existed between affinity for macrophages and adjuvant activity which indicated that the affinity of a bacterial fraction for the macrophage surface was accompanied by a low ability to enhance anti-ovalbumin antibody production. It was observed that a positive correlation existed

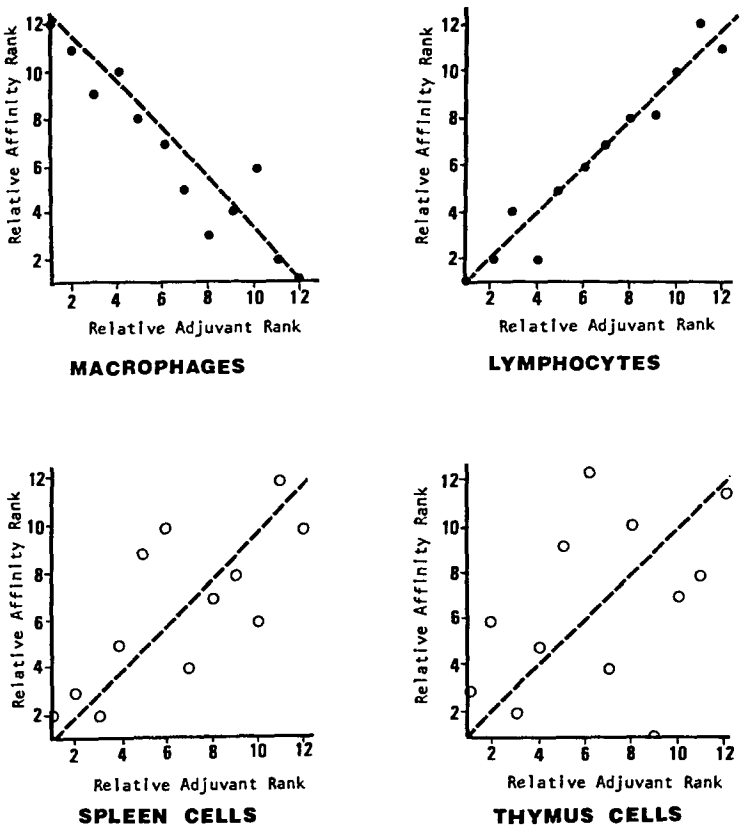

Fig. 1. The rank correlation of the lipopolysaccharides and mycobacterial glycopeptides for affinity to macrophages, peritoneal lymphocytes, thymocytes and spleen cells and immunopotentiating activity. The line drawn at 45° represents a perfect correlation; this correlation is either positive (lymphocytes, thymocytes and spleen cells) or negative (macrophages). The adjuvant rank for the compounds on the *x*-axis are as follows: lipopolysaccharide fractions, 1, *S. typhi* NCTC 8393; 2, *E. coli* NCTC 8623; 8, *E. coli* B (ether extraction); 9, *S. typhimurium* NCTC 5710; 10, *E. coli* ATCC 12014; 11, *Sh. flexneri;* 12, *E. coli* B (EDTA extraction), and mycobacterial fractions: 3, ST 210; 4, ST 82; 5, ST 213; 6, ST 211; 7, ST 208. The *y*-axis represents the rank for the relative affinity for cell membranes.

between adjuvant activity and affinity for peritoneal lymphocyte ($r_s = +0.97$, $P < 0.0005$) and whole spleen cells ($r_s = +0.76$, $P < 0.005$) (Fig. 1 and Table V). The increased affinity of the bacterial fractions for spleen cell and peritoneal lymphocyte membranes was associated with an ability to stimulate the humoral antibody response. For example, *S. typhi* NCTC 8393 lipopolysaccharide was an efficient adjuvant and had relative affinity values of 1.13 and 1.04 for lymphocytes and spleen cells, respectively, but only 0.67 for macrophages. Similarly, the lipopolysaccharide from *E. coli* B (EDTA extraction) was the poorest adjuvant with a relative affinity value of 1.60 for macrophages but only 0.65 and 0.79 for lymphocytes and spleen cells, respectively. Examination of the other cell types revealed that no strong correlations existed between adjuvant activity and affinity for erythrocytes ($r_s = +0.53$, $P < 0.05$) thymus cells ($r_s = +0.41$, $P > 0.05$) or bone-marrow cells ($r_s = +0.15$, $P > 0.05$).

TABLE V

CORRELATION BETWEEN ADJUVANT ACTIVITY AND AFFINITY FOR CELL MEMBRANES

The values quoted are those for five mycobacterial glycopeptides or six lipopolysaccharides compared with standard *E. coli* lipopolysaccharides. Data are the Spearman rank correlation coefficient (r_S) of the affinity of the bacterial fractions for the cells and the adjuvant activity of the fractions.

Sample size		Erythrocytes	Thymus cells	Bone-marrow cells	Peritoneal lymphocytes	Peritoneal macrophages	Spleen cells
5	Mycobacterial glycopeptides	+0.77	+0.20	+0.28	+0.77	—0.94	+0.60
6	Lipopolysaccharides	+0.26	+0.61	—0.07	+0.95	—0.85	+0.86
11	Mycobacterial glycopeptides and lipopolysaccharides	+0.53 $P > 0.05$	+0.41 $P > 0.05$	+0.15 $P > 0.05$	+0.97 $P < 0.0005$	—0.94 $P < 0.0005$	+0.76 $P < 0.005$

Discussion

In this study, an attempt was made to determine whether the natural affinity for mammalian cell membranes was related to the inherent immunopotentiating activity of lipopolysaccharides from Gram-negative bacteria and mycobacterial glycopeptides.

The binding of various bacterial components to different mammalian cells was measured and the results were expressed in terms of relative affinity values which ranged from 0.40 to 1.60. Macrophages tended to bind or take up approx. 10-times more of the bacterial fractions than either erythrocytes or peritoneal lymphocytes (Table I); however, this was probably a reflection of the scavenger role of macrophages. For each mammalian cell type, there was a range of relative affinity values which was dependent upon the bacterial fraction (Table II). For example, relative affinity values for lymphocytes varied from 0.59 for *Sh. flexneri* lipopolysaccharide to 1.13 for *S. typhi* NCTC 8393 lipopolysaccharide. This range indicated either that the mammalian cells carried different numbers of receptors for each bacterial component or that there were few receptors on the cell surface that corresponded to a particular chemical configuration in the bacterial fractions. It has already been shown that lipopolysaccharide (or lipid A) can combine with a wide variety of mammalian cells [5,26,27] and that various lipopolysaccharide species exhibited different levels of biological activity [28,29]. Mansheim et al. [29] suggested that these activities were due to the different chemical compositions of lipopolysaccharide but they could also be due to contamination with other bacterial components [30]. It is also interesting to note that not only were all the lipopolysaccharide preparations able to compete with the standard lipopolysaccharide for the available receptor sites on mammalian cells but so were the mycobacterial fractions [5,6].

The polymeric nature of the lipopolysaccharides and glycopeptides and their amphiphilic characteristics cause difficulties in quantitatively determining the interaction between these fractions and the cell membranes. The surface area of the actual cells is also variable. It was for these reasons that the competitive binding technique was used. The surface area of the erythrocyte is approx. 140 μm^2 so in this instance it was possible to calculate the comparative quantities of the fractions binding to 1.0 μm^2 of membrane. These values are shown in Table II and it is significant that the lipopolysaccharide of *S. typhi* which has the highest value was also the most efficient adjuvant preparation. In addition, the rough lipopolysaccharide of *E. coli* B extracted with diethyl ether was more adjuvant-active in smaller amounts. This could be due to a predominance of the lipid-containing moiety previously shown by Davies et al. [5] to bind irreversibly to the membrane.

The cell types from the various lymphoid organs were studied in an attempt to determine whether the affinity exhibited by a particular fraction was critical for a good immunopotentiating response. Examination of erythrocytes, thymus cells and bone-marrow cells revealed that the binding of the bacterial fractions to these cells did not appear to correlate well with the ability to stimulate a humoral immune response (r_s = +0.53, +0.41 and +0.15, respectively). However, examination of the data for spleen cells showed a positive

correlation ($r = +0.76$, $P < 0.005$) with immunopotentiating activity and the data for peritoneal lymphocytes revealed an almost perfect correlation ($r_s = +0.97$, $P < 0.0005$). The slight difference between the correlation coefficients for spleen cells and peritoneal lymphocytes might be due to the comparative levels of T and B lymphocytes. However, Raff and Owen [31] indicated that 35—40% of peritoneal exudate lymphocytes were T cells which is an equivalent value to that found for the spleen. Conversely, macrophages exhibited an almost perfect negative correlation ($r_s = -0.94$, $P < 0.0005$). When the glycopeptides and lipoolysaccharides were analysed separately, the results still produced the same type of correlation; a negative correlation being obtained for macrophages with both lipopolysaccharide ($r_s = -0.85$) and mycobacterial fractions ($r_s = -0.94$), and positive correlations for spleen cells ($r_s = +0.86$ and +0.60 for lipopolysaccharide and mycobacterial fractions. respectively) and peritoneal lymphocytes ($r_s = +0.95$ and +0.77). Hence, from these data it appears that both lipopolysaccharides and the mycobacterial fractions may cause immunopotentiation by similar mechanisms, in a saline injection medium. The best adjuvant-active compounds when compared to poor adjuvant-active compounds have a relatively low affinity for macrophages and a high affinity for peritoneal lymphocytes. However, it should be remembered that on average nearly 10-times more of the bacterial fraction was absorbed by macrophages, although engulfment and subsequent digestion may reduce the effective level of the bacterial adjuvant component.

It has been observed that lipopolysaccharide can elicit various effects both in vitro and in vivo, including a mitogenic effect on B cells [32,33] and enhancement of immunoglobulin production [7,8]. It was generally agreed that the B cell was the direct target of the lipopolysaccharide when acting as an immunostimulator [34—37]. However, there is an increasing number of reports in the literature proposing that lipopolysaccharide enhancement of the humoral response is dependent on the T cell [38—40]. In order to account for these contrasting reports, Shinohara and Kern [41] proposed a model in which the requirement for T cells appeared to be related to a B cell differentiation event, whereas B cells were the direct target for the mitogenic activity of lipopolysaccharide. On the other hand, Newburger et al. [42] suggested that the action of lipopolysaccharide either directly on T cells or through a macrophage mediator, could account for the major part of the adjuvant effect. The present report extends these observations by showing that the immunopotentiating activity of lipopolysaccharide in a saline medium was directly related to its ability to bind to the cell membranes of a population of lymphocytes and was indirectly related to its ability to bind to macrophages. Furthermore, the immunopotentiating activity of the mycobacterial glycopeptides appeared to be mediated through a similar mechanism.

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