Physicochemical Surface Properties and Phagocytosis by Polymorphonuclear Leucocytes of Different Serogroups of *Salmonella*

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Salmonella isolates belonging to different serogroups have been analysed with respect to physicochemical surface properties and interaction with human polymorphonuclear granulocytes (PMNs). Most (22/34) recent isolates of the different serotypes showed hydrophilic surface properties and little if any negative charge accompanied by resistance to phagocytosis by PMN similar to the old laboratory S strains *Salmonella typhimurium* 395MS and *Salmonella minnesota* S99 (main group). However, all isolates belonging to the serogroups C1 (5 isolates), E4 (2), O43 (1), and one out of three E1 isolates (C1/E4 group) differed from the main group. In aqueous biphasic partition in dextran-polyethyleneglycol (PEG) systems the bacteria in the main group accumulated in the PEG-rich phase to 55–97%, those in the C1/E4 group to less than 10%, and R-mutants only to 1–2%.

The bacteria in the C1/E4 group displayed a negative surface charge and a susceptibility to phagocytosis by PMNs that were greater than those for strains in the main group but much lower than those shown by the R-mutants. Bacteria belonging to serogroup C1 also displayed a significant susceptibility to hydrophobic interaction. The results are discussed in relation to the pathogenicity of salmonella.

INTRODUCTION

The structure of the outer membrane (Nikaido, 1980; Osborn & Wu, 1980) and the surface properties (Edebo *et al.*, 1980) of certain species of *Salmonella* like *S. typhimurium* and *S. minnesota* have been studied extensively in S strains and in different R-mutants. The S strains showed hydrophilic surface properties and little charge, whereas their R-mutants displayed more hydrophobic properties and negative charge. Furthermore the S strains were more resistant to phagocytosis by professional phagocytes (Stendahl & Edebo, 1972) and were more virulent (Edebo & Normann, 1970).

The genus *Salmonella* comprises approximately 2000 serotypes. The early serotypes were given Linnean binomial names related to disease such as *S. typhi* (human typhoid), *S. choleraesuis* (hog cholera), *S. typhimurium* (mouse typhoid), but later the host dependance of the pathogenicity of several species was shown to be broader. The pathogenicity of salmonella is dependent on the endotoxic properties of the lipid A portion of the outer membrane lipopolysaccharide (LPS), and on the structure of the O-antigenic polysaccharide chains peripheral to lipid A in the LPS which in some species have been shown to prevent phagocytosis and to

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Abbreviations: FITC, fluorescein isothiocyanate; HIC, hydrophobic interaction chromatography; IEC, ion exchange chromatography; PEG, polyethyleneglycol; P-PEG, palmitate-PEG; PMN, polymorphonuclear leucocytes; S-PEG, sulphonate-PEG; TMA-PEG, bistrimethylamino-PEG; TPP, two-phase partitioning.

promote virulence. In addition to the O antigen, some strains, notably *S. typhi*, show the presence of Vi antigen at the surface. The Vi antigen, which is a highly polymerized acidic polysaccharide consisting of repeating units of *O*- and *N*-acetyl-D-galactosaminuric acid, also promotes virulence by preventing phagocytosis. Vi antigens closely related to that of *S. typhi* have been found in *S. paratyphi* C and in occasional strains of *S. dublin*. These antigens are closely related to the K antigens of *Escherichia coli* which also are acidic polysaccharides and prevent phagocytosis (Öhman *et al.*, 1981).

In the genus *Salmonella* the base composition is 50–52 mol % G + C and the strains (including *arizona*) are at least 70% related to each other as determined by DNA/DNA hybridization (Crosa *et al.*, 1973). The metabolic properties which have been used for several years in the isolation and identification from clinical specimens are also similar. Also the antigenic surface structure is well established and extensively used for classification. Less is known about the physicochemical surface properties which may be related to pathogenicity in the different strains. This investigation has examined the physicochemical surface properties and phagocytosis of a representative selection of salmonella serotypes (species).

METHODS

Bacterial strains. *Salmonella typhimurium* 395MS and 395MR 10 (chemotype Rd) and *S. minnesota* S99 (S strain), R345 (chemotype Rb), R595 (chemotype Re) are laboratory strains that have been described elsewhere (Kihlström & Magnusson, 1980). Thirty-four salmonella isolates recently cultured from clinical specimens were kindly supplied by Dr A. A. Lindberg (Swedish Salmonella reference laboratory, National Bacteriological Laboratory, Stockholm, Sweden).

Cultivation and radioactive labelling of bacteria. Bacteria were inoculated into 10 ml glucose broth (glucose 1 g, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.6 g, NaCl 1.8 g, peptone (Difco) 10 g, beef extract (Difco) 5 g, distilled water 1000 ml) containing 25 μl L-[^3H]leucine (37 MBq ml^{-1} NET-460; New England Nuclear), in 100 ml bottles and were incubated on a rotary shaker at 37 °C for 16 h. Organisms were harvested by centrifugation (4000 g, 10 min) washed twice in saline buffer with 0.01 M-sodium phosphate (PBS, pH 7.3) and were suspended to 5×10^8 bacteria ml^{-1} in indicated buffers for testing the physicochemical surface properties.

Two-phase partitioning (TPP). The TPP system contained 4.4% (w/w) PEG and 6.2% (w/w) dextran T500 in 0.03 M-Tris buffer pH 7.0. This mixture was prepared in a separatory funnel from stock solutions of 20% (w/w) PEG 6000 (Union Carbide, N.Y.), 20% (w/w) dextran T500 (Pharmacia), 0.1 M-Tris buffer and distilled water. After mixing thoroughly the phases were allowed to separate at 4 °C overnight. The bottom phase and the top phase were collected separately. In order to analyse the charge and hydrophobicity of the bacterial surface, positively charged bistrimethylamino [$(\text{CH}_3)_3\text{N}^+$]-PEG (TMA-PEG), negatively charged sulphonate [SO_3^-]-PEG (S-PEG), and palmitate-PEG (P-PEG) were added to the systems (Kihlström & Magnusson, 1980). The hydrophobic change of the partition of one strain, was calculated as the sum of the increase in the top phase and the decrease in the bottom phase (Kihlström & Magnusson, 1980), and the significance estimated with a *t*-test with two degrees of freedom (Bailey, 1981).

For a TPP test, 2 ml of bottom phase and 2 ml of top phase were pipetted into graduated tubes and 0.1 ml bacterial suspension was added. The tubes were inverted 20 times for mixing and kept at 4 °C for 45 min to separate the phases. After recording the volumes of the bottom phase and the total system, 0.5 ml samples were taken from each of the top and the bottom phase. The remainder was mixed, and another 0.5 ml sample was taken. After dilution with 2 ml water and addition of 5 ml Aquasol, the radioactivity of the samples was counted in a beta-scintillation counter (Isocap 300, Searle-Nuclear Chicago, Ill., U.S.A.). The partition of bacteria was calculated from the volumes of the phases and the radioactivity (c.p.m.) of the samples from them.

Hydrophobic interaction chromatography (HIC). HIC was performed (Kihlström & Magnusson, 1980) on Octyl-Sepharose CL-4B (Pharmacia). A high ionic strength solution is desired as suspension medium in order to promote hydrophobic interaction. We employed an intermediate concentration, 0.35 M- $(\text{NH}_4)_2\text{SO}_4$ in a 0.01 M-sodium phosphate buffer, pH 7.0, to reduce the tendency of cells to aggregate. The Sepharose stock material was suspended in an equal volume of ammonium sulphate/phosphate buffer and 2 ml was loaded into a Pasteur pipette. The height of the gel column was 4 cm. After washing with the starting solution, 0.1 ml of the bacterial suspension was carefully added on top of the column. Elution was performed with successive 2 ml volumes of 0.35 M- $(\text{NH}_4)_2\text{SO}_4$ /0.01 M-phosphate buffer pH 7.0, 0.35 M- $(\text{NH}_4)_2\text{SO}_4$ /0.01 M-phosphate buffer pH 7.0 plus 0.1% Triton X-100, and 95% ethanol. A 0.5 ml sample was taken from each eluate and mixed with 2 ml water and 5 ml Aquasol; c.p.m. values were determined with a beta-counter. As a control, a parallel test was run on a similar Sepharose CL-4B column.

Ion-exchange chromatography (IEC). IEC on DEAE-Sephacel (Pharmacia) was performed (Kihlström &

Magnusson, 1980) in a manner similar to HIC in Pasteur pipettes. A volume of 0.1 ml bacteria suspended in 0.15 M-NaCl/0.01 M-sodium phosphate buffer pH 7.0, was loaded on to the column. Elution was done with 2 ml volumes of increasing salt concentrations: 0.15 M-NaCl, 2.1 M-NaCl and 4.1 M-NaCl. Eluates were processed as above for beta-counting.

Phagocytosis. Phagocytosis was tested *in vitro* by adding fluorescein isothiocyanate (FITC)-labelled bacteria to monolayers of human polymorphonuclear leucocytes (PMN) and observing the cells in a combined phase-contrast and UV-epifluorescence microscope (Hed, 1977). For FITC-labelling, bacteria (2.5×10^9 ml⁻¹) were suspended in 0.2 M-sodium carbonate-bicarbonate buffer, pH 9.5, mixed with FITC-solution (1.5 mg ml⁻¹ in the same buffer), and kept at room temperature for 30 min. After washing twice with PBS and once with Krebs-Ringer phosphate buffer with 10 mM-glucose, pH 7.3, the bacteria were suspended to a concentration of 5×10^7 ml⁻¹ in Krebs-Ringer buffer. Freshly taken human blood dropped directly from the finger-tip on to a microscopic slide was the source of PMNs. After 30 min at 37 °C the blood clot was rinsed away, 0.1 ml of the bacterial suspension (5×10^7 ml⁻¹) added, and the slide incubated for 1 h at 37 °C.

Fluorescence quenching of non-ingested bacteria with crystal violet (0.8 mg ml⁻¹) was used to discriminate between extracellular and intracellular bacteria. One hundred PMNs were observed and the total number of associated (observed in the absence of crystal violet) and ingested (observed with crystal violet) bacteria in conjunction with each PMN, was counted. It was noted that extracellular salmonella S-type bacteria were less easily quenched than R-type bacteria. After heat-killing the extinction of the fluorescence was more complete.

Haemagglutination by bacteria. The presence of type 1 fimbriae and P-fimbriae was tested for by agglutination of guinea-pig and human erythrocytes, respectively (Duguid & Old, 1980; Öhman *et al.*, 1982). From a suspension of erythrocytes 1.5% (v/v) in PBS, pH 7.3, 75 µl was mixed with 25 µl bacteria (1.2×10^9 ml⁻¹) in microtiter plates and rocked gently for 45 min at room temperature. Haemagglutination was observed with the naked eye.

RESULTS

Partition in dextran-PEG two-phase systems

In the basic dextran-PEG system without substituted PEG, R-mutants accumulated mainly at the interface and in the bottom phase (Table 1) such that very few organisms (1–2%) appeared in the top phase (Fig. 1). In contrast, most (24/36) of the clinical isolates were found mainly (55–98%) in the top phase (Fig. 1). However, some of the clinical isolates showed less than 10% partition into the top phase. Partition to the bottom phase consistently appeared in certain serogroups, viz. in all of five serogroup C1 isolates, the two E4 isolates, and the one *S. houten* (O43), isolate tested. One out of three serogroup E1 isolates (*S. muenster*) also showed this type of partition. This group is called the C1/E4 group. Two out of seven serogroup B isolates, and one serogroup K isolate (*S. cerro*), showed intermediate partition into the top phase (i.e. between 25 and 36%).

Addition of positively charged TMA-PEG changed the partition of R-mutants from the bottom phase and interface towards the top phase (Table 1), indicating a predominantly negative surface charge. In contrast, little change of partition was observed with the laboratory S strains and most of the clinical isolates. A small effect of TMA-PEG was observed on some strains particularly on those belonging to the C1/E4 group. When the significance of the change of partition by TMA-PEG was calculated for the individual isolates by Student's *t*-distribution with two degrees of freedom (Bailey, 1981) the significance was rarely greater than $P < 0.05$ but a clustering of significant values appeared in the C1/E4 group. Thus, when the substitution by TMA-PEG was calculated as the sum of the increase in the top phase and the decrease in the bottom phase, the C1/E4 showed a difference of 16.3% (± 0.76 S.E.M.), whereas the remaining S strains (main group) differed by only 2.2% (± 2.1 S.E.M.). The difference in response of these two groups of strains was highly significant ($P < 0.001$) as tested with a *t*-test (Bailey, 1981).

For most strains negatively charged S-PEG had no effect on partition though some effect was observed with individual isolates. When the effect was calculated as the sum of increase in top phase and decrease in bottom phase the effect on the C1/E4 group (8.6% ± 1.9 S.E.M.) was not significantly different from that on the main group (6.0% ± 1.4 S.E.M.). The effect of P-PEG was greater on the C1/E4 group (9.3% ± 2.3 S.E.M.) than on the main group (3.6% ± 1.4 S.E.M.). However, the difference was barely significant ($P \approx 0.05$). The difference was highly significant ($P < 0.001$) when only the C1 strains were grouped (13.5% ± 1.5 S.E.M.). The effect of P-PEG was still greater on the R-mutants with the most incomplete LPS.

Table 1. Partition of salmonella in different dextran-PEG two-phase systems

The partition is given as a percentage of added radioactivity. Duplicate tests were performed and the mean was calculated. In the basic system the $\pm$ values indicate ranges about the mean. Similar ranges were observed with the substituted PEGs but these are not shown. The significance (P value) of the effect of replacing PEG by three substituted PEGs was calculated by Student's t -test ($-$, $P \geq 0.05$)

Strain	Serotype/chemotype	Serogroup	Basic system:			S-PEG:			TMA-PEG:			P-PEG:		
			Top	Bott.		Top	Bott.	P	Top	Bott.	P	Top	Bott.	P
395MS	<i>S. typhimurium</i> /S	B	95.2 $\pm$ 1.3	2.9 $\pm$ 0.3		98	2	—	94	3	—	96	3	—
395MR10	<i>S. typhimurium</i> /Rd	B	2.3 $\pm$ 0.1	61.1 $\pm$ 0.5		3	51	<0.05	101	2	<0.001	12	41	<0.05
S2887	<i>S. typhimurium</i>	B	85.0 $\pm$ 2.0	1.5 $\pm$ 0.1		95	1	—	90	2	—	86	1	—
S2894	<i>S. typhimurium</i>	B	85.3 $\pm$ 0.5	1.9 $\pm$ 0.4		86	2	—	84	1	—	86	2	—
S2871	<i>S. heidelberg</i>	B	83.1 $\pm$ 3.9	3.3 $\pm$ 0.2		84	3	—	78	3	—	84	3	—
S2825	<i>S. saint-paul</i>	B	55.0 $\pm$ 0.1	4.7 $\pm$ 0.1		66	3	<0.01	52	6	—	73	4	<0.001
S2863	<i>S. agona</i>	B	36.0 $\pm$ 0.5	6.3 $\pm$ 0.5		52	5	<0.01	63	4	<0.01	33	4	—
S2843	<i>S. derby</i>	B	25.2 $\pm$ 0.5	10.4 $\pm$ 0.8		33	9	<0.05	46	13	<0.05	39	9	<0.05
S2824	<i>S. thompson</i>	C1	4.9 $\pm$ 0.1	58.5 $\pm$ 2.4		5	46	<0.05	7	43	—	5	45	—
S2860	<i>S. infantis</i>	C1	9.3 $\pm$ 0.5	37.0 $\pm$ 2.0		13	32	—	11	22	<0.05	13	27	—
S2905	<i>S. infantis</i>	C1	2.7 $\pm$ 0.1	51.5 $\pm$ 4.6		3	50	—	3	32	<0.05	2	33	—
S2832	<i>S. virchow</i>	C1	2.9 $\pm$ 0.2	18.7 $\pm$ 1.2		9	8	<0.01	11	10	<0.05	6	13	—
S2828	<i>S. braenderup</i>	C1	3.3 $\pm$ 1.1	38.1 $\pm$ 2.1		3	26	<0.05	9	27	<0.01	3	25	—
S2862	<i>S. newport</i>	C2	80.2 $\pm$ 2.7	4.2 $\pm$ 1.9		84	3	—	71	3	—	80	3	—
S2822	<i>S. hadar</i>	C2	90.0 $\pm$ 3.9	2.7 $\pm$ 0.4		88	2	—	89	3	—	88	3	—
S2821	<i>S. manhattan</i>	C2	77.4 $\pm$ 3.6	3.8 $\pm$ 0.3		76	2	—	73	2	—	71	2	—
S2856	<i>S. litchfield</i>	C2	82.8 $\pm$ 2.6	3.5 $\pm$ 0.1		80	3	—	82	3	—	87	3	—
S2865	<i>S. bovis morbillicans</i>	C2	85.3 $\pm$ 1.8	3.4 $\pm$ 0		89	3	—	90	3	—	88	3	—
S2628	<i>S. haardt</i>	C3	78.7 $\pm$ 2.6	4.5 $\pm$ 1.4		86	4	—	81	3	—	83	5	—
S2812	<i>S. enteritidis</i>	D1	82	3		81	2	—	72	2	—	83	4	—
S2819	<i>S. enteritidis</i>	D1	58.4 $\pm$ 3.4	5.1 $\pm$ 0.7		69	5	<0.05	62	5	—	66	5	<0.05
S2897	<i>S. panama</i>	D1	58.3 $\pm$ 4.6	2.7 $\pm$ 0.6		63	4	—	58	4	—	60	4	—
S2805	<i>S. anatum</i>	E1	73.0 $\pm$ 0	2.9 $\pm$ 0.2		89	2	<0.05	92	2	<0.01	76	2	—
S2811	<i>S. muenster</i>	E1	1.8 $\pm$ 0	36.2 $\pm$ 3.4		2	25	—	2	18	<0.05	1	24	—
S2820	<i>S. falkensee</i>	E1	68.7 $\pm$ 2.1	3.6 $\pm$ 0.3		88	2	<0.01	79	3	<0.05	73	3	—
S2810	<i>S. krefeld</i>	E4	4.6 $\pm$ 0.5	31.7 $\pm$ 3.0		5	23	—	7	20	—	4	25	—
S2915	<i>S. senftenberg</i>	E4	3.5 $\pm$ 0.2	48.1 $\pm$ 0.1		5	49	—	5	36	<0.01	5	55	<0.01
S1497	<i>S. huttigfoss</i>	I	96.2 $\pm$ 1.9	2.4 $\pm$ 0.1		90	2	—	98	2	—	91	2	—
S114	<i>S. hull</i>	I	98.0 $\pm$ 0	2.9 $\pm$ 0.1		91	2	—	89	2	—	96	2	—
S709	<i>S. granlo</i>	J	89.1 $\pm$ 1.0	2.5 $\pm$ 0.1		93	2	<0.05	93	2	—	96	2	—
S2804	<i>S. cerro</i>	K	33.3 $\pm$ 1.2	7.2 $\pm$ 0.2		42	8	—	73	4	<0.05	60	6	<0.05
S99	<i>S. minnesota</i> /S	L	75.0 $\pm$ 5.0	3.0 $\pm$ 0.4		86	3	—	83	4	—	82	3	—
R345	<i>S. minnesota</i> /Rb		2.4 $\pm$ 0.4	14.5 $\pm$ 0.6		3	11	<0.05	73	3	<0.001	11	9	<0.05
R595	<i>S. minnesota</i> /Re		1.1 $\pm$ 0.5	28.4 $\pm$ 3.6		1	25	—	79	2	<0.01	13	7	<0.05
S2124	<i>S. minnesota</i>	L	65.1 $\pm$ 0.3	1.2 $\pm$ 0.1		84	2	<0.05	83	2	<0.01	78	3	<0.05
S407	<i>S. alachua</i>	O35	76.8 $\pm$ 0.6	3.2 $\pm$ 0.1		93	2	—	74	3	—	84	4	—
S1354	<i>S. johannesburg</i>	O40	90.2 $\pm$ 1.5	2.8 $\pm$ 1.5		99	2	<0.05	89	2	—	92	2	—
S200	<i>S. offa</i>	O41	94.5 $\pm$ 0.5	1.9 $\pm$ 0.1		99	2	—	92	2	—	93	2	—
S409	<i>S. houten</i>	O43	8.7 $\pm$ 1.0	8.8 $\pm$ 0.2		11	8	—	22	9	<0.05	12	7	—

Fig. 1. Hydrophobic interaction chromatography on Octyl-Sepharose in relation to partition into the top phase in the basic dextran-PEG two-phase system. Each strain (isolate) is represented as a bar. Percentage elution from Octyl-Sepharose is shown as bars (the first elution step, open bar; the second elution step, filled bar; the third elution step, stippled bar and the fourth elution step, filled bar). The strains (isolates) are ranked according to recovery in the top phase (shown at the top of the diagram by the strain number). The exact positions of overlapping bars are shown by triangles below the abscissa.

Table 2. *Chromatography of salmonella on DEAE-Sephacel (ion exchange) and Sepharose CL-4B (control)*

For the individual strains/isolates the duplicate tests were performed and the mean was calculated. For the DEAE-Sephacel data the $\pm$ values indicate ranges about the mean. Similar ranges were observed with Sepharose CL-4B, but these are not shown. The bacteria in the serogroups C1, E4 and O43, plus the *S. muenster* (E1) strain were grouped together, and the mean and s.d. calculated. The radioactivity is given as the sum in per cent of the added radioactivity.

	DEAE-Sephacel				
	Radioactivity (%) in elution step:				Recovery (%)
	1	2	3	4	
<i>S. typhimurium</i> 395MS	77.0 $\pm$ 1.8	17.1 $\pm$ 1.7	1.8 $\pm$ 0.3	4.1 $\pm$ 0.1	90 $\pm$ 2
<i>S. minnesota</i> S99	71.3 $\pm$ 1.9	21.7 $\pm$ 2.1	2.5 $\pm$ 0.3	4.4 $\pm$ 0.1	84 $\pm$ 3
<i>S. typhimurium</i> 395MR10	0.8 $\pm$ 0.1	33.5 $\pm$ 1.8	5.7 $\pm$ 0.1	60.1 $\pm$ 1.8	73 $\pm$ 0
<i>S. minnesota</i> R345	1.5 $\pm$ 0.9	43.5 $\pm$ 2.7	4.5 $\pm$ 0.9	50.5 $\pm$ 2.8	73 $\pm$ 2
<i>S. minnesota</i> R595	1.3 $\pm$ 0.1	27.0 $\pm$ 3.4	7.5 $\pm$ 1.8	64.2 $\pm$ 4.8	73 $\pm$ 5
<i>S. agona</i> (B)	65.0 $\pm$ 2.4	26.0 $\pm$ 1.4	3.0 $\pm$ 1.3	6.0 $\pm$ 0.6	79 $\pm$ 1
<i>S. derby</i> (B)	58.4 $\pm$ 1.3	21.4 $\pm$ 0.3	3.6 $\pm$ 0.4	16.6 $\pm$ 0.6	66 $\pm$ 2
<i>S. cerro</i> (K)	61.2 $\pm$ 1.1	22.0 $\pm$ 0.6	4.0 $\pm$ 0.6	12.7 $\pm$ 0.1	82 $\pm$ 0
Serogroup C1, E4, O43 and <i>S. muenster</i> (E1) (9 isolates)	62.1 $\pm$ 8.5	21.6 $\pm$ 6.3	4.2 $\pm$ 1.5	12.1 $\pm$ 4.5	79.4 $\pm$ 7.0

	Sepharose CL-4B				
	Radioactivity (%) in elution step:				Recovery (%)
	1	2	3	4	
<i>S. typhimurium</i> 395MS	95	4	1	0	89
<i>S. minnesota</i> S99	86	12	1	1	84
<i>S. typhimurium</i> 395MR10	40	44	4	11	80
<i>S. minnesota</i> R345	18	49	15	19	80
<i>S. minnesota</i> R595	27	39	25	9	68
<i>S. agona</i> (B)	63	33	3	1	88
<i>S. derby</i> (B)	38	55	5	2	80
<i>S. cerro</i> (K)	59	39	2	1	93
Serogroup C1, E4, O43 and <i>S. muenster</i> (E1) (9 isolates)	50.5 $\pm$ 8.6	43.5 $\pm$ 7.7	4.4 $\pm$ 1.4	1.6 $\pm$ 1.0	84 $\pm$ 4

Hydrophobic interaction chromatography on Octyl-Sepharose

After chromatography on Octyl-Sepharose recovery of the R-mutants R10, R345 and R595 was very low in the first (3.4–9.2%) and second (3.8–6.3%) eluting steps; most of the organisms (49–68%) were recovered after elution with ethanol, although a substantial proportion (25–37%) remained in the column after elution (Fig. 1). In control experiments in which these strains were passed through Sepharose CL-4B columns, most of the bacteria (66–85%) were recovered in the first two elution steps (Table 2); thus the hydrophobic interaction of the R-mutants was great. In contrast the two laboratory S strains *S. typhimurium* 395MS and *S. minnesota* S99 showed little if any interaction, since these strains eluted with yields of 86% and 82%, respectively, from Octyl-Sepharose in elution step 1 (Fig. 1). Control tests on Sepharose CL-4B gave 95% and 86% elution of these strains in elution step 1 (Table 2). Another isolate, S2628 (*S. haardt*) which yielded 87% in the first elution step from Octyl-Sepharose (Fig. 1), is not considered here because this value was larger than the yield from Sepharose CL-4B (67%).

For the remaining clinical isolates the yield in the first elution step contained 29–71% (Fig. 1); these values were little different from the control tests on Sepharose CL-4B. The yield from the second elution step after addition of another 2 ml buffer was, however, conspicuously lower after chromatography on Octyl-Sepharose (4–19%, mean $\pm$ s.d. = 11.0 $\pm$ 3.2) than on Sepharose CL-4B (15–58%, mean $\pm$ s.d. = 36.1 $\pm$ 10.4). The combined totals of the first two elution steps ranged from 36–83% (mean $\pm$ s.d. = 62.7 $\pm$ 12.6) on Octyl-Sepharose, and from 73–98%

Fig. 2

Fig. 3

Fig. 2. Number of bacteria associated per PMN (extracellular + intracellular) in relation to the partition of the bacteria into the top phase of the basic dextran-PEG two-phase system. ■, R-mutants; ▼, the C1/E4 group, i.e. serogroups C1, E4 and O43 and *S. muenster* S2811 (E1); ▲, *S. agona* S2863 (B), *S. derby* S2843 (B), and *S. cerro* S2804 (K); ●, the old laboratory S strains and remaining clinical isolates. The vertical bars join duplicate determinations. When no bar appears the two determinations are within the area of the symbol.

Fig. 3. Number of bacteria ingested per PMN versus the partition into the top phase. Symbols as for Fig. 2.

(mean $\pm$ s.d. = 92.3 ± 4.9) on Sepharose CL-4B indicating a small hydrophobic interaction. For the C1/E4 group the sums of the first two fractions on Octyl-Sepharose was 44–72% (mean $\pm$ s.d. = 60.9 ± 7.5) not significantly different from the main group.

Ion exchange chromatography

When bacterial suspensions were passed through DEAE-Sepharcel columns, recovery of the R-mutants in the first elution step was only 0.8–1.5% (Table 2), whereas the corresponding value for S bacteria and clinical isolates ranged from 48–82% (*S. litchfield*, 26%, and *S. alachua*, 39%, were exceptions). However, an unusually large yield was obtained in the second elution step, indicating only minor retardation. After elution with 4.1 M-NaCl 51–64% of the R-mutants remained in the gel, whereas the corresponding fraction of S bacteria was 4–28%; again *S. litchfield* (28%) and *S. alachua* (21%) were two of the three strains showing the greatest retention in the column. The different groups of S strains and clinical isolates showed considerable overlapping around slightly different means (Table 2). In summary the order with respect to free flow (non-retention) on DEAE-Sepharcel was: laboratory S strains > most clinical isolates > *S. agona*, *S. derby*, *S. cerro* > group C1/E4 $\gg$ laboratory R-mutants. In all groups retention was greater by DEAE-Sepharcel than by Sepharose CL-4B.

Association with PMN

The three R-mutants showed extensive association with the PMNs giving a range of 7.1–9.5 organisms per PMN (Fig. 2), and 63–80% of these, i.e. 4.5–7.6 bacteria per PMN were ingested (Fig. 3). The association of the laboratory S strains and the clinical isolates showed great differences among the strains. Values ranged from 0.15–6.6 bacteria per PMN, with only few organisms being ingested (2.5–20%). The nine isolates of the C1/E4 group, which were not

recovered from the top phase in a dextran-PEG two-phase system (Fig. 3), were more liable to phagocytosis than 24 strains for which recovery in the top phase was more than 50%. Calculated according to Wilcoxon's rank sum test this difference is highly significant ($P < 0.001$).

Haemagglutination test for fimbriae

Among 16 strains tested (all C1, E4 and O43 strains) none agglutinated human erythrocytes, indicating absence of P-fimbriae. Only *S. krefeld* showed weak agglutination with guinea-pig erythrocytes. Thus, under the growth conditions used in the present experiments neither type 1 fimbriae were detected by haemagglutination (Duguid & Old, 1980).

DISCUSSION

As found previously (Edebo *et al.*, 1980; Kihlström & Magnusson, 1980) the laboratory S strains *S. typhimurium* 395MS and *S. minnesota* S99 showed hydrophilic properties and absence of ionized groups, whereas the R-mutants *S. typhimurium* 395MR10, *S. minnesota* R345 and *S. minnesota* R595 demonstrated hydrophobic properties and negative charge. The present experiments included chromatography on Sepharose CL-4B as a control for interaction with the dextran matrix. Some retardation was observed with the R-mutants on the control gel, but there was a considerable increase in the retention of the R-mutants on Octyl-Sepharose CL-4B and on DEAE-Sephacel, so binding to the hydrophobic and positively charged groups, respectively, appeared significant. Aggregation of the R bacteria in 0.3 M-(NH₄)₂SO₄ or weak interaction with the Sepharose beads might have contributed to retardation in the Sepharose CL-4B column.

Most of the clinical isolates behaved like the laboratory S strains in all partition and chromatography tests. However, isolates belonging to the serogroups C1 and E4 (the C1/E4 group), did not conform to this pattern. They accumulated in the bottom phase in the dextran-PEG two-phase system like R bacteria but differed from them in being less influenced by TMA-PEG and P-PEG. No such differences among the clinical isolates were evident in the interaction with Octyl-Sepharose CL-4B and DEAE-Sephacel. The surface structure(s) responsible for the behaviour in phase partition is not known. The similar partition of bacteria and LPS (Edebo *et al.*, 1980) and the agreement among the five serogroup C1 isolates suggest that the chemical nature of the LPS is responsible. This may also be the case for the two serogroup E4 isolates and *S. houten* S409 (O43). However, differences in partition within the serogroups E1 and B demonstrate that the immunochemical properties of the LPS is not the sole determinant of the physicochemical surface properties. Chromatography of the clinical isolates on Octyl-Sepharose showed some retardation compared to the laboratory strains *S. typhimurium* 395MS and *S. minnesota* S99. This retardation was greater than on Sepharose CL-4B. Several of the clinical isolates also associated more avidly with PMNs than the laboratory S strains indicating that adhesive surface structures may be exposed. Öhman *et al.* (1981, 1982) recently showed that increased affinity of *E. coli* strains for Octyl-Sepharose correlated with increased exposure of hydrophobic type 1 fimbriae. Although haemagglutination experiments did not detect any type 1 fimbriae under our experimental conditions, it cannot be ruled out that adhesive structures of this kind may be present in our clinical isolates, since Duguid *et al.* (1976) found that 85% of strains of *S. typhimurium* from natural sources contained these structures. Quantitative differences with respect to fimbriae, LPS and O-antigenic determinant polymerization as well as additional outer membrane proteins may affect the results of several of the tests performed (Stendahl *et al.*, 1973a, b).

The virulence of *S. typhimurium* after injection into mice is dependent on the mouse strain (Hormaeche, 1979; Plant & Glynn, 1982) and the quantity of the S(O)-antigen repeat unit of the bacteria (Edebo & Normann, 1970). Larger quantities of S-antigen also correlate with hydrophilic surface properties and absence of charge (Edebo *et al.*, 1980) and with resistance to phagocytosis (Stendahl & Edebo, 1972).

The virulence of different salmonella serogroups and serotypes has been elegantly investigated in genetic transfer experiments by Mäkelä, Valtonen and coworkers. Isogenic

transductants with the O-antigen 1,4,12, were most virulent. Bacteria with the O-antigen 1,9,12, were less virulent and bacteria with the O-antigen 6,7, were nearly avirulent (Mäkelä *et al.*, 1973; Valtonen, 1970; Valtonen *et al.*, 1975). Also three fresh human faecal isolates of salmonella group C (O-6,7) were completely avirulent ($LD_{50} > 10^7$; Valtonen, 1977) for the mice. These results showed that the chemical composition of the S-specific repeating carbohydrate portion is of great importance for virulence. In contrast, mutations in *S. typhimurium* leading to loss of two major outer membrane proteins did not change the virulence (Valtonen *et al.*, 1977). *Salmonella typhimurium* hybrid strains containing O-6,7 antigen were cleared more rapidly from the blood than O-4,12 sister hybrids (Valtonen, 1977) and phagocytosed more rapidly by a macrophage-like cell line *in vitro* in a complement-dependent process (Liang-Takasaki *et al.*, 1982). Our experiments showed that, in the absence of complement the ingestion by PMNs of the clinical isolates of the O-6,7 bacteria was significantly greater than that of the main group. Furthermore, these bacteria were more liable to hydrophobic interaction with P-PEG. The chemical composition of the O-6,7 LPS is notably different from that of other serogroups of salmonella (Lindberg & Le Minor, 1983). It contains mainly mannose polymerized in 1 $\rightarrow$ 2 linkages. This polysaccharide might expose an area deficient in hydroxyl groups with increased liability to hydrophobic interaction.

The above results indicate that qualitative differences between the LPS of different serogroups influence the pathogenicity. Little data comparing the pathogenicity of different species (serotypes) is, however, available. In a classical paper on the taxonomy of salmonella, Kaufmann (1930) examined the oral pathogenicity for mice. Three species that showed oral pathogenicity for mice in his investigation accumulated in the top phase (58–95%, mean = 81, S.D. $\pm$ 11, n = 6) of our dextran-PEG two-phase system, whereas three species that did not kill the mice were not recovered from the top phase (3–5%, mean 3.8, S.D. $\pm$ 0.8, n = 3). This correlation is, however, not significant (P = 0.1). Neither were all isolates of serogroup C1 non-pathogenic for mice. In different oral feeding experiments of rats *S. oranienburg* (6,7 : m, t : -), showed no difference to four other salmonella serotypes including *S. typhimurium* (Ruitenber *et al.*, 1971). Thus, the relationship between LPS structure and pathogenicity is equivocal. The increase in virulence for chicken and mice on converting the O antigen of *S. cholerae-suis* from 6,7 to 6,17 by phage may be involved (Smith & Parsell, 1974). Properties affecting the multiplication within the host, like defects in the synthesis of thymine, aromatic amino acids and enterochelin as well as attachment fimbriae and the enterobacterial common antigen are also known to influence the virulence (Lindberg, 1980). With the exceptions of *S. typhi* and *S. paratyphi* A-C most salmonellae are considered to cause mild enteritis in general. For a long time the serological response in *S. typhi* and *S. paratyphi* infections has been recognized, e.g. by bacterial agglutination tests. When more sensitive serological methods were used to study a food-borne epidemic with *S. enteritidis*, there was a regular, time-dependent antibody response (Edebo *et al.*, 1981) indicating that the bacteria or their LPS penetrated far enough to stimulate the immunological apparatus even in the mild cases. *Salmonella enteritidis* showed hydrophilic surface properties and resistance to phagocytosis. No data are as yet available about the human antibody response to the C1/E4 group to support further differences in pathogenicity.

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