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Effect of Enterobacterial Common Antigen on Mouse Virulence of *Salmonella typhimurium*

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A series of nearly isogenic O-4,12 and O-6,7 *Salmonella typhimurium* strains differing in regard to the enterobacterial common antigen (ECA) were constructed by conjugation. When tested in intraperitoneal infection of mice, the O-4,12 strains containing ECA were more virulent than their O-4,12 sister recombinants without ECA ($P = <0.001$). The same difference could be shown with ECA-positive and ECA-negative *S. typhimurium* derivatives, whose O antigens were of the group C type (O-6,7). The ECA-positive and ECA-negative O-4,12 strains did not differ in their growth rates in broth or clearance rates in vivo.

The enterobacterial common antigen (ECA) is shared by bacteria of the family *Enterobacteriaceae* (7) and probably is a part of the cell envelope, as is lipopolysaccharide (LPS), an important virulence factor (14). The chemical composition of ECA has been the subject of conflicting reports (4, 6), but all data are most consistent with it having a polysaccharide (or oligosaccharide) part. This would also fit best with the demonstration (11, 12) that genes of LPS synthesis participate in the synthesis of ECA. The genetic findings have made it possible to construct nearly isogenic strains of *Salmonella* differing only in regard to ECA. We have now used such strains to study the possible role of ECA as a virulence factor and report here data that show that ECA-positive strains of *Salmonella typhimurium* are more virulent in intraperitoneal infection of mice than are ECA-negative mutants.

MATERIALS AND METHODS

Bacterial strains and genetic methods. The bacterial strains and their main properties are listed in Table 1. Bacterial cultivation and genetic procedures were performed according to standard methods (9, 16).

ECA determination. ECA determination was based on the indirect hemagglutination test (19), using sheep erythrocytes and anti-*Escherichia coli* O14 rabbit serum as anti-ECA. This serum had a titer of 1:1,000 with cells sensitized by extracts of ECA-positive strains, 1:10 by extracts of ECA-negative strains, and intermediate values when ECA trace strains were used (11).

Virulence test. The 50% lethal dose values were determined from 10-day survival in groups of 10 mice after intraperitoneal injection of 0.5 ml/mouse from a series of 10-fold dilutions of overnight broth cultures (18). The significance was calculated from pooled numbers of survivors as described previously (18). The mice used were (CBA × C57Bl/6) F1 hybrids in all experiments. As previously shown (17, 18), in this system 10-day survival data are as reliable as 30-day data.

Measurement of the growth rate. The growth rates of different strains of bacteria are expressed in terms of generations per hour. The growth took place in flasks containing 100 ml of complete liquid medium (Lab Lemco [Oxoid] broth, 8.0 g; glucose, 1.0 g; NaCl, 3.5 g; K_2HPO_4, 3.68 g; KH_2PO_4, 1.32 g; water, 1,000 ml; pH 7.1) on a shaker at 37 C for a total period of 40 h. The bacteria were kept in the exponential growth phase by diluting them into fresh prewarmed medium every 8th h. Care was taken not to use too small inocula (not less than 5,000 bacteria). At each time suitable dilutions were plated on nutrient agar plates for counting colony formers. The number of generations was calculated from final colony counts and dilution data.

Clearance test. The clearance test (5) was performed by injecting 0.2 ml (about 5×10^7 bacteria) of an exponentially growing broth culture into the central tail vein of the mice. Blood samples (0.05 ml) were taken after 0.5, 1, 2, 3, and 5 min from the retro-orbital venous plexus into 5 ml of distilled water. They were plated immediately in suitable dilutions for enumeration of viable bacteria. The clearance rate of bacteria from the blood per minute was calculated from these data. Each strain was tested in three mice.

RESULTS

Principles for preparation of the strains. We hope that the following list of five principles concerning the genetic determination of ECA and LPS will facilitate understanding the procedures in constructing the ECA$^+$, ECA$^-$, and ECA^{tr} strains.

(P1) *rfe*$^+$ genes close to *ilv* are always neces-

TABLE 1. *Salmonella strains used*

Strain	Species[a]	Line	LPS	ECA	Relevant genotype[b]					Source
					rfb	*rfe*[c]	*ilv*[d]	*his*[e]	Other	
SH1606	mv	HfrH14 Ky129	6,7	+	C+	C+	C+	C+	*leu*−	From HfrH14 (9)
SH3879	tm	IH2	4,12	+	B+	B+	B−	B−		From IH2 (17)
SH5454	tm	HfrK1-2	R	−	C+	B−	B−	B+	*ser*−	From HfrK1-2 (16)
SH5492	tm	IH2	4,12	+	B+	B+	B+	B−		Fig. 1
SH5493	tm	IH2	4,12	−	B+	B−	B+	B−		Fig. 1
SH5494	tm	IH2	4,12	−	B+	B−	B+	B−		Fig. 1
SH5495	tm	IH2	4,12	+	B+	B+	B+	B−		Fig. 1
SH5516	tm	IH2	4,12	+	B+	B+	B+	B−		Fig. 1
SH5517	tm	IH2	4,12	+	B+	B+	B+	B−		Fig. 1
SH5521	tm	IH2	4,12	−	B+	B−	B+	B−		Fig. 1
SH5522	tm	IH2	4,12	−	B+	B−	B+	B−		Fig. 1
SH6405	tm	IH2	6,7	tr	C+	B+	B−	C+		Fig. 1
SH6416	tm	IH2	6,7	+	C+	C+	C+	C+		Fig. 1
SH6417	tm	IH2	6,7	+	C+	C+	C+	C+		Fig. 1
SH6418	tm	IH2	6,7	+	C+	C+	C+	C+		Fig. 1
SH6422	tm	IH2	6,7	tr	C+	B+	C+	C+		Fig. 1
SH6423	tm	IH2	6,7	tr	C+	B+	C+	C+		Fig. 1
SH6424	tm	IH2	6,7	tr	C+	B+	C+	C+		Fig. 1

[a] mv, *S. montevideo* of group C_1; tm, *S. typhimurium* of group B.
[b] Origin of genes from group B or C is indicated by C+, etc.
[c] B− = *rfe-4274*.
[d] B− = *ilv-1141*.
[e] B− = *his-5892*.

sary for ECA synthesis. All *rfe*− strains are ECA− (11, 12).

(P2) The O-antigenic part of LPS is specific for each O group and its structure is determined by *rfb* genes close to *his*. Thus, the B-*rfb*+ genes of *Salmonella* group B determine O antigens 4,12 and the C-*rfb*+ genes of group C determine O antigens 6,7 (9).

(P3) Groups B and C also differ from each other in respect to the role of *rfe*+ genes in O antigen synthesis. *rfe*+ genes are needed for O-6,7 synthesis but not for O-4,12 synthesis; so B-*rfe*− strains with C-*rfb*+ are rough (R) with defective LPS, whereas B-*rfe*− strains with B-*rfb*+ are smooth with O-4,12 (10).

(P4) C-*rfe*+ alone suffices for ECA synthesis in both groups B and C (11).

(P5) B-*rfe*+ is somehow dependent on the function of B-*rfb*, so that the combination of B-*rfe*+ with C-*rfb*+ results in minimal amounts of ECA detectable by hemagglutination (= phenotype ECAtr) (11).

The same mouse virulent *his*−*ilv*− derivative (SH3879) of *S. typhimurium* line IH2 (a recent isolate from human diarrhea) was used as the parent for all the strains to be compared (17).

ECA+ and ECA− derivatives with O-4,12. In cross I we used SH3879 (O-4,12, B-*rfb*+ B-*rfe*+) as recipient and an Hfr strain of *S. typhimurium* (SH5454) with a mutation in its *rfe* genes (B-*rfe*−) as donor (Fig. 1). Because the *rfe* region is closely linked to *ilv* (11) we selected *ilv*+ recombinants and tested them for ECA. As expected, most of these recombinants were ECA− (with B-*rfe*− from the donor), whereas some were ECA+ (B-*rfe*+ of the recipient) according to P1. All were O-4,12 as determined by the B-*rfb*+ of the recipient. Three strains of each kind were used for virulence determination.

The B-*rfe*− genotype of the ECA-negative recombinants was further ascertained by using one of the recombinants as recipient with a group C donor (cross II, Fig. 1). *his*+ recombinants were expected to have inherited the donor C-*rfb*+, genes closely linked to *his*. They all were rough, indicating the genotype C-*rfb*+ B-*rfe*− according to P3.

ECA+ and ECAtr derivatives with O-6,7. In cross III (Fig. 1) we used SH3879 again as recipient and *S. montevideo* (group C, SH1606) as donor to get recombinants with the donor C-*rfb*+. Most *his*+ recombinants were O-6,7 (determined by C-*rfb*+) and all these were ECAtr, as is consistent with the inferred genotype B-*rfe*+ (from recipient) C-*rfb*+ (from donor) (P5). To get the corresponding ECA+ strain, one of these recombinants, SH6405, was used as a recipient in a cross (cross IV, Fig. 1) with the same group C donor (SH1606). *ilv*+ recombinants were selected as in cross I. Some of these retained their original B-*rfe*+ and the phenotype O-6,7 ECAtr. The others had acquired the C-*rfe*+ from the donor and were O-6,7 ECA+ (P4).

Virulence tests. ECA+, ECA−, or ECAtr sis-

ter recombinants were compared for their mouse virulence after intraperitoneal inoculation (Table 2). Of those with the O antigen 4,12, the ECA-positive strains were more virulent than their ECA-negative sisters; the average

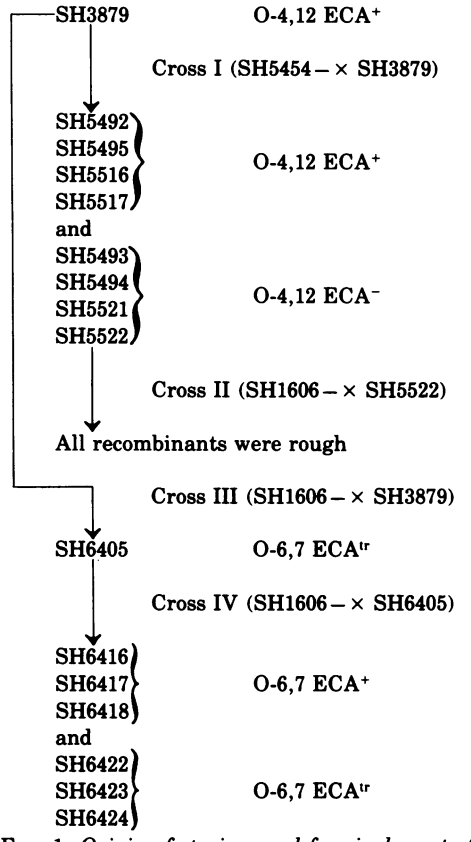

FIG. 1. *Origin of strains used for virulence tests. For further details, see Table 1.*

50% lethal dose for the three ECA-positive strains was 9×10^4 and for the three ECA-negative strains it was 10^6. The difference is highly significant ($\chi^2 = 29{,}32$; $P < 0.001$).

The difference in average 50% lethal dose values between the ECA$^+$ and ECA^{tr} O-6,7 sister recombinants was similarly about 10-fold (Table 3). The ECA$^+$ derivatives had a 50% lethal dose of 7×10^6, and for ECA^{tr} derivatives it was more than 3×10^7. This difference in virulence is highly significant ($\chi^2 = 10{,}63$; $P \sim 0.001$).

Growth rates. The growth rates of the O-4,12 strains with or without ECA were measured in vitro. All strains gave very similar results irrespective of the presence or absence of ECA, ranging from 2.11 to 2.18 generations per h in ECA-positive strains and from 2.11 to 2.16 generations/h in ECA-negative strains (Table 4).

Since the growth rates were so similar, a possible small difference would not be detected by this method but might be revealed in a competition situation. We therefore grew together, in a similar set up and starting from equal inocula, pairs of ECA-positive and ECA-negative O-4,12 strains (Table 4). The colonies of the two strains on the counting plates could be presumptively identified on the basis of slight differences in colony morphology; they were ascertained by ECA determination. There was no sign of take-over by any strain.

Clearance rates. The clearance rates of these sister O-4,12 ECA-positive and ECA-negative derivatives of *S. typhimurium* were determined to find out whether the difference in clearance rate could explain the observed differences in virulence (Fig. 2). The clearance test is a sort of in vivo measure of the resistance of the bacterial strains to phagocytosis. No sta-

TABLE 2. *Virulence of O-4,12 ECA$^+$ and O-4,12 ECA$^-$ derivatives of S. typhimurium*

Strain	LPS	LD_{50}[a]	No. of deaths in groups of 10 mice[b]		
			5×10^5[c]	5×10^4	5×10^3
ECA$^+$					
SH5492	4,12	2×10^5	9	1	0
SH5495	4,12	3×10^4	9	8	0
SH5516	4,12	3×10^4	9	6	1
Avg		9×10^4			
ECA$^-$					
SH5493	4,12	7×10^5	4	0	0
SH5494	4,12	2×10^6	1	0	0
SH5521	4,12	8×10^5	3	0	0
Avg		1×10^6			

[a] LD_{50}, 50% lethal dose.

[b] Significance of the difference in virulence calculated from numbers of dead versus surviving mice between ECA$^+$ and ECA$^-$ derivatives is highly significant ($P < 0.001$).

[c] Dose (bacteria per mouse).

TABLE 3. *Virulence of O-6,7 ECA$^+$ and O-6,7 ECA^{tr} derivatives of S. typhimurium*

Strain	LPS	LD_{50}	No. of deaths in groups of 10 mice[b]		
			2×10^7 [c]	2×10^6	2×10^5
ECA$^+$					
SH6416	6,7	6×10^6	10	0	0
SH6417	6,7	6×10^6	10	0	0
SH6418	6,7	1×10^7	6	0	0
Avg		7×10^6			
ECA^{tr}					
SH6422	6,7	4×10^7	3	0	0
SH6423	6,7	3×10^7	4	0	0
SH6424	6,7	1×10^7	7	0	0
Avg		3×10^7			

[a] LD_{50}, 50% lethal dose.
[b] The difference in virulence calculated from numbers of dead versus surviving mice between ECA$^+$ and ECA^{tr} derivatives is highly significant ($P \sim 0.001$).
[c] Dose (bacteria per mouse).

TABLE 4. *Growth rates of sister ECA$^+$ and ECA$^-$ strains (both O-4,12) in mixed culture*[a]

Expt	ECA$^+$			ECA$^-$		
	Strain	Growth rate[b] (generations/h)	No. of colonies[c] ($\times 10^{-32}$)	Strain	Growth rate (generations/h)	No. of colonies ($\times 10^{32}$)
1	SH5492	2.12	273	SH5493	2.12	256
2	SH5492	2.11	198	SH5493	2.16	325
		(2.12)			(2.14)	
3	SH5516	2.16	921	SH5521	2.14	477
4	SH5516	2.18	1,258	SH5521	2.11	235
		(2.17)			(2.13)	
5	SH5517	2.15	639	SH5522	2.11	170

[a] Exponential growth, 40 h at 37 C in broth.
[b] Calculated from these data; average values of each strain in different experiments are in parentheses.
[c] Number of colonies of each type after 40 h of mixed growth, corrected to identical inocula of 10,000 bacteria of each type (which in reality varied between 10,000 and 30,000).

tistically significant differences in the clearance rates between the O-4,12 ECA-positive and ECA-negative derivatives of either strain were found. They all remained in the blood for the test period as efficiently as do normal, smooth, virulent strains. The fast clearance of a rough derivative is also shown in Fig. 2 for comparison.

DISCUSSION

These data demonstrate that the absence (or reduced amount) of ECA lowered the mouse virulence of *S. typhimurium* by intraperitoneal inoculation irrespective of LPS (O-4,12 or O-6,7). Although the difference was not more than 10-fold, it was highly significant ($P < 0.001$).

A similar fairly small, but significant, difference in virulence has been recorded as due to the quality of the O antigen of smooth bacteria: in isogenic pairs of strains, O-4,12 strains were more virulent than O-9,12 strains (17, 18). The difference between O-4,12 and O-6,7 strains is larger. It is seen here by comparing the ECA-positive O-4,12 and O-6,7 strains although they are not isogenic (they differ also in respect to the origin of their *ilv-rfe* genes). We have, however, also prepared isogenic pairs of ECA-positive O-4,12 and O-6,7 strains and found them to have a 100-fold difference in virulence (unpublished observation).

In the paper by Valtonen (18), 4,12 and 6,7 strains were also compared for virulence; at that time, however, it was not realized that the strains also differed in respect to ECA.

Since ECA is apparently a component of the bacterial cell envelope, it was thought possible that its absence might affect the growth rate of the bacteria, and slower growth would reduce virulence. However, the growth rates measured in vitro were rather similar (ranging from 2.11 to 2.18 generations/h) in all the ECA-positive and ECA-negative O-4,12 strains tested.

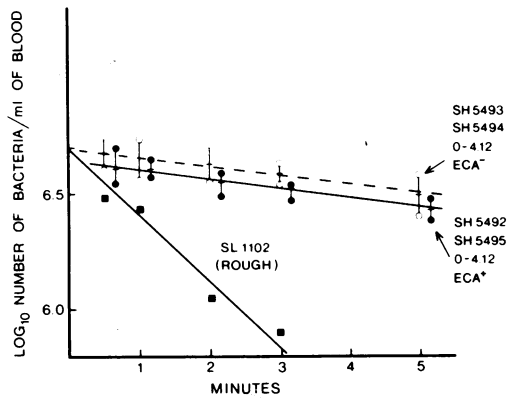

FIG. 2. *Clearance rate of different Salmonella strains from the blood of mice after intravenous injection. Symbols: Solid line and ●, ECA-positive strains; dotted line and ○, ECA-negative strains; ■, a rough strain, SL1102 (5, 17). The vertical bar between the circles shows the scatter and the small horizontal line on it is the mean value from tests with three mice each. The solid and dotted lines represent these mean values.*

It would also seem possible that strains missing the ECA component of their cell envelopes might be more susceptible to phagocytosis or the lytic effect of complement, as are LPS-defective R strains (15). The in vivo clearance test used in the present study could not show differences between the ECA-positive and ECA-negative O-4,12 strains, suggesting that phagocytosis is not a discriminating mechanism. However, this conclusion is tentative only, because of the relative insensitivity of the test used.

The role of immunity might be expected to be less significant because mice develop only minimal titers of humoral antibodies in response to immunization with ECA (2). However, ECA antibodies passively transferred (1) or due to immunization (3) transiently protect mice during experimental *Salmonella* infections. In human infections ECA antibodies appear to have little relevance (8).

We feel that the now demonstrated role of ECA as a virulence determinant and the possibility for more exact experimental work using isogenic ECA-positive and -negative strains warrant a continuation of studies, e.g., in respect to possible protection through ECA-specific immunization.

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