IMMUNOLOGICAL STUDY OF THE O-ANTIGENS OF STREPTOMYCIN-DEPENDENT MUTANTS OF SALMONELLA

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В в	*В в*	V, v	Т т	*Т т*	T, t
Г г	*Г г*	G, g	У у	*У у*	U, u
Д д	*Д д*	D, d	Ф ф	*Ф ф*	F, f
Е е	*Е е*	Ye, ye; E, e*	Х х	*Х х*	Kh, kh
Ж ж	*Ж ж*	Zh, zh	Ц ц	*Ц ц*	Ts, ts
З з	*З з*	Z, z	Ч ч	*Ч ч*	Ch, ch
И и	*И и*	I, i	Ш ш	*Ш ш*	Sh, sh
Й й	*Й й*	Y, y	Щ щ	*Щ щ*	Shch, shch
К к	*К к*	K, k	Ъ ъ	*Ъ ъ*	"
Л л	*Л л*	L, l	Ы ы	*Ы ы*	Y, y
М м	*М м*	M, m	Ь ь	*Ь ь*	'
Н н	*Н н*	N, n	Э э	*Э э*	E, e
О о	*О о*	O, o	Ю ю	*Ю ю*	Yu, yu
П п	*П п*	P, p	Я я	*Я я*	Ya, ya

*ye initially, after vowels, and after ъ, ь; e elsewhere. When written as ë in Russian, transliterate as yë or ë. The use of diacritical marks is preferred, but such marks may be omitted when expediency dictates.

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RUSSIAN AND ENGLISH TRIGONOMETRIC FUNCTIONS

Russian	English
sin	sin
cos	cos
tg	tan
ctg	cot
sec	sec
cosec	csc
sh	sinh
ch	cosh
th	tanh
cth	coth
sch	sech
csch	csch
arc sin	$\sin^{-1}$
arc cos	$\cos^{-1}$
arc tg	$\tan^{-1}$
arc ctg	$\cot^{-1}$
arc sec	$\sec^{-1}$
arc cosec	$\csc^{-1}$
arc sh	$\sinh^{-1}$
arc ch	$\cosh^{-1}$
arc th	$\tanh^{-1}$
arc cth	$\coth^{-1}$
arc sch	sech^{-1}
arc csch	csch^{-1}
rot	curl
lg	log

GREEK ALPHABET

Alpha	A	α	𝛂		Nu	N	ν	
Beta	B	β			Xi	Ξ	ξ	
Gamma	Γ	γ			Omicron	O	o	
Delta	Δ	δ			Pi	Π	π	
Epsilon	E	ε	ϵ		Rho	P	ρ	𝛒
Zeta	Z	ζ			Sigma	Σ	σ	ς
Eta	H	η			Tau	T	τ	
Theta	Θ	θ	𝛝		Upsilon	T	υ	
Iota	I	ι			Phi	Φ	φ	ϕ
Kappa	K	𝛋	κ	𝛞	Chi	X	χ	
Lambda	Λ	λ			Psi	Ψ	ψ	
Mu	M	μ			Omega	Ω	ω	

IMMUNOLOGICAL STUDY OF THE O-ANTIGENS OF STREPTOMYCIN-DEPENDENT MUTANTS OF SALMONELLA

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In recent years attempts have been undertaken to use streptomycin-dependent mutants of enterobacteria for the preparation of live vaccines. It was shown that such mutants of Shigella and Salmonella are weakly virulent for laboratory animals and stimulate a high level of specific protection (Mel et al., 1965; Sergeyev et al., 1967; Mikhaylov et al., 1968). In connection with this the need appears for a study of the antigenic composition and comparison of the data of antigenic analysis with virulence and the protective activity of such mutants.

In a preceding work (Yedvabnaya et al., 1969) it was shown that the streptomycin-dependent mutant S. enteritidis 921/3 did not synthesize the sugars of the determinant groups of O-antigen - the antigen complex consisted basically of base structure (Luderitz et al., 1966). During the further study of O-antigens of different genetically related variants streptomycin-dependent mutants of S. enteritidis it was shown that in the structure of their O-antigens there is a defect which in separate mutants is expressed to a

different degree. Thus in some mutants, according to paper chromatography, the O-antigen lacks tyvelose, in others - tyvelose and rhamnose, in the third - additionally mannose. There is doubtless interest in the immunological analysis of the indicated mutants. For study we selected 2 mutants: in one of them (921/3) the defect in the structure of the O-antigen was expressed considerably (there was no tyvelose, rhamnose and mannose), in the other (921/2) - in the polysaccharide component only tyvelose was not revealed. Mutant 921/3 was isolated in 1966, and for 3 years it was reinoculated on media with streptomycin. Mutant 921/2 was isolated in 1969.

In the work the established goal was to study the immune response in respect to O-antigens, defective in the structure of the polysaccharide component, to determine their activity in the test of passive skin anaphylaxis, and also to determine the toxicity of the lipopolysaccharides isolated from streptomycin-dependent mutants of S.enteritidis.

Both mutants were found in the S-form, they were weakly virulent for rats (LD_{50} more than $4 \cdot 10^6$ cells) and mice (LD_{50} strain 921/3 - 10^9, strain 921/2 - $7 \cdot 10^{10}$ cells).

The O-antigen of mutant 921/3 in the reaction of passive hemagglutination did not react with O9- and O12-antisera, but reacted with polyvalent salmonellose serum. The O-antigen of mutant 921/2 reacted with O9-antiserum just as the initial O-antigen. From the cells of mutants, and also initial virulent strain No. 921 the lipopolysaccharides were extracted by the method of Westphal et al. (1952).

Dynamics of the immune response were studied in rats of the August line and mice of line BALB/c. The animals were immunized with heated vaccine (heating of the culture of the initial strain No. 921 at 60° for an hour and the addition of merthiolate to

1:10,000), with the live cells of mutants or with the cell walls of the corresponding strains. The sera of the immunized animals were fractionated on a column with Sefadex gel G-200 for differentiation of IgM- and IgG-antibodies. A 3×90 cm column was used. The titers of O-antibodies in the serum and fractions were determined with the help of the reaction of passive hemagglutination, using sheep erythrocytes sensitized with activated lipopolysaccharide.

The activity of antibodies ($\overline{A}$) in the fractions (in each test tube of eluate) was estimated according to the initial activity, referred to a unit of concentration of protein (E in mg/ml). After some conversions the following formula was derived:

$$\overline{A}=\frac{2^n}{E_{нач}},$$

where n - number of dilutions with double step, $E_{нач}$ - the initial concentration of protein in a fraction, determined on a calibration curve.

Passive skin anaphylaxis was studied in experiments on guinea pigs 250-400 g in weight. The animals were given intracutaneously 0.05 ml of antiserum to the corresponding O-antigens and in 20-24 hours - intravenously a solution of lipopolysaccharide in a 0.25% solution of Evans' bluing. The toxicity of the isolated lipopolysaccharides was determined in experiments on mice using the standard method.

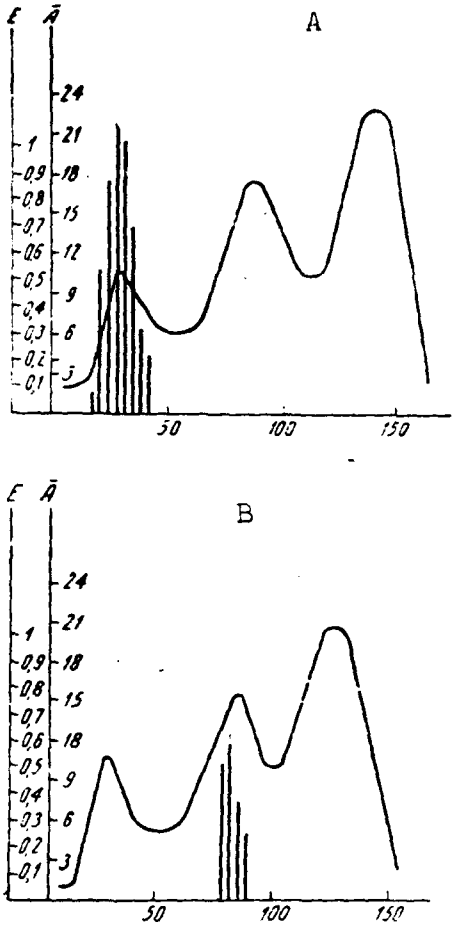
For the purpose of studying the immune response into the cushions of the extremities of rats $4 \cdot 10^9$ cells of mutant 921/2 or 921/3 or of heated vaccine was introduced doubly. The interval between immunizations was 2 weeks. In each group there were 10 animals. Sera from each individual were investigated, and for fractionation a mixture of sera from 5-10 animals was used. The dynamics of formation of antibodies was observed for 30 days after

the double immunization. After the single immunization in the blood of rats antibodies to the O-antigen of the initial strain were revealed in dilutions of 1:40-1:320 and to the O-antigen of mutants - in dilutions of 1:10-1:80. The secondary immunization stimulated the synthesis of O-antibodies according to the secondary type.

The analysis of serological activity of the 19S and 7S-fractions of serum of singly and doubly immunized rats showed the following. On the 7th day (maximum of antibodies) after 2 immunizations with the initial strain the activity of antibodies was localized in the 19S-fraction, and after the second immunization with the mutant 921/3 - in the 7S-fraction (see the figure). After a single immunization with the initial strain on the 7th day O-antibodies were also detected in the 19S-fraction. After a single immunization with mutant 921/3 in the serum of rats the level of O-antibodies was very low, therefore in the fractions the antibodies were not exposed in view of the significant dilution of serum in the process of gel filtration.

The immunization of rats with mutant 921/2 stimulated the synthesis of O-antibodies, the dynamics of formation of which was very nearly the same as during immunization with the initial strain.

For the purpose of a more detailed immunological study of the defective O-antigen of mutant 921/3 the rats were initially immunized with the heated vaccine (strain No. 921), and for the second time - with the mutant, and vice versa. It turned out (see the table) that if initially the rats were immunized with the initial strain No. 921, and for the second time - with mutant 921/3, the antibodies to normal O-antigen were exposed in the 19S-fraction, and the antibodies to O-antigen 921/3 were not detected in even one of the fractions. If initially the mutant was introduced, and the second time - the initial strain, then the antibodies to the initial O-antigen were detected mainly in the 19S-fraction (65 units) and in an insignificant measure - in



Gel filtration of the sera of the rats, immunized doubly with the cell walls of the initial strain No. 921 (A) and mutant 921/3 (B).
Vertical lines - activity of the antibodies ($\bar{A}$) of fractions, 1st peak - IgM, 2nd peak - IgG, 3rd peak - albumin; E - optical density of fractions at 280 nm.

the 7S-fraction (7 units). Antibodies to O-antigen 921/3 were exposed in lower titers than antibodies to the initial O-antigen: in the 19S-fraction the activity of antibodies to O-antigen 921/3 comprised 19 units, in the 7S-fraction - 1 unit. In the immunization of rats with cell walls of the corresponding strains analogous results were obtained.

In experiments on mice the following scheme is used: the animals received the antigens intravenously three times, the interval between immunizations was 15 days, period of observation - 30 days. The mice of one series were given heated vaccine (1st group), the live culture of mutant 921/2 (2nd group), mutant 921/3 (3rd group) in doses of $3 \cdot 0^8$, $5 \cdot 10^8$ and $5 \cdot 10^8$ cells for each immunization. In the other series of experiments the mice were immunized with the cell walls of the initial strain (1st group), mutant 921/2 (2nd group), mutant 921/3 (3rd group) in doses of 60, 100 and 100 μg of dry weight for each immunization.

Each group of mice consisted of 50 animals. The experiment was repeated twice. In the reaction of passive hemagglutination the mixture of sera from 3-10 animals of each group was

investigated. For fractionation a mixture of sera from 15-20 animals of one group was used.

Determination of the activity of antibodies in 19S- and the 7S-fractions of the sera of rats and mice, immunized with heated vaccine of S. enteritidis (strain in No. 921) and with a live culture of mutant 921/3.

Immunization		Type of animal	Activity of antibodies (in $\bar{A}$) to the O-antigens of strain-mutants in test tubes with the maximum content of protein			
			19S-fraction		7S-fraction	
first	second		strain No. 921	mutant 921/3	strain No. 921	mutant 921/3
Strain No. 921	Mutant 921/3	Mice	0	3	3	6
		Rats	4	3	3	0
Mutant 921/3	Strain S. 921	Mice	43	3	4	37
		Rats	49	14	7	1

In the experiments on mice the qualitative difference in the immune response in respect to the O-antigen of the initial strain and the O-antigen of mutant 921/3 was also revealed. Antibodies to O-antigen 921/3 were revealed in the blood of mice after the first, and to the initial antigen - after the second immunization. Antibodies to the O-antigen of mutant are concentrated mainly in the 7S-fraction of serum, antibodies to the initial O-antigen - in both fractions, but predominantly in the 19S-fraction, i.e., data are obtained which are similar to the results of experiments on rats (see the figure).

In experiments on mice as on rats, the cross immunization with the initial strain and mutant 921/3 was conducted (see the table). During the first immunization the introduction of the initial strain, and during the second - mutant 921/3 stimulated a weak immune response to O-antigen. In this case the antibodies to O-antigen 921/3 were revealed only in the 7S-fraction. Primary immunization with mutant and secondary with the initial strain stimulated intensive antibody formation. In this case the antibodies to the initial O-antigen were localized predominantly in

the 19S-fraction, and antibodies to the O-antigen of the mutant - in the 7S-fraction. The activity of antibodies to O-antigen 921/3 in the 7S-fraction of the serum of mice was significantly higher than in the analogous fraction of the serum of rats.

It should be noted that during the primary immunization of mice antibodies to normal O-antigen were not detected in the reaction of passive hemagglutination; however, after preliminary sensitization with defective O-antigen 921/3 the primary introduction of normal antigen stimulated the synthesis of O-antibodies.

During the immunization of mice with freshly isolated mutant 921/2 or its cell walls the dynamics of formation of O-antibodies was almost identical to the dynamics of formation of O-antibodies after immunization with the initial strain or its cell walls.

The study with the help of the test of passive skin anaphylaxis was conducted only with the lipopolysaccharide of mutant 921/3, since the response to the introduction of the O-antigen of this mutant differed most strongly from the response to the O-antigen of the initial strain. In the experiments hyperimmune rabbit sera were used. The titer of serum to the O-antigen of the initial strain was equal to 1:2560-1:5120, to defective O-antigen it was equal to 1:160-1:320. The results of the determination of the skin reaction testified that the defective lipopolysaccharide turned out to be inactive, and the lipopolysaccharide of the initial strain caused in pigs in response to the introduction of homologous antigen a distinct skin reaction: in dilution of 1:2 - up to 20×20 mm, in dilution 1:5 - 10×8 mm. Thus, with the loss of the sugars of determinant groups the lipopolysaccharide was deprived of the capacity to cause a skin reaction in guinea pigs.

The lipopolysaccharides extracted from the cells of the initial strain and mutants turned out to be toxic for mice to an equal degree: the LD_{50} of lipopolysaccharide 921/3 and lipopolysaccharide 921 was equal to 0.088 mg. However, in skin tests on

guinea pigs a somewhat higher toxicity of lipopolysaccharide of the initial strain is revealed in comparison with lipopolysaccharide 921/3.

On the basis of the investigations conducted it is possible to make the conclusion that the mutation in a streptomycin-dependent locus of Salmonella leads to a more or less expressed defect in the synthesis of the lipopolysaccharide complex of the cell wall, i.e., O-antigen. However, in freshly isolated mutants this defect is less significant than in the mutants which are stored on nutrient medium with streptomycin for a long time (3 years). The O-antigen of such mutants did not contain the sugars which form determinant groups, but the specificity of O-antigen did not disappear completely, and in the nature of growth of the colonies mutant 921/3 was found in the S-form. The immune response in respect to the defective O-antigen, which consists basically of base structure, differed qualitatively from the immune response in respect to normal O-antigen according to the formation of IgM- and IgG-antibodies. At the same time defective lipopolysaccharide was toxic for mice to the same degree as initial, which testified to the insignificant role of the polysaccharide component of O-antigen in the manifestation of its toxicity.

Freshly isolated mutant 921/2, in the antigen of which, according to chromatography, the end sugar tyvelose was absent, in serological and immunogenic activity did not differ from the initial.

According to the data of Luderitz et al. (1966), tyvelose determines the specificity of the factor of O9-antigen. However, despite the fact that in O-antigen 921/2 tyvelose is not revealed, it reacted with monoreceptor O9-serum. In connection with this two assumptions are possible: probably the factor of O9-antigen is determined not only by tyvelose, but also by another sugar, or in the lipopolysaccharide of mutant 921/2 there is a certain amount of tyvelose, not exposed by the method of paper chromatography, but detected by serological test.

Thus the full or significant loss of end sugar (tyvelose) was not reflected substantially in the dynamics of formation of O-antibodies.

Conclusions

1. During the immunization of mice and rats with O-antigen of the streptomycin-dependent mutant 921/3 of S.enteritidis, the polysaccharide component of which consisted basically of base structure, in animals mainly IgG-antibodies were synthesized, whereas during immunization with O-antigen of the initial strain predominantly IgM- and in an insignificant measure IgG-antibodies were synthesized.

2. The dynamics of formation of antibodies to the O-antigen of freshly isolated streptomycin-dependent mutant 921/2, in the polysaccharide component of which only the end sugar tyvelose is not revealed, did not differ from the dynamics of the formation of antibodies to normal O-antigen.

3. Lipopolysaccharide 921/3, which did not contain sugar of the determinant groups, was not active in the reaction of passive skin anaphylaxis in guinea pigs.

4. The lipopolysaccharides extracted from the mutants were toxic for mice to the same degree as the lipopolysaccharides of the initial strain.

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A study was made of the formation of IgM- and IgG-antibodies in rats of August strain and of mice of BALB/c strain immunized with heated vaccine prepared from wild strain 921 S. enteritidis, or with the living culture of streptomycin-dependent mutant or the corresponding cell walls. It was revealed that in immunization with O-antigen of the 921/3 mutant, whose polysaccharide component consisted chiefly of the basis structure, IgG-antibodies were the ones to be chiefly synthesized, and after the adminnistration of O-antigen of the initial strain — chiefly IgM - and an insignificant amount of IgG-antibodies were synthesized.

Dynamics of formation of IgM- and IgG-antibodies against the O-antigen of the freshly isolated mutant 921/2, in whose polysaccharide component only tyvelose was absent, failed to differ from the dynamics of antibody formation against the O-antigen of the wild strain. Lipopolysaccharide of the 921/3 mutant proved to be inactive in the passive skin anaphylaxis test in guinea pigs. Lipopolysaccharides extracted from the cells of mutants were as toxic for mice as the lipopolysaccharide of the initial strain.