Immunochemical and Biochemical Studies on Phage-mediated Antigenic Conversion in *Shigella flexneri*

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In the genus *Salmonella*, Iseki and Sakai (1953) found that an alteration of somatic antigen of E group *Salmonella* was induced by the lysogenization with a temperate phage *epsilon*. Since then, the similar type of antigenic conversion by certain bacteriophages was detected in several other strains of *Salmonella* (Iseki and Kashiwagi 1955, 1957, Harada 1956, Baron *et al.* 1957, Le Minor *et al.* 1961) and in *Shigella flexneri* (Matsui 1958, Iseki and Hamano 1959, Okada *et al.* 1960).

The somatic antigen of *Shigella flexneri* consists of type antigens (major antigens) and group antigens (minor antigens) as described by Edward and Ewing. Two temperate phages ϕ_I and ϕ_{IV_1} are involved in *Sh. flexneri*. By the lysogenization with ϕ_I , strains 2a, 2b, 3a,** 4a, VX and VY, which have no type antigen I, are converted to have the type antigen I, and by the lysogenization with ϕ_{IV_1} , strains 1a, 1b, 2a and 3b, which have no type antigen IV₁, are converted to have the type antigen IV₁.

Other phages which have the ability of antigenic conversion were found in *Sh. flexneri* by Okada (1963).

The mechanism of the antigenic conversion by a phage is considered that the synthetic pathways of the somatic antigens are altered by the injected phage DNA. Several workers conducted the immunochemical and biochemical studies of bacterial polysaccharide to determine the structure of antigenic determinants and to analyse the mechanism of antigenic conversion by a phage. On the polysaccharides of *Salmonella*, studies were made by Staub *et al.* (1959), Iseki *et al.* (1959, 1962), Stocker *et al.* (1960), Tinelli and Staub (1960a, 1960b), Robbins and Uchida (1962) and Uchida *et al.* (1963).

Iseki *et al.* (1962) reported that the polysaccharides of strains 1c, 2a, 3b and 4c of *Sh. flexneri* consist of glucose, galactose, heptose (?), hexosamine and N-acetyl-

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** The cells of the strain 3a are infected by and lysogenized only with the phage from Hiruma strain (I:—).

hexosamine. They proposed the following tentative structures of determinants: α -glucosyl-(1 $\rightarrow$ 4)-[galactose or glucose]- for the type antigen I, II and IV_I, and β -glucosyl-(1 $\rightarrow$ 6)-[galactose or glucose]- for the type antigen III.

The present paper describes the results of quantitative estimation of the constituent sugars in *Shigella* polysaccharides, and of the characterization of the determinants of the group antigens in *Sh. flexneri* by the serological methods. These studies were carried out to investigate the mechanism of antigenic conversion with phages ϕ_I and ϕ_{IV_I} .

Materials and Methods

Strains: The strains used are listed in Table 1.

Immune sera: Immune sera against type and group antigens were prepared by the method of Edward and Ewing (1961) with some modification (Table 2).

Table 1. Strains of *Sh. flexneri* and their antigenic structure

Strain	Abbrev.	Antigenic structure
EW 8	1a	I : 4
EW 9	1b	I : 4, 6
Variant (ϕ_I)*	1c	I : 7, 8
EW 10	2a	II : 3, II: 3, 4
EW 40	2b	II : 7, 8
EW 14	3a	III : 6, 7, 8
B 6	3b	III : 3, 4, 6
EW 19	4a	IV _I : 3
Variant (ϕ_{IV_I})*	4c	IV _I : 3, 6
EW 12	VX	— : 7, 8
EW 13	VY	— : 3, 4

* Lysogenic strains

Table 2. Preparation of factor sera

Factor serum	Immunizing strain	Absorbing strain
Anti-I	1c	2a, 3a, 4c and VX
Anti-II	2a	1a, 3b, 4c and VX
Anti-III	3b	1a, 2a, 4c and VX
Anti-IV _I	4c	1b, 2a, 3b and VX
Anti-3	2a (II: 3)	1a, 2b and VX
Anti-4	2a (II: 3, 4)	2a (II: 3) and VX
Anti-6	4c	4a, VX and VY
Anti-7, 8	1c	1a, 2a and VY

Polysaccharides: All of the chemical and serological studies were carried out on bacterial polysaccharides which were prepared as follows. Cells of *Sh. flexneri* were grown for about 24 hours at 37°C on nutrient agar in large baking dishes. Cells were dried with ethanol and acetone. These dried cells were deproteinized with 5% trichloroacetic acid, and centrifuged. The supernatant was dialyzed against water for 4 to 5 days, the non-dialyzable fraction was poured into 5 volumes of ethanol, and centrifuged. The precipitate was dissolved into distilled water, dialyzed and lyophilized. Three grams of polysaccharide preparation were obtained from 100 grams of dried cells.

Acid hydrolysis: Acid hydrolysis was usually carried out with N H₂SO₄ or 2N HCl in a sealed tube for 6 hours at 100°C. The detection of N-acetylhexosamine

was made under mild conditions using N/20 H_2SO_4 for 40 minutes at 100°C . H_2SO_4 and HCl were neutralized with $\text{Ba}(\text{OH})_2$ and NaOH respectively.

Paper chromatography: Composing sugars of the polysaccharide were detected by the method of paper chromatography. Descending paper chromatography was carried out with *n*-butyl acetate-pyridine-water (4:3:1) or *n*-butanol-pyridine-water (6:4:3), and ascending paper chromatography with *n*-butanol-acetic acid-water (4:1:2). Sugar spots were detected with AgNO_3 - NaOH (Trevelyan *et al.* 1950) and aniline hydrogen phthalate (Partridge 1949). Hexosamine and N-acetylhexosamine were detected by the method of Partridge (1948) and Salton (1959), respectively.

Quantitative analysis: Quantitative determinations of constituent sugars were performed as follows. Total reducing sugars were determined by the method of Fujita and Iwatake (1931). Hexosamine was determined by the spectrophotometric method of Boas (1953). Pentose was determined by the method of Gibbons (1955) and glucose by the enzymatic method of Huggett and Nixon (1957). Hexose and pentose were simultaneously determined as described by Fernell and King (1953). Total acetyl groups were determined by the method of Ludswieg and Dorfman (1960) and O-acetyl group by the method of Hestrin (1949).

Stability of the type antigen: Stability of the type antigen against acid and alkali was examined by the inhibition test. The hemagglutination reaction between factor serum and polysaccharide-coated rabbit red cells was inhibited with hydrolyzed *Shigella* polysaccharide. When the antigenicity of the polysaccharide was lost by hydrolysis, this reaction was not inhibited.

Analysis of antigenic determinants: The method of the inhibition test was used for analysis of determinants of the group antigen as made in the determination of the type antigen (Iseki *et al.* 1962). This test is based on the principle that a sugar which inhibits the antigen-antibody reaction, corresponds to a sugar of the determinants of the antigen. In this experiment, precipitation and hemagglutination reaction between the bacterial polysaccharide and the factor serum were inhibited by authentic sugars.

In the procedure of precipitation inhibition, a 10% sugar solution was mixed with a factor serum (1:1) of 4-fold precipitation titers, kept for 2 hours at 25°C , and centrifuged. The supernatant was examined for the precipitation with a polysaccharide solution. In the procedure of inhibition of hemagglutination, 2-fold serial dilution of 10% sugar was mixed with factor serum (1:1) of 4-fold agglutination titers, kept for 2 hours at 25°C . Polysaccharide-coated red cells were added to these mixtures and the minimum amount of the sugar which inhibited the reaction was determined. The preparation of the polysaccharide-coated red cells was done with the procedure described by Keogh *et al.* (1947, 1948) with some modification. Five tenth percent polysaccharide solution was mixed with 2.5% (v/v) rabbit red cells (1:2), kept at 37°C for one hour, and centrifuged. The cells were washed 2 times with

0.75% NaCl and diluted to 2.5% suspension of the red cells. The polysaccharide and the red cells were dissolved in 0.75% NaCl solution.

Results

Acid hydrolysis of polysaccharides: The time course for the release of reducing sugars during acid hydrolysis was determined to facilitate the following qualitative and quantitative analysis. The results are shown in Fig. 1. The amount of reducing sugars which is released during acid hydrolysis reaches maximum after 6 hours with 2 N HCl at 100°C.

Fig. 1. Time course of the release of reducing sugars from *Shigella* VX polysaccharide.

Paper chromatography: Polysaccharides of strains 1a, 2a, 3a, 4a and VX of *Sh. flexneri* were hydrolyzed with 2 N HCl or N H₂SO₄ for 6 hours at 100°C and constituent sugars were detected on paper chromatograms.

Regardless of the kind of type antigens, polysaccharides extracted from five strains contain galactose, glucose, rhamnose, hexosamine, N-acetylhexosamine and another one which seems to be heptose. The results are the same as those described by Iseki *et al.* (1962). In addition to those reported by them, ribose was also detected, which may be derived from nucleic acid.

Quantitative assay: The quantitative determination of sugars in polysaccharides was carried out under the same conditions as in the case of acid hydrolysis. The results are shown in Table 3. The ratios of hexosamine, glucose and galactose to methylpentose in the strains 1a and 2a are approximately similar with each other. The ratios of these sugars in 4a and VX are similar. Particularly, for 3a polysaccharide, the ratio of hexosamine to methylpentose is 5.5, which is a relatively small value, and that of glucose to methylpentose is 8.2, which is the highest value of glucose among five strains. The contents of hexosamine and glucose are complementary

Table 3. The result of quantitative determination of sugars in *Shigella* polysaccharides

Strain	Total Reducing sugar (as glucose)	Methyl-pentose (as rhamnose)	Hexosamine (as glucosamine)	Glucose	Galactose	Pentose (as ribose)
	μM	μM	μM	μM	μM	μM
1 a	3.50	1.18 (10)	0.65 (5.5)	0.56 (4.7)	1.07 (9.1)	0.10 (0.8)
2 a	2.36	0.79 (10)	0.48 (6.1)	0.45 (5.7)	0.85 (10.8)	0.09 (1.1)
3 a	3.46	1.02 (10)	0.56 (5.5)	0.84 (8.2)	1.34 (13.1)	0.12 (1.2)
4 a	1.56	0.44 (10)	0.36 (8.2)	0.19 (4.3)	0.66 (15.0)	0.07 (1.6)
VX	2.72	0.80 (10)	0.62 (7.8)	0.34 (4.3)	1.03 (12.9)	0.05 (0.6)

Figures indicate the amount of sugars (μM) per mg. of polysaccharide.

Figures in parentheses indicate the ratio of sugars to methylpentose.

each other in the strains 3 a, 4 a and VX. The ratio of hexosamine to glucose is 1:1 in 1 a and 2 a polysaccharides, 3:4 in 3 a and 2:1 in 4 a and VX.

The amount of total acetyl groups in polysaccharides is listed in Table 4 together with the amount of O-acetyl group. The amount of acetyl groups in polysaccharides of the strains 1 a, 2 a, 4 a and VX is approximately similar to the amount of hexosamine in the strain.

Table 4. Total amount of acetyl groups and O-acetyl group in the polysaccharides

Acetyl group	Strain				
	1 a	2 a	3 a	4 a	VX
Total acetyl groups	0.53	0.49	0.70	3.30	0.51
O-acetyl group	—	—	0.32	—	—

Figures indicate the amount of acetyl group (μM) per mg. of polysaccharide. —; Detectable amount of O-acetyl group is not found.

O-acetyl group was found only in the strain 3 a.

Loss of antigenicity of polysaccharide with acid or alkali treatment: Antigenicity of type antigens of polysaccharides were lost by the treatment with acid or alkali, the results being shown in Table 5.

The antigenicity of the type antigens of the strains 1 c, 2 a and 4 c was more stable than that of 3 b against the treatment with alkali. This fact may indicate that the determinants of the type antigen III are very sensitive to mild alkali hydrolysis.

Nature of determinants of group antigen: The determinants of the group antigens were inferred from the results of the inhibition test. The results are shown in Table 6.

N-acetyl-D-galactosamine strongly inhibited the monofactor 3, 4 and 6 immune systems, while the monofactor 7, 8 systems were inhibited by D-glucose, N-acetyl-D-galactosamine, D-galactose and others. Maltose inhibited the monofactor 4 and 7, 8 systems and trehalose and cellobiose also inhibited the 7, 8 systems.

From the above serological findings an assumption may be proposed that the

Table 5. Loss of antigenicity of polysaccharides by the treatment with acid or alkali

Treatment		Polysaccharide																	
		1 c (I)				2 a (II)				3 b (III)				4 c (IV _I)					
acid	Min.																		
	0	—	—	—	—	—	+	—	—	—	—	—	+	—	—	—	—	—	+
	30	—	±	+	+	+	+	—	—	—	±	+	+	—	+	+	+	+	+
	60	—	+	+	+	+	+	—	+	+	+	+	+	—	—	—	+	+	+
	120	+	+	+	+	+	+	—	+	+	+	+	+	—	—	+	+	+	+
alkali																			
	0	—	—	—	—	—	+	—	—	—	—	—	+	—	—	—	—	—	+
	30	—	—	+	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+
	60	—	—	+	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+
	120	—	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+

Acid treatment was carried out by N/10 CH₃COOH at 100°C, and alkali treatment by N/10 NaOH at 100°C.
—; no inhibition of reaction, +; inhibition of reaction

Table 6. Inhibition test by sugars of hemagglutination of polysaccharide-coated rabbit red cells by factor sera against group antigens

Sugar		Factor serum			
		Anti-3	Anti-4	Anti-6	Anti-7, 8
A	D-Galactose	—	—	—	27.7
	D-Glucose	—	—	—	6.9
	D-Glucosamine	46.4	—	—	—
	N-Acetyl-D-galactosamine	11.3	22.6	11.3	22.6
	Methyl- α -D-glucoside	—	—	—	6.4
	Salicine	—	34.8	—	17.4
B	Maltose	—	27.7	—	3.5
	Cellobiose	—	—	—	29.5
	Trehalose	—	—	—	6.6
	Raffinose	—	—	—	16.8

Figures indicate the minimum amount of sugars (μ M/0.1 ml.), which inhibit the reaction. —; Inhibition of the reaction was not observed by the sugars less than 30 μ M/0.1 ml. (in the column A), or 50 μ M/0.1 ml. (in the column B).
The following sugars were also used in this test, but they did not inhibit the reaction: 50 μ m/0.1 ml. of D-Mannose, L-Rhamnose, D-Galactosamine, N-Acetyl-D-glucosamine, α -Ethyl-N-acetyl-D-glucosaminide, β -Ethyl-N-acetyl-D-glucosaminide and 30 μ M/0.1 ml. of Lactose and Melibiose.

determinants of the group antigens 3, 4 and 6 would be N-acetyl-D-galactosamine and those of the group antigen 7, 8 would be α -glucosyl-(1 $\rightarrow$ 4)-(glucose or N-acetyl-D-galactosamine)-. The latter is similar to those of the type antigens I, II and IV_I.

Discussion

There are several reports of the studies on the mechanism of phage mediated conversion of antigens. The present study deals with immunochemical and biochemical assay of the polysaccharides of *Sh. flexneri*.

Constituent sugars of the polysaccharides are galactose, glucose, rhamnose, hexosamine, N-acetylhexosamine, ribose and another sugar which seems to be heptose.

As a result of quantitative assay, some differences in the ratios of the constituent sugars were observed among polysaccharides from different strains. Methylpentose, galactose and pentose are in constant ratios in all polysaccharides, but the ratios of hexosamine and glucose to methylpentose are different as 5.5 and 4.7 in strain 1 a, 6.1 and 4.5 in 2 a, 5.5 and 8.2 in 3 a, 8.2 and 4.3 in 4 a and 7.8 and 4.3 in VX.

It is a matter of question whether these differences in hexosamine and glucose ratios are related to differences in the structure of antigens. Robbins and Uchida (1962) described that the glucose contents of lipopolysaccharides 3, 10 and 3, 15 of *Salmonella* E group depend on the growing conditions of the organisms. The extraction of polysaccharide from different *Shigella* strains was carried out with the same medium and method for extraction, so that the differences in sugar contents may be considered to depend on the differences in polysaccharide structures.

The antigens of type 3 (3 a and 3 b) strains are so alkali sensitive that the cells lose the type antigen III and group antigen 6 by treatment with 0.5% NaOH for one hour at 0°C (Okada *et al.* 1958). When 3 b polysaccharide was treated with N/10 NaOH for 30 minutes at 100°C, the antigenicity of type antigen III was completely lost. Thus, the determinants of type antigen III and group antigen 6 are considered to be sensitive against mild alkali treatment.

In the determination of acetyl groups, it was found that the 3 a polysaccharide contained O-acetyl group, while the polysaccharides of other strains did not. The fact that the antigenicity of the type antigen III is very sensitive to alkali may be due to the presense of O-acetyl group in 3 a polysaccharide, and it is considered that presumably this O-acetyl group is a part of the determinants of the type antigen III. In *Salmonella* polysaccharide, the presence of O-acetyl group was reported by Kotelko *et al.* (1961). Recently, Uchida *et al.* (1963) reported that O-acetyl group was found to be a part of the determinant of O-antigen 10 in *Salmonella*.

The analysis of antigenic determinants is possible by inhibition test. Iseki *et al.* (1962) reported that the end determinants of the type antigens were α -glucosyl-(1 $\rightarrow$ 4)-(galactose or glucose)- for the type antigens I, II and IV_I, and β -glucosyl-(1 $\rightarrow$ 6)-(galactose or glucose)- for the type antigen III, because glucose and maltose completely inhibited the monofactor I, II and IV_I systems, and the monofactor III systems were inhibited by gentiobiose.

Consequently, the end determinants of the group antigens 3, 4, 6 and 7, 8 were

analyzed by the same method. The end-determinants of the group antigens 3, 4 and 6 might be N-acetyl-D-galactosamine. The differences in the results of the inhibition test of three antigens are not large enough to characterize their own determinants. However, the monofactor 4 systems were also inhibited by maltose. The end determinants of the group antigens 7, 8 were detected as α -glucosyl-(1 $\rightarrow$ 4)-(glucose or N-acetyl-D-galactosamine)- which is similar to those of the type antigens I, II and IV_I.

A preliminary study of the antigenic determinants was performed to confirm the results of the inhibition test. Six grams of 1c polysaccharides were treated with N/20 H₂SO₄ for 30 minutes at 100°C, and applied to a charcoal-Celite column. The column was eluted with water and serial concentrations of ethanol. These fractions were assayed for carbohydrate by the paper chromatography. The fraction which was eluted with water contained hexose and N-acetylhexosamine, and some oligosaccharides were detected in ethanol fractions.

After reisolation of these oligosaccharides on the paper, sugars were examined for constituent sugars and degrees of polymerization. It was demonstrated that all oligosaccharides consisted of glucose and that the degrees of polymerization were 2 to 5. Therefore, the second sugar of determinant groups of the type antigen I may be glucose.

On the other hand, from biochemical and immunochemical studies on the polysaccharides of O-antigen of *Salmonella* E group, it was shown that the determinants 10, 15 and 34 appear to be an acetylated α 1, 6-D-galactosyl-1, 4 (or 1, 5)-D-mannosyl-L-rhamnose, β -1, 6-D-galactosyl-1, 4 (or 1, 5)-D-mannosyl-L-rhamnose, and α -1, 4-D-glucosyl-D-galactose site, respectively (Iseki *et al.* 1962, Robbins and Uchida 1962 and Uchida *et al.* 1963). Uchida *et al.* (1963) discussed some modifications which take place on the determinants by lysogenization with phages ϵ_{13} , and ϵ_{34} .

In *Sh. flexneri*, studies on the oligosaccharides extracted from 1c and 2a polysaccharides have been made by Sekijima and Yamamoto (1963), and they discussed the differences in the determinants of the type antigens I and II.

The nature of the antigenic conversion by ϕ_I and ϕ_{M_I} are inferred from the present data as follows. The antigenic conversion of type antigen II and IV_I to I, or I and II to IV_I is considered to be due to exchange in the arrangement of the inner part sugars, because no differences are found among analyzed end determinants of I, II and IV_I; they are all the same and may be α -glucosyl-(1 $\rightarrow$ 4)-(galactose or glucose)-. The conversion of III to I or IV_I may be due to the change in the configuration of the end part from β -glucosyl-(1 $\rightarrow$ 6)- site to α -glucosyl-(1 $\rightarrow$ 4)-site.

The conversion of VX (- : 7, 8) to 1c (I : 7, 8), or VY (- : 3, 4) to 1a (I : 4) seems to be resulted from acquisition of biosynthetic processes for the type antigen I. The group antigens 7, 8 and 4 do not change. While the group antigen 3 is lost when cells of strain VY convert to have the type antigen I. Therefore the site of the group antigen 3 may participate in the formation of the type antigen I. This is

similar to the case in the antigenic conversion with phage ϵ_{15} in *Salmonella* E group, where the appearance of new O-antigen 15 accompanies with the loss of O-antigen 10.

Summary

Immunochemical and biochemical studies were carried out on the polysaccharides of *Shigella flexneri* in which phage mediated conversion of the type antigen had been observed.

Descending and ascending paper chromatography was carried out to confirm the constituent sugars of the polysaccharides. The polysaccharides consist of glucose, galactose, heptose (?), hexosamine and N-acetylhexosamine.

Quantitative determinations of sugars in the polysaccharides of the strains 1a, 2a, 3a, 4a and VX were carried out. Methylpentose, galactose and pentose were present in approximately the same ratios in all polysaccharides, but hoxosamine and glucose were detected in various ratios. The ratio of hexosamine to glucose is 1:1 in 1a and 2a, 3:4 in 3a, and 2:1 in 4a and VX. The amount of acetyl group is approximately similar to the amount of hexosamine, however in 3a polysaccharide O-acetyl group was also detected.

Stability of various antigens of the polysaccharides against acid and alkali was compared. Antigenicity of the type antigen III was very sensitive against alkali.

The antigenic determinants of the group antigens 3, 4 and 6 would be N-acetyl-D-galactosamine, and the antigenic determinants of 7, 8 would be α -glucosyl-(1 $\rightarrow$ 4)-[glucose or N-acetyl-D-galactosamine]- which is similar to those of the type antigens I, II and IV₁.

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