CHANGES IN IMMUNODIFFUSION PATTERNS AND IN NUCLEIC ACID CONTENT OF *STAPHYLOCOCCUS AUREUS* GROWN IN THE PRESENCE OF A NUCLEIC ACID FLUOROCHROME

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ABSTRACT

SONEA, S. (University of Montreal, Montreal, Canada), J. DE REPENTIGNY, AND A. FRAPPIER. Changes in immunodiffusion patterns and in nucleic acid content of *Staphylococcus aureus* grown in the presence of a nucleic acid fluorochrome. J. Bacteriol. **84**:1056–1060. 1962.—When grown in the presence of acridine orange, coagulase-positive *Staphylococcus aureus* exhibits changes in the number and the shape of its precipitin bands; these are more pronounced with commercial horse staphylococcal antitoxin than with normal human γ-globulin. Simultaneously, there is an increase in the content of nucleic acids, especially deoxyribonucleic acid. Consequently, when grown in the presence of this nucleic acid fluorochrome, pathogenic staphylococci become similar to nonpathogenic strains in their antigenic structure and nucleic acid content. The bacterial population becomes a mixture of red and green fluorescing cells of different sizes. After centrifugation, most of the large and red fluorescing cells are found in the top layer, which shows more changes in antigenic composition and in nucleic acid content than the bottom layer. In subcultures grown in the absence of acridine orange, the cells revert to the original composition of the pathogenic strains.

Nucleic acid content in a number of *Staphylococcus aureus* strains is apparently related to the presence or absence of antigenic virulence factors, such as hemolysins and coagulase (Catlin and Cunningham, 1958; de Repentigny, Sonea, and Frappier, Can. J. Microbiol., *in press*). On the other hand, acridines bind preferentially with nucleic acids (Lerman, 1961; Peacock and Skerrett, 1956). β-Hemolysin-producing staphylococcal strains, when grown on sheep-blood agar in the presence of acridine orange, do not cause hot-cold hemolysis; the loss or the inactivation of this antigenic enzyme occurs simultaneously with a rise in staphylococcal nucleic acid content, expressed as percentage dry weight of washed cells (de Repentigny, Sonea, and Frappier, *in press*).

We purposely modified the bacterial nucleic acids by growing different strains in the presence of acridine orange, to modify the synthesis of antigenic virulence factors. The changes have been studied with immunodiffusion techniques, as there are no intracellular visible particles which could be modified by this nucleic acid fluorochrome, as there are in yeasts (Ephrussi, 1953) and protozoa (Werbitzki, 1910).

MATERIALS AND METHODS

We studied the in vitro and in vivo properties of eight coagulase-positive *S. aureus* strains previously described (Frappier, Sonea, and Panisset, 1955; de Repentigny, Sonea, and Frappier, 1961, *in press*). Four strains (A3, A4, A5, and A9) were of animal origin and the others were from human infections. The well-known α-toxin-producing Wood 46 strain was also included as a reference. The cultures were grown in Roux bottles for 17 hr at 37 C, in the dark, on 150 ml of nutrient agar (Difco) covered with 15 ml of nutrient broth (Difco). Acridine orange (Schmid and Co., Stuttgart, Germany) was added to a final concentration of 100 mg/liter or of 1 mg/liter.

The bacterial cells were centrifuged and twice washed with distilled water, before analysis. Nucleic acid contents, expressed as percentages of cell dry weight, were determined according to the techniques of Burton (1956) and Ceriotti (1955). In our experimental conditions, the presence of acridine orange in the cells did not alter appreciably the determinations of nucleic acids. The Outchterlony technique (Ionagar; Oxoid) was used to determine the number of precipitin bands occurring between the washed

TABLE 1. *Changes in the number of precipitin bands and in the nucleic acid content of Staphylococcus aureus cells grown in the presence of acridine orange*

Strain	Acridine orange added (mg/liter)	Ouchterlony* no. of lines		Nucleic acids (% of dry cells)		Ouchterlony* no. of lines		Nucleic acids (% of dry cells)		Ouchterlony* no. of lines		Nucleic acids (% of dry cells)	
		Horse antitoxin	Human γ-globulin	DNA	RNA	Horse antitoxin	Human γ-globulin	DNA	RNA	Horse antitoxin	Human γ-globulin	DNA	RNA
		Expt 1				*Expt 2*				*Expt 3*			
A3	—†	9 (6)	8 (3)	6.3	12.2	11 (9)	5 (5)	5.0	11.0	10 (8)	6 (5)	8.0	15.6
	1									7 (5)	6 (5)	6.7	13.3
	100	4 (3)	5 (3)	12.5	20.3	4 (4)	5 (5)	9.0	18.6	4 (3)	7 (6)	8.9	16.3
	—‡	9 (6)	7 (3)	4.9	11.1	7 (5)	6 (6)	4.7	10.4				
		Expt 4				*Expt 5*				*Expt 6*			
A5	—†	5 (5)	7 (6)	6.0	11.0	7 (6)	5 (5)	5.0	10.9	8 (6)	5 (3)	6.0	10.6
	1	6 (5)	6 (6)	6.5	11.2								
	100	4 (4)	5 (5)	10.3	15.7	5 (5)	5 (5)	13.5	25.0	5 (4)	4 (3)	19.7	19.6
	—‡					5 (4)	5 (5)	5.7	11.1	7 (4)	5 (3)	4.0	10.0
		Expt 7				*Expt 8*				*Expt 9*			
A9	—†	9 (6)	8 (7)	4.8	11.2	9 (7)	6 (6)	5.2	14.3	9 (7)	6 (5)	6.0	9.6
	1	10 (6)	7 (6)	8.4	14.6								
	100	7 (5)	6 (5)	8.8	18.7	6 (5)	6 (6)	9.0	20.6	3 (3)	5 (4)	11.6	13.1
	—‡					8 (5)	5 (5)	4.7	11.2	2 (2)	2 (2)	5.3	13.2
		Expt 10				*Expt 11*				*Expt 12*			
S18	—†			5.8	11.5		6 (6)	15.1	15.1	3 (3)		5.1	12.3
	1			9.4	12.7		8 (8)	10.8	12.6	6 (5)		6.1	13.8
	100			10.7	13.5		4 (3)	18.6	14.6	1 (1)		7.0	10.6
		Expt 13				*Expt 14*				*Expt 15*			
S34	—†	8 (5)	4 (4)	7.5	13.7	6 (6)	5 (4)	4.3	9.8	9 (7)	4 (2)	5.7	11.0
	1	9 (5)	5 (4)	7.9	15.8								
	100	5 (4)	4 (3)	9.8	13.2	3 (3)	6 (3)	11.9	13.8	4 (3)	4 (2)	14.3	16.9
	—‡					7 (5)	5 (5)	7.7	12.9	10 (7)	4 (2)	4.2	11.5
		Expt 16				*Expt 17*				*Expt 18*			
S817	—†			7.0	11.4	2 (2)	7 (4)	8.2	9.3	2 (2)	7 (5)	7.6	10.6
	1			9.8	11.2	4 (3)	7 (5)	14.2	17.8	5 (5)	7 (4)	11.1	13.2
	100			14.2	12.2	3 (3)	6 (4)	12.2	17.2	2 (2)	6 (4)	11.7	12.1
		Expt 19				*Expt 20*				*Expt 21*			
Wood	—†			11.2	16.4			4.3	14.8	5	6	4.5	13.6
	1			13.0	17.6		6 (5)	8.3	15.1	6 (5)	7 (5)	8.6	18.1
	100			20.7	24.0			16.0	21.8		5 (5)	11.6	21.7

* In parentheses, the number of lines common to Wood 46 strain grown without acridine orange.

† Control cells grown without acridine orange.

‡ Subcultured without acridine orange, starting with the culture grown in the presence of 100 mg/liter of acridine orange.

bacterial cells and different staphylococcal antitoxins or normal human γ-globulin (Institute of Microbiology and Hygiene of the University of Montreal, Montreal, Canada). A suspension containing 5 mg of dry cells/ml was used in each agar well. All these cultures were also examined with a fluorescence microscope.

RESULTS

The number of precipitating antigens was modified in *S. aureus* cells grown in the presence of different concentrations of acridine orange (Table 1). The changes were more obvious with an antitoxin (Connaught Medical Research Laboratories, Toronto, Canada) than with a normal human γ-globulin. At the 100 mg/liter concentration, a decrease in the number of lines was observed, but there was a slight increase for most strains with the 1 mg/liter concentration. There were also differences in the shape of precipitin bands for the cells grown in the presence of acridine orange (Fig. 1 through 4).

Also, the nucleic acid content of *S. aureus* generally showed an increase, which was higher with 100 than with 1 mg of acridine orange/liter. This increase was larger for deoxyribonucleic acid (DNA) than for ribonucleic acid (RNA) in 20 of 21 experiments. In one of three experiments, respectively, with strains S18, S34, and A3, the RNA content appeared to be unchanged (Table 1).

When examined under a fluorescence microscope, these modified staphylococcal cells exhibited a mixture of red and green fluorescing cells of different sizes, especially with the 100 mg of acridine orange/liter. After centrifugation in Hopkins tubes, most of the red fluorescing cells were recovered in the top layer, and the green fluorescing cells in the bottom layer. The number of precipitin lines and the nucleic acid content were determined in the two layers. The top layer gave the smallest number of precipitin bands, some of which were of a different shape (Table 2; Fig. 3 and 4).

When diffused against staphylococcus β-antitoxin (kindly supplied by J. F. Morgan, Department of National Health and Welfare, Laboratory of Hygiene, Ottawa, Canada), the strains grown in the presence of 100 mg of acridine orange/liter gave only one precipitin line instead of two (Fig. 5 and 6).

Nucleic acid content was much higher in the top layer than in the bottom layer (Table 2). DNA increased more than RNA, so that the RNA to DNA ratio decreased. The sum of DNA plus RNA was considerably increased in the top layer only.

When modified *S. aureus* populations from total cultures, or from either of the two layers, were subcultured without acridine orange, the

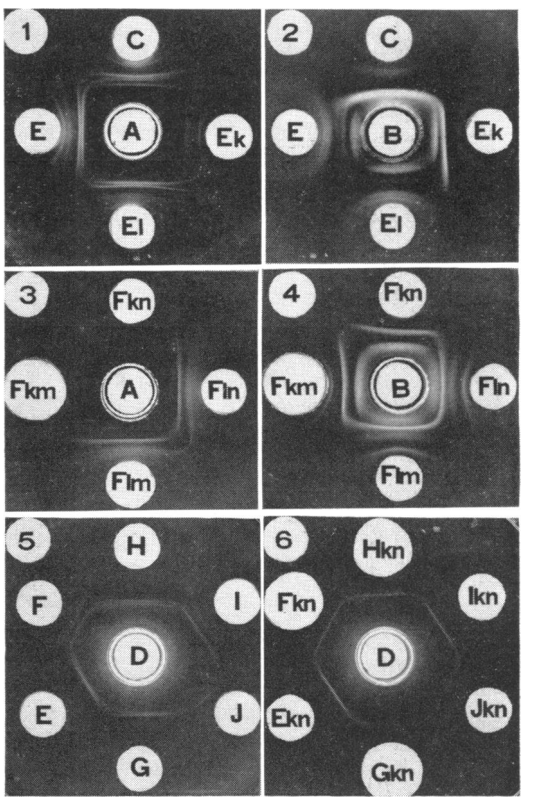

FIG. 1 to 6. *Changes in the immunodiffusion patterns of Staphylococcus aureus, when grown in the presence of acridine orange.* A = *horse commercial Staphylococcus antitoxin;* B = *normal human γ-globulin;* C = *strain Wood 46 cells;* D = *Staphylococcus β-antitoxin;* E = *strain A3 cells;* F = *strain A4 cells;* G = *strain S18 cells;* H = *strain S34 cells;* I = *strain A9 cells;* J = *strain A5 cells;* k = *after growth in the presence of 100 mg of acridine orange/liter;* l = *after growth without acridine orange, subcultured from a culture grown in the presence of acridine orange;* m = *bottom layer;* n = *top layer. Fig. 1 and 2. Whole culture's sediment. Fig. 3 and 4. Top and bottom layers of culture's sediment. Fig. 5 and 6. Reaction of several strains with purified Staphylococcus β-antitoxin.*

TABLE 2. *Changes of the number of precipitin bands and of the nucleic acid content in two fractions obtained by centrifuging cultures of Staphylococcus aureus grown in the presence of acridine orange (100 mg/liter)*

Strain	Fraction	Ouchterlony no. of lines		Nucleic acids			
				Dry cells (%)		Ratio	Total
		Horse antitoxin	Human normal γ-globulin	DNA	RNA	RNA/DNA	RNA+DNA
A3	Control*	8	6	8.9	12.2	1.37	21.1
	Top layer	4	4	*29.5*	*24.6*	*0.83*	*54.1*
	Top layer subculture*	6	5	7.1	10.3	1.46	17.4
	Bottom layer	*5*	4	11.5	14.0	1.22	25.5
	Bottom layer subculture*	7	5	7.5	10.3	1.39	17.8
A4	Control*	11	7	5.6	10.3	1.84	15.9
	Top layer	*1*	5	*13.6*	*17.0*	*1.25*	*30.6*
	Top layer subculture*	10	5	6.3	11.3	1.79	17.6
	Bottom layer	*3*	6	5.5	10.0	1.82	15.5
	Bottom layer subculture*	10	6	5.6	11.2	1.98	16.8
A5	Control*	5	5	5.9	11.1	1.88	17.0
	Top layer	4	4	*22.2*	*24.7*	*1.11*	*46.9*
	Top layer subculture*	6	4	6.4	9.1	1.42	15.5
	Bottom layer	4	4	5.9	11.8	2.0	17.7
	Bottom layer subculture*	7	4	5.9	9.6	1.62	15.5
A9	Control*	10	5	6.9	9.5	1.37	16.4
	Top layer	3	4	*21.0*	*19.0*	*0.90*	*40.0*
	Top layer subculture*	10	6	8.2	11.5	1.41	19.7
	Bottom layer	3	5	7.1	8.9	1.25	16.0
	Bottom layer subculture*	10	6	5.1	9.5	1.86	14.6
S34	Control*	6	6	7.5	9.6	1.98	17.1
	Top layer	1	6	*14.8*	*13.7*	*0.92*	*28.5*
	Top layer subculture*	5	7	6.7	10.0	1.49	16.7
	Bottom layer	2	5	5.6	7.7	1.37	13.3
	Bottom layer subculture*	5	7	7.8	11.1	1.42	18.9

* Cells grown without acridine orange.

number of lines and the nucleic acid content returned to normal (Tables 1 and 2; Fig. 1 to 4).

DISCUSSION

The precipitating antigens and the nucleic acids of pathogenic staphylococcal strains grown in the presence of acridine orange are temporarily modified, becoming similar to those observed previously with nonpathogenic strains grown without acridine orange (de Repentigny et al., 1962*a*).

This could be explained by the selective binding

of acridine orange with nucleic acids (Beers, Hendley, and Steiner, 1958; Lerman, 1961; Peacock and Skerrett, 1956), followed by a change in the synthesis of antigens. The disappearance of one precipitin line with β-antitoxin seems to be related to the loss of hot-cold hemolysis on sheep-blood agar when staphylococcal strains are grown in the presence of acridine orange (de Repentigny et al., *in press*).

Fukui, Lawton, and Nortlock (1961) have already studied the relation of nucleic acids to protein synthesis through the action of nucleic acid antimetabolites in *Pasteurella pestis*. In mammals, antigenic differences were also found with the fluorescent-antibody technique, among benign, malignant, or premalignant cells or among cells of the same premalignant tumor (Nairn, 1962). In our work, the changes produced by acridine orange could also be explained by a selective action in favor of nonpathogenic cells, as suggested by the increased heterogeneity of the bacterial population.

As penicillin and chloramphenicol in sub-bacteriostatic doses in the culture media have not produced in our laboratories such changes in *S. aureus* nucleic acid content, or in its precipitating antigens, the action of acridine orange does not seem to be due to unfavorable growth conditions or to transformation to L forms (Klieneberger-Nobel, 1960; Marston, 1961).

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LITERATURE CITED

BEERS, R. F., JR., D. D. HENDLEY, AND R. F. STEINER. 1958. Inhibition and activation of polynucleotide phosphorylase through the formation of complexes between acridine orange and polynucleotides. Nature **182**:242-244.

BURTON, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315-323.

CATLIN, B. W., AND L. S. CUNNINGHAM. 1958. Studies of extracellular and intracellular bacterial deoxyribonucleic acids. J. Gen. Microbiol. **19**:522-539.

CERIOTTI, G. 1955. Determination of nucleic acids in animal tissues. J. Biol. Chem. **214**:59-70.

EPHRUSSI, B. 1953. Nucleo-cytoplasmic relations in microorganisms. Clarendon Press, Oxford, England.

FRAPPIER, A., S. SONEA, AND M. PANISSET. 1955. Le pouvoir pathogène des staphylocoques. I. Etude comparative de 40 souches à l'aide de quatre méthodes d'infection expérimentale. Rev. Can. Biol. **14**:152-172.

FUKUI, G. M., W. D. LAWTON, AND R. P. MORTLOCK. 1961. Synthesis of virulence factors by Pasteurella pestis. J. Bacteriol. **81**:656-660.

KLIENEBERGER-NOBEL, E. 1960. L-forms of bacteria. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria. Academic Press, Inc., New York.

LERMAN, L. S. 1961. Structural considerations in the interaction of DNA and acridines. J. Mol. Biol. **3**:18-30.

MARSTON, J. 1961. Fluorescence microscopy of staphylococcal L-Forms. Bacteriol. Proc., p. 82.

NAIRN, R. C. 1962. Fluorescent protein tracing. E. and S. Livingstone, Edinburgh.

PEACOCKE, A. R., AND J. N. H. SKERRETT. 1956. The interaction of aminoacridines with nucleic acids. Trans. Faraday Soc. **52**:261-279.

REPENTIGNY, J. DE, S. SONEA, AND A. FRAPPIER. 1961. Etude microfluorométrique des microorganismes. I. La fluorescence primaire (autofluorescence). Ann. Inst. Pasteur **101**: 353-366.

WERBITZKI, F. W. 1910. Uber blepharoblastlosce Trypanosomen. Centr. Bakteriol. Parasitenk. Abt. I, Orig. **53**:303-315.