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DIFFERENTIATION BY IMMUNODIFFUSION AND BY QUANTITATIVE IMMUNOFLUORESCENCE BETWEEN 5-FLUOROURACIL-TREATED AND NORMAL CELLS FROM A TOXINOGENIC *STAPHYLOCOCCUS AUREUS* STRAIN

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ABSTRACT

DE REPENTIGNY, J. (University of Montreal, Montreal, Quebec, Canada), S. SONEA, AND A. FRAPPIER. Differentiation by immunodiffusion and by quantitative immunofluorescence between 5-fluorouracil-treated and normal cells from a toxinogenic *Staphylococcus aureus* strain. J. Bacteriol. **88**:444-448. 1964.—Immunodiffusion and quantitative immunofluorescence can both detect antigenic changes produced by 5-fluorouracil (FU) in *Staphylococcus aureus* Wood 46 strain. When FU is added to the cultures in their logarithmic phase of growth, a number of bacterial antigens are no longer detectable by immunodiffusion and the intensity of the total immunofluorescence of bacteria is diminished; thus, these antigens are either profoundly modified or no longer synthesized. Uracil and, less effectively, thymine can reverse the FU inhibitory effect on the synthesis of antigens, and the number of precipitin lines remains closer to controls. The immunochemical approach provides a new way of obtaining information on the action of this pyrimidine analogue on metabolic processes in pathogenic bacteria. Microscopic quantitative immunofluorescence seems to be adaptable to give indirect information on changes in the metabolism or synthesis or antigens of a single bacterial cell.

The well-known pyrimidine competitive analogue, 5-fluorouracil (FU), has been used by many authors to produce changes in the synthesis of cellular macromolecules, e.g., enzymes (Gros and Naono, 1961), antibodies (Sterzl, 1961), cell-wall constituents (Rogers and Perkins, 1960), and nucleic acids (Cohen et al., 1958), but these studies have been restricted almost exclusively to biochemical investigations. With the exception of the negative results of Gros and Naono (1961), no immunochemical approach has been attempted to add supplementary information about the changes observed in the synthesis of antigenic macromolecules when the metabolism of growing bacterial cells is modified by FU. Using *Staphylococcus aureus*, whose antigenic structure is well known to be related to virulence (Howard, 1954), we had previously applied immunodiffusion to detect changes in the synthesis of the antigens of this species when its nucleic acids are modified by acridine orange (Sonea, de Repentigny, and Frappier, 1962). More recently, Pontieri and Plescia (1962) also showed by immunodiffusion techniques the effects of 8-azaguanine on *Escherichia coli* antigens. In the present work, we applied immunodiffusion to the study of changes produced by FU in the synthesis of antigenic macromolecules of a toxinogenic strain of *S. aureus*. Simultaneously, we used a quantitative immunofluorescence technique because it probably evaluates mostly structural or nondiffusible bacterial antigens (de Repentigny, Sonea, and Frappier, 1963).

MATERIAL AND METHODS

We used the Wood 46 strain of *S. aureus*, producing alpha toxin and other precipitating antigens. It was grown in a semisynthetic nutrient medium whose composition excluded nucleic acid metabolites so that FU action could be easily obtained. Its composition was as follows: K_2HPO_4, 2 g; Casamino Acids (Difco), 15 g; $MgSO_4 \cdot 7H_2O$, 90 mg; $CaCl_2 \cdot 2H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; Sigma 7-9 (Sigma Chemical Co., St. Louis, Mo.), 24.2 g; thiamine, 0.02 mg; nicotinic acid, 0.2 mg; distilled water, 1 liter; the final pH was 7.4. This solution was sterilized in an autoclave; 0.5% glucose (50% sterile solution) was added aseptically. We added 65 μg of FU per ml of culture at the beginning of its logarithmic phase of

growth. No attempts were made to isolate the few possible mutants. Control cultures without FU or with FU and uracil or thymine were made simultaneously under identical conditions. Nucleic acids contents, expressed as percentages of bacterial cell dry weight, were also determined according to the techniques of Burton (1956) and Ceriotti (1955). The Ouchterlony technique (Ionagar; Oxoid) was used to determine the number and pattern of precipitin bands occurring between washed bacterial cells and *Staphylococcus* antitoxin. A suspension containing 5 mg of dry cells per ml was used in each circumferential agar well; undiluted commercial *Staphylococcus* antitoxin (Connaught Medical Research Laboratories, Toronto, Canada) was used in the central well; there were no refills. A quantitative immunofluorescence technique (de Repentigny and Sonea, 1962) was used to measure the intensity of specific fluorescence on equal volumes of bacteria before and after staining with fluorescent antitoxin.

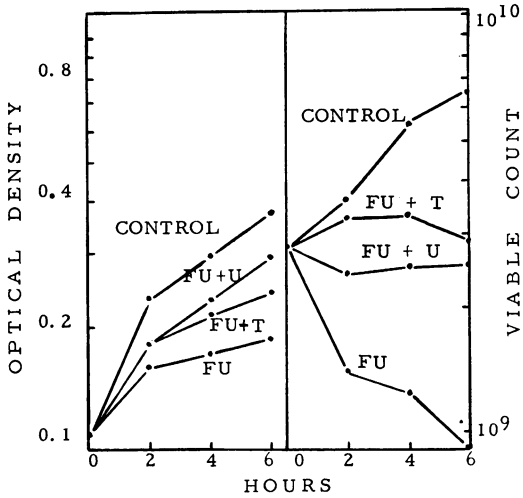

FIG. 1. *Effect of 65 μg/ml of 5-fluorouracil (FU) on the growth and multiplication of Staphylococcus aureus Wood 46 strain in the described culture medium, and its reversal by uracil (U) or thymine (T).*

RESULTS

Growth curves (Fig. 1) show, as an effect of adding FU, a decrease in the total number of bacteria and more so in the number of viable cells, similar to those previously obtained with other bacteria by Cohen et al. (1958). Uracil or thymine partially prevent these inhibitions.

The following changes occurred when FU was added to the cultures as compared with the controls (Table 1): (i) Growth diminished as seen by opacity determinations according to the known effect of FU. (ii) The nucleic acids contents (percentages of dry washed cells) dimin-

TABLE 1. *Changes in Staphylococcus aureus Wood 46 strain when grown in the presence of 5-fluorouracil**

Expt	Estimation of bacterial protoplasm (per cent opacity)		Nucleic acid content (per cent of dry cells)				Antigen estimation with *Staphylococcus* antitoxin		
			DNA		RNA		No. of precipitin lines†		Immunofluorescence intensities‡
	FU	Control	FU	Control	FU	Control	FU	Control	
A	69	73	3.4	5.3	8.0	11.5	4	11	$\frac{367}{1,493}$
B	52	67	3.7	5.2	9.0	11.0	4	11	$\frac{539}{1,103}$
C	64	74	3.9	5.1	7.5	9.0	7	9	$\frac{593}{645}$
D	52	70	4.1	6.9	12.7	16.5	7	12	—
E	55	64	3.5	5.2	8.6	9.1	8	14	—
F	42	66	3.9	5.9	8.4	8.0	0	11	—

* Amount of 5-fluorouracil (FU) used was 65 μg per ml of culture.

† After 10 days of diffusion at room temperature.

‡ Expressed as ratio of $\frac{\text{FU}}{\text{control}}$. Immunofluorescence intensity = total fluorescence intensity after staining with fluorescent antibodies minus primary fluorescence intensity. (The numbers represent the results given in 0.0001 microlumen for each experiment.)

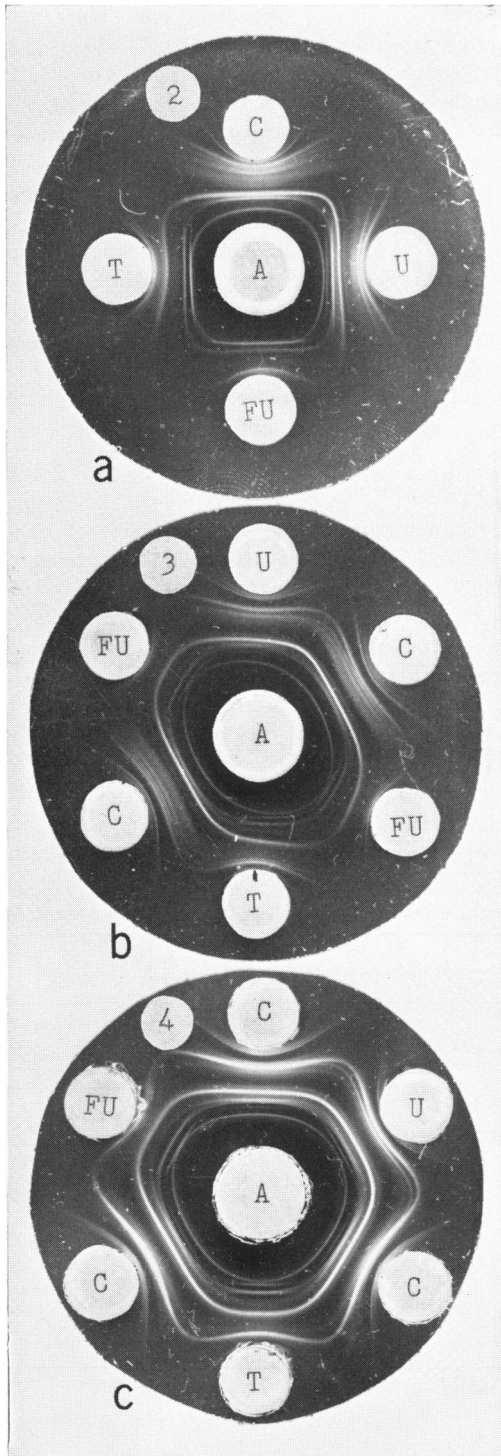

FIG. 2. *Effect of 65 μg/ml of 5-fluorouracil (FU) on the precipitin lines, and its reversal by uracil or thymine when added to a growing culture of Staphylococcus aureus Wood 46. The plates in b and c are from two different experiments (see Table 2). The precipitin bands are more intense in c than in b. A, Staphylococcus antitoxin; C, control culture; FU, culture in the presence of FU; U, culture in the presence of FU plus uracil; T, culture in the presence of FU plus thymine.*

TABLE. 2. *Variations of nucleic acids content and of the number of precipitin lines in relation to the ability of uracil or thymine to reverse the effect of 5-fluorouracil*

Expt	Conditions of growth*	DNA†	RNA†	No. of precipitin bands‡
1	Control	5.6	12.8	13
	FU	3.3	9.6	8
	FU + U	2.4	9.6	12
	FU + T	4.4	8.8	10
2	Control	4.6	8.4	13
	FU	2.8	7.4	6
	FU + U	2.8	8.7	11
	FU + T	4.2	7.8	8

* FU, 5-fluorouracil; FU + U, 5-fluorouracil + uracil; FU + T, 5-fluorouracil + thymine.

† Expressed as percentage of dry cells.

‡ After 10 days of diffusion at room temperature.

ished, more so in the case of deoxyribonucleic acid (DNA). (iii) The number of precipitating antigens, as observed by the Ouchterlony technique, decreased. The shape of the precipitin lines also changed (Fig. 2). When this FU effect was reversed by uracil or thymine, the number of precipitin lines increased up to normal with uracil and to an intermediate level with thymine (Fig. 2). (iv) The specific intensity of fluorescence added by fluorescein-coupled *Staphylococcus* antitoxin decreased.

When the inhibitory effect of FU on the synthesis of macromolecules was reversed by uracil or by thymine, the nucleic acid contents were modified (Table 2) according to the differential metabolic incorporation of these two substances in ribonucleic acid (RNA) and DNA (Cohen et al., 1958); the numbers of precipitating antigens vary accordingly.

DISCUSSION

The study of Cohen et al. (1958) on the influence of FU on *E. coli* growth curves through modifications of DNA and RNA synthesis was

the basis of our hypothesis in the present work. With our *S. aureus* Wood 46 strain, we obtained similar effects either on inhibition of growth or on reversal of FU action by uracil or thymine. These results are different from those obtained by Gause and Kochetkova (1962) with another *S. aureus* strain, in which thymine could not reverse FU action. We assume, by analogy with the work of Rogers and Perkins (1960), that, by altering the synthesis of cell-wall mucopeptides, FU could modify also the cellular permeability leading eventually to the death of the cells. These changes in the bacterial growth and in the synthesis of some macromolecules of FU-modified *S. aureus* cells were supplemented in our study by observations on changes in antigenic macromolecules. It would be possible to identify the precipitating antigens which disappear, as done by Oeding and Haukenes (1963). Immunodiffusion techniques, because of their extraordinary ability to distinguish between molecules, offer the advantage of finer definition than other methods (Crowle, 1961). The supplementary information brought by immunodiffusion is even more important in the case of the complex antigenic structures of pathogenic microorganisms. FU inhibits or alters the synthesis of cellular antigens normally found in pathogenic *S. aureus* cells which become antigenically closer to nonpathogenic staphylococci (Howard, 1954).

Quantitative immunofluorescence results supplement our immunodiffusion data (Table 1) by giving information mostly on the structural antigens localized at the surface of the microbial cells (de Repentigny et al., 1963). Part of the microfluorometric changes caused by FU may be related to the already described FU-produced modifications in the synthesis of a cell-wall mucopeptide of *S. aureus* (Rogers and Perkins, 1960). With technical improvements, quantitative immunofluorescence could be used to study specifically metabolic changes in the surface antigens of isolated microbial cells.

The use of immunochemical methods in studying the effects of inhibitory substances (e.g., pyrimidine analogues) on the synthesis of macromolecules of pathogenic bacteria may help in establishing some new correlations between virulence and metabolic pathways in microorganisms.

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