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Cellular Sites for the Competence-provoking Factor of Streptococci

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Immune globulins against competent cells of group H streptococci, strains Challis and Wicky, inhibited genetic transformation to streptomycin resistance when added to competent cultures. Antibodies against noncompetent cells did not inhibit transformation of competent cells. Strain Challis is spontaneously highly transformable. Strain Wicky is very poorly transformable but can be converted to high transformability with the exocellular competence-provoking factor (CPF) produced by strain Challis. Globulins against noncompetent cells of strain Challis and Wicky also inhibited transformation when added to noncompetent cultures prior to conversion to competence. Antibodies against cells of the related strain Blackburn, however, did not inhibit transformation under any circumstances. It is concluded that, although globulins prepared against competent cells block the deoxyribonucleic acid receptor sites present in these cells, the globulins prepared against noncompetent cells prevent conversion to competence by blocking the access of CPF to specific cellular sites for this factor. Strain Blackburn seems not to contain CPF-receptive sites and is, therefore, nontransformable.

The ability of some bacterial strains to absorb deoxyribonucleic acid (DNA) from related organisms and to become genetically transformed has been designated competence (12). Transformable streptococci produce an exocellular product which converts noncompetent cells to the state of competence (8).

Competent cells contain an antigen specific for the competent state (2, 5, 13). This antigen appears after the competence-provoking factor (CPF) has acted on noncompetent cells. The action of CPF may involve either "unmasking" of a hidden, pre-existing antigen or synthesis de novo. Regardless of which process takes place during conversion, one would expect that CPF must attach itself to specific cellular sites in order to perform conversion. Obviously, the cellular sites for CPF should be present in noncompetent cells but may also be present in competent cells. If this were the case, the attachment of CPF to the cell, with consequent conversion to competence, should be prevented by antibodies against noncompetent cells and possibly also against competent cells. In previous experiments, however, no significant inhibition of conversion was observed when antibodies against either noncompetent or competent cells were added prior to addition of CPF and were then removed before treatment of the cells with DNA (5).

As later revealed, the failure to demonstrate cellular sites for CPF in the earlier experiments was caused by the use of unsuitable concentrations of antibodies and of CPF. However, when the concentrations of the immune globulins were increased and the amounts of CPF were reduced, conversion to competence was inhibited by antibodies against both competent and noncompetent cells. These results indicate the presence of specific cellular sites for the competence-provoking factor.

MATERIALS AND METHODS

Test organisms. The organisms used were group H hemolytic streptococci received from the Central Public Health Laboratories, Colindale, London, England. Strain Challis produced large amounts of exocellular CPF and was highly transformable. Strain Wicky did not produce measurable amounts of CPF but occasionally yielded a few transformants. However, transformability of Wicky cultures was increased by 1,000- to 10,000-fold upon action of CPF derived from cultures of strain Challis (4).

Strain Blackburn was not transformable when tested under a variety of cultural conditions and at various stages of growth. It was also impossible to convert cells of this strain to competence with CPF preparations from cultures of the naturally transformable strains Challis and 3437/48. These preparations were very effective in converting cultures of strain Wicky to the state of competence.

Media. For CPF production and spontaneous transformation, strain Challis was cultivated in medium ET 3 (8), which was sterilized by membrane filtration (Millipore Corp., Bedford, Mass.). Noncompetent cells of this strain were produced by growth in the same medium heated at 115 to 117 C for 60 min. Strain Challis did not produce CPF in the heated medium, and the cells remained noncompetent throughout the period of growth (4). They could, however, be converted to competence with CPF prepared in the filter-sterilized medium.

Strain Wicky was grown in Brain Heart Infusion (Difco) supplemented with 1.5% hog serum heated for 30 min at 62 C to destroy deoxyribonuclease activity. The same medium was used for converting Wicky to competence with CPF.

CPF preparations. At the time of expected maximal competence, or 1 to 2 hr later (4), a culture of strain Challis was centrifuged, and the supernatant fluid was sterilized by membrane filtration. CPF was next precipitated by the addition of 300 g of ammonium sulfate per liter of filtrate. After 2 hr at 4 C, the sediment was collected and redissolved in one-fortieth volume of distilled water. Insoluble debris was removed by centrifugation; the clear solution was sterilized by membrane filtration and stored in 1-ml volumes at −25 C.

Noncompetent cells were converted to competence with this crude preparation of CPF.

Preparation of vaccines and immune globulins. For production of antibodies, rabbits were injected with either competent or noncompetent cells. The vaccines were prepared in the following way. The naturally transformable strain Challis was grown in 1 liter of medium ET3, sterilized by membrane filtration. With a starting inoculum of 5×10^5 to 6×10^5 viable units per ml and a generation time of about 30 min, the peak of competence appeared after 2 hr and 45 min of growth. At that time, a 2-ml sample was withdrawn and exposed to DNA to test transformability, which indicates competence. The total culture was treated with Formalin to a final concentration of 0.25% and was left at room temperature overnight. On the next day, the cells were collected by centrifugation, washed three times, resuspended in 20 ml of physiological saline, and dispensed in 1-ml volumes.

Noncompetent cells of the above strain were grown in the heat-sterilized medium ET3, tested for absence of competence, killed with Formalin, washed, and resuspended in saline to a final optical density equal to that of the suspension of competent cells.

Wicky cells were grown for 2.5 hr in Brain Heart Infusion supplemented with 1.5% hog serum and converted to competence with a saturating concentration of CPF which acted for 25 min.

Noncompetent cells of this strain were grown in the same medium for 3 hr, but treatment with CPF was omitted.

Before addition of Formalin, samples of the Wicky cultures were tested for the presence or absence of competence.

All vaccines were kept at −25 C until used.

Rabbits were injected with the above vaccines for a period of 6 weeks.

Each week, three 1-ml injections were given on successive days followed by a resting period of 4 days. The rabbits were bled 10 days after the last injection. Rabbit sera occasionally inhibit transformation by nonspecific factors, and, therefore, globulin fractions containing specific antibodies were prepared as described by Strauss et al. (11). Globulins were also made from antisera against cells of strain Blackburn. Globulin fraction from serum of the rabbits before immunization and also from serum of nonimmunized rabbits served as controls. The globulins from 5 ml of serum were dissolved in 1 ml of physiological saline and sterilized by filtration.

Transforming DNA. The method of extraction of DNA has been previously described (9). Streptomycin-sensitive cells of strains Challis and Wicky were transformed to streptomycin resistance with DNA extracted from a mutant of strain Challis which grew readily in the presence of 2 mg/ml of the drug. The recipients were sensitive to 25 μg/ml of streptomycin, and transformants were scored on blood-agar plates which contained 250 μg/ml of the antibiotic.

Transformation procedures. Transformation mediated by CPF involves converting noncompetent cells to competence and then treating them with transforming DNA. This operation is followed by a period of growth necessary for phenotypic expression of the newly acquired character.

The experiments reported here consisted in using antibodies to inhibit the two initial steps in transformation, namely, conversion to competence and uptake of DNA. This required some modifications of the usual procedure, which, for convenience, are reported in the description of the experiments.

RESULTS

Effect of immune globulins on transformation of competent cultures. Cultures of strains Challis and Wicky were started with an initial inoculum of 5×10^5 to 6×10^5 viable units per ml. A viable unit of strain Challis contained an average of 2.6 cells and of strain Wicky about 3.0 cells. After 105 min of growth in a water bath at 37 C, 2 ml of the crude CPF preparation, diluted 1:25 in medium, was added to 18 ml of culture to convert the cells to the state of competence. After CPF had acted for 10 min, 1-ml samples were withdrawn and mixed with 0.1 ml of the various globulin preparations as indicated in Table 1. After 15 min, the culture in each tube was treated with 5 μg of transforming DNA per ml; the DNA was allowed to act for 10 min, after which nonabsorbed DNA was destroyed by the addition of deoxyribonuclease.

The cultures were then grown for the next 110 min to allow phenotypic expression of the streptomycin-resistance character. Serial dilutions of the culture (0.2-ml volumes) were spread over the surface of blood-agar plates containing streptomycin for assay of the number of transformants. The total viable count was determined at the

TABLE 1. *Inhibition of competent cells by antibodies against competent cells*[a]

Designation of globulin	Globulin	Dilution of globulin in the culture[b]	Percentage of transformants in cultures of strains	
			Challis	Wicky
30	Against competent Challis cells	1:10	0.002	0.009
		1:250	0.013	0.065
		1:1,250	1.23	1.31
		1:1,750	3.10	4.32
31	Against competent Wicky cells	1:10	0.015	0.22
		1:100	0.028	0.36
		1:500	2.83	3.65
	Normal globulin	1:10	2.89	3.91
		1:100	2.75	4.13
	Nil (control)		2.59	3.37

[a] The globulins were added after conversion to competence and before addition of DNA. The numbers of viable units per milliliter, at the time of DNA addition, varied in the Challis cultures between 2.82×10^6 and 3.41×10^6, and in the Wicky cultures between 3.30×10^6 and 3.72×10^6.

[b] Undiluted globulin is a preparation concentrated fivefold with respect to the original serum.

moment of DNA addition by plating dilutions of the culture. The percentage of transformants is expressed in terms of this viable count.

The presence of immune globulins did not affect the rate of growth, for cultures without globulin and also those containing normal globulin had numbers of viable units at the time of DNA addition similar to those of cultures containing immune globulins.

The results show that globulins prepared against competent cells were very effective in inhibiting the transformation of cultures that had previously been rendered competent by the addition of CPF (Table 1). On the other hand, the globulins against noncompetent cells and the immune globulin against cells of strain Blackburn had no effect on the transformation of competent cultures (Table 2). In other words, the globulins against competent cells blocked DNA uptake, whereas those against noncompetent cells did not.

Inhibition of conversion to competence by globulins against noncompetent cells. The aim of these experiments was to find out whether the globulins against noncompetent cells of Challis and Wicky, and the globulin against cells of strain Blackburn, would inhibit transformation when added prior to treatment of cells with CPF.

From 105-min cultures of strains Challis and Wicky, 0.9-ml samples were withdrawn and treated with 0.1 ml of undiluted globulins. After 15 min, 0.1-ml portions of CPF, diluted 1:150, were added to each tube. The concentration of CPF was thus six times lower than in the previous experiments. After 10 min, the cultures were exposed for 10 min to DNA and then were handled as described before. The results shown in Table 3 indicate that each of the three "noncompetent globulins" added before conversion reduced significantly the percentage of transformants. Since these globulins did not affect transformation when added after conversion, it seems reasonable to conclude that they block conversion. When saturating concentrations of CPF were used, inhibition was low or insignificant. Most important, however, is the fact that the globulin against the cells of strain Blackburn, which was nontransformable in many tests under a variety of cultural conditions, did not affect transformation at all and behaved like a globulin from a nonimmunized normal rabbit.

Effect on transformation of "competent globulins" added to noncompetent cultures and removed before treatment with DNA. As in the previous experiment, 0.9-ml samples of young cultures of the strains Challis and Wicky were supplemented with 0.1-ml amounts of undiluted "competent globulins." After 15 min, the samples were treated with CPF, which was allowed to act for 10 min. Since "competent globulins" block DNA

TABLE 2. *Transformation of competent cells in the presence of antibodies against noncompetent cells*[a]

Designation of globulin	Globulins	Percentage of transformant in cultures of strains	
		Challis	Wicky
36	Against noncompetent Wicky cells	2.07	2.51
37	Against noncompetent Wicky cells	1.84	2.73
38	Against noncompetent Challis cells	1.72	2.58
40	Against noncompetent Blackburn cells	1.98	3.19
	Normal globulin (control)	1.87	2.52
	Nil (control)	1.78	2.13

[a] The globulins were added after conversion to competence and before addition of DNA. The final concentration of globulin in each culture was 10%. The numbers of viable units per milliliter, at the time of DNA addition, varied in the Challis cultures between 2.29×10^6 and 2.56×10^6 and in the Wicky cultures between 3.30×10^6 and 3.72×10^6.

TABLE 3. *Inhibition of transformation by antibodies against noncompetent cells added before conversion to competence*[a]

Designation of globulins	Globulins	Percentage of transformants in cultures of strains			
		Challis		Wicky	
		Expt 1	Expt 2	Expt 1	Expt 2
36	Against noncompetent Wicky cells	0.15	0.13	0.31	0.18
37	Against noncompetent Wicky cells	0.012	0.014	0.024	0.01
38	Against noncompetent Challis cells	0.12	0.13	0.39	0.21
40	Against cells of strain Blackburn	0.88	0.71	1.34	0.54
	Normal globulin	0.63	0.58	1.31	0.45
	Nil (control)	0.72	0.61	1.25	0.45

[a] The globulins were added before conversion to competence. The final concentration of globulin in each culture was 10%. The numbers of viable units per milliliter, at addition of DNA, varied in the Challis cultures between 1.89×10^6 and 2.49×10^6 and in the Wicky cultures between 3.01×10^6 and 3.45×10^6.

uptake, nonadsorbed antibodies were removed by centrifugation before addition of DNA. The cells, after washing, were resuspended in fresh medium, treated with DNA, and allowed to grow for phenotypic expression as already described. The controls with "noncompetent" and normal globulin were handled in the same way.

The results reported in Table 4 indicate that the "competent globulins" under these conditions inhibited transformation approximately to the same extent as the "noncompetent" ones.

DISCUSSION

Previous experiments indicated a direct correlation between synthesis of CPF and development of competence. Cultural conditions adverse to synthesis of CPF were also adverse to transformation, and the efficiency of transformation ran parallel to the amount of CPF accumulated in the culture (4). CPF which has been purified by ammonium sulfate precipitation and Sephadex gel chromatography is sensitive to proteolytic enzymes, and, when injected into rabbits, stimulates the production of antibodies which inhibit completely the capacity of CPF to perform conversion (*unpublished data*). These data indicate that CPF is a protein.

TABLE 4. *Effect on transformation of antibodies against competent cells added before conversion to competence and removed before addition of DNA*[a]

Designation of globulins	Globulins	Percentage of transformants in cultures of strains	
		Challis	Wicky
30	Against competent Challis cells	0.11	0.13
31	Against competent Wicky cells	0.16	0.21
36	Against noncompetent Wicky cells	0.11	0.14
	Normal globulin	0.68	0.82

[a] Each globulin was added to a final concentration of 10%. The numbers of viable units per milliliter, at addition of DNA, varied in the Challis cultures between 1.78×10^6 and 2.23×10^6 and in the Wicky cultures between 2.23×10^6 and 2.63×10^6.

The process of conversion to competence is time- and temperature-dependent (8). In addition, a given nonsaturating amount of CPF can be used to effect conversion of several batches of noncompetent cells. It seems likely, therefore, that the process of conversion is enzymatic in nature.

Experiments with pneumococci (14), streptococci (6), and *Haemophilus influenzae* (10) have shown that development of competence requires special cultural conditions and undisturbed protein synthesis. Clearly, the acquisition of competence is a complicated process which finally, as shown in Tables 1 and 2, results in the appearance of an antigen specific for the competent state. Studies with radioactive DNA point to this antigen being the cellular site which binds DNA (3, 7, 13).

It seems reasonable to suppose that the CPF must attach itself to the cell in order to perform conversion, i.e., to change the antigenic structure of the cell. The results in Table 3 show that the "noncompetent globulins," which do not affect uptake of DNA, nevertheless inhibit transformation. This inhibition may be caused by competition for specific cellular sites between the antibodies against the cell antigens and the CPF. The antibodies present in the culture before the addition of CPF combine with cell antigens and may prevent the access of CPF. The same conclusion may be drawn from the data in Table 4, although

in this case it is impossible to exclude the possibility that the "competent globulins" may cause some inhibition by blocking of DNA-receptive sites as they appear during the action of CPF.

Other reasons for immune inhibition of conversion may be considered. As mentioned before, the extracellular CPF is itself antigenic, and its ability to perform conversion is completely blocked by anti-CPF antibodies. The competent cells used for immunization of rabbits, although washed three times in saline, could contain some bound CPF which would stimulate production of anti-CPF antibodies. However, such a supposition seems most unlikely with regard to the noncompetent cells, since they were not treated with CPF and were totally nontransformable when exposed to DNA.

This leaves us with the hypothesis that uptake of DNA by streptococci involves an enzymatic type of reaction. The latter seems to require a genetically controlled capacity to synthesize CPF, together with a specific structure on the cell surface which binds CPF. On this basis, the strains used in these experiments may be characterized as follows: strain Challis produces CPF and contains cellular sites for CPF; strain Wicky, although receptive to CPF of other transformable streptococci, does not produce measurable amounts of CPF, and is, therefore, "spontaneously" very poorly transformable; strain Blackburn is noncompetent and cannot be converted to competence by CPF of other streptococci because it neither produces CPF nor contains receptive sites for CPF.

Colman and Williams (1) have shown that strains of streptococci of the same serological groups may have different cell wall structures. Six strains of group H streptococci had three different cell wall patterns. Similar variation was observed among strains of *Streptococcus sbe*, which also belonged to the serological group H. It seems, therefore, that correlation of cell wall structure with studies of DNA uptake may throw some additional light on the nature of transformability.

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