STEPWISE INTRATYPE TRANSFORMATION OF PNEUMOCOCCUS FROM R TO S BY WAY OF A VARIANT INTERMEDIATE IN CAPSULAR POLYSACCHARIDE PRODUCTION

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INTRODUCTION

Previous studies of pneumococcal transformation have been concerned in the main with the conversion of R organisms derived from one specific type to S organisms of heterologous type. The present investigations are concerned with intratype conversion or transformation of pneumococci. A stable variant of pneumococcus Type II, intermediate between the classical R and S forms, has been isolated. It has been demonstrated that the variant produces a certain amount of specific capsular polysaccharide (SSSII) although it does not possess a demonstrable capsule nor does it form colonies with a smooth surface similar to those of S organisms. The gene-like material controlling the formation of SSSII has been separated from the intermediate variant and transferred *in vitro* to an R strain of pneumococcus which thereby assumes certain characteristics of the intermediate. The transformed R strain retains its rough colonial form, produces SSSII, but has no demonstrable capsule. In addition, the intermediate has been converted *in vivo* to a fully encapsulated strain of pneumococcus Type II.

A variety of variants intermediate between the R and S forms of pneumococci have been described before. Blake and Trask (1) in 1923 noted the presence of intermediates derived from pneumococcus Type I and described 5 of these in detail 10 years later (2). Of the 5 intermediate variants derived from Type I by serial cultivation in broth containing Type I antipneumococcal serum, 2 were relatively stable on cultivation in broth, but both of these reverted to fully encapsulated Type I S on mouse passage. Blake and Trask (2) likewise described an intermediate originating from pneumococcus Type II. The occurrence of intermediate variants has also been reported by Dawson (3), Neufeld and Levinthal (4), Klumpen (5), Paul (6), and by others.

It has been customary in the past to consider that cultivation of S pneumococci in media containing homologous anticapsular antibodies suppresses in some manner the polysaccharide-synthesizing mechanism, and that con-

versely, the reversion of R pneumococci to the homologous S form on cultivation in anti-R serum involves reactivation of capsular synthesis. In the light of modern knowledge it seems more probable that R and intermediate mutants occur in cultures of pneumococci of various specific types and that these mutants have a selective advantage when the predominantly S cultures are grown in the presence of anti-S serum. Similarly, some, but not all, cultures of R pneumococci contain a small number of S and intermediate forms or else occasionally throw off S mutants which are selected when cultivated in anti-R serum or injected into mice. That the degradation from S to R may involve a stepwise loss of characters is indicated by earlier reports (1–6). The present studies describe a specifically directed, stepwise restoration from R to intermediate and then to the fully encapsulated S form.

Materials and Methods

1. Preparation of Transforming Extracts.—In previous methods for the preparation of transforming extracts (7) the bacterial cells obtained by centrifuging young broth cultures of encapsulated pneumococci have served as the starting material. Very large amounts of broth culture must be used to obtain even a modest amount of active transforming extract. In the present method advantage was taken of the mass culture technique in which an excess of glucose is added to the medium and the acid produced during the course of growth is neutralized intermittently with NaOH. A much greater yield of bacterial cells per volume of medium can be obtained under these circumstances. In addition, by observation of the rate of glucose utilization, the transforming material can be harvested from bacteria in the more active phase of growth.

One liter of nutrient broth prepared from fresh beef heart infusion and containing 1 per cent neopeptone was seeded with 15 cc. of the supernatant of an 18 hour rabbit blood broth culture of pneumococci. After incubation at 37°C. for approximately 16 hours, the organisms were all, or virtually all, Gram-positive. Glucose was then added to a final concentration of 0.5 per cent and 1 cc. of 0.5 per cent phenol red in 95 per cent alcohol was added as indicator of acid production (0.0005 per cent phenol red). Following the addition of glucose, incubation was continued for a period of 3 to 6 hours depending on the growth characteristics of the strain of pneumococcus. During the last 15 minutes the culture was grown in the presence of 0.001 molar sodium citrate to inhibit destruction of the transforming principle (8). Growth was stopped when the slope of the curve for the addition of alkali began to fall off. At this time sodium citrate was added to a concentration of 0.1 M. Sodium desoxycholate was then added to a concentration of 0.05 per cent. The broth culture, which had been creamy with cells, cleared within approximately 3 minutes and became only moderately opalescent and somewhat viscous depending on the type of pneumococcus that was lysed.

An attempt to grow pneumococci in the presence of a small amount of sodium citrate (0.0001 M) was made but abandoned because the growth rate was reduced to about one-third that obtained in citrate-free medium.

To the desoxycholate-lysed culture, one volume of alcohol was added with shaking whereupon a white fibrous precipitate separated out. The mixture was centrifuged and the supernatant discarded. The precipitate was dissolved in 100 cc. of 0.1 M sodium chloride solution containing 0.1 M sodium citrate. Protein was removed by repeated shaking with a mixture of 20 cc. of chloroform and 5 cc. of octyl alcohol in a shaking machine. After the third chloroform treatment only a very slight film of protein-chloroform gel was visible at the inter-

face following centrifugation. The transforming principle was then precipitated with two volumes of alcohol. The active principle was partially purified by dissolving the precipitate in saline and reprecipitating with two volumes of alcohol. After the second precipitation the active fraction appeared as a dense white fibrous mass.

A difficulty encountered through addition of 0.1 M sodium citrate is the precipitation of large amounts of crystalline sodium citrate when one volume of alcohol is added and the mixture allowed to stand overnight in the cold. The active fraction becomes entrapped in the crystalline deposit. This difficulty can be avoided by completing the separation and partial purification of the desoxyribonucleic acid fraction during the course of a single day, and avoiding storage in the ice box during these stages.

2. Transformation Reactions in Vitro.—A method previously described (7) was used with minor modifications. One drop of a 10^{-4} dilution of an 18 hour blood broth culture of the R or intermediate variant of Type II pneumococcus was seeded into charcoal-absorbed broth containing an appropriate amount of transforming principle and 10 per cent anti-R pleural fluid which had been heated previously at 60° for 30 minutes. The volume of broth, chest fluid, and transforming principle was 2 cc. in all. After 24 hours' incubation, transfers were streaked on the surface of blood agar plates and subcultures of the supernatant were made in 10 per cent anti-R serum broth with or without further addition of transforming principle. The supernatants of all cultures were examined carefully for turbidity as an indication of the occurrence of transformation. In all instances the experiments were set up in duplicate.

3. Transformation Reactions in Vivo.—The technique followed was that described originally by Griffith (9) in which mice are injected subcutaneously with small amounts of living R forms mixed with large amounts of heat-killed smooth organisms.

To obtain a mass growth of smooth organisms, a liter of neopeptone infusion broth was seeded with smooth pneumococci and incubated at 37°C. overnight. The next morning 10 gm. of glucose was added and the culture was incubated for 6½ hours with intermittent neutralization. During the last half-hour the culture grew in the presence of 0.001 M sodium citrate. The culture was then centrifuged in the presence of approximately 0.1 M sodium citrate and the pneumococci were resuspended in 0.1 M sodium citrate–normal saline solution. The organisms were killed by heating at 65°C. for 30 minutes in a water bath. Pyrex tubes were half filled with the bacterial suspension. The mouth of each tube was flamed thoroughly and the tubes immersed in the water bath to the lip. The suspension of heat-killed organisms was streaked on blood agar plates, inoculated into broth and injected into mice. There was no evidence of living pneumococci from any of these tests.

White mice of the CFW strain were injected subcutaneously in the lower abdominal region with 0.1 cc. of an 18 hour blood broth culture of R pneumococci and the suspension of heat-killed S organisms in an amount sufficient to make the mixture as turbid as possible and still allow injection through a No. 25 hypodermic needle. A total volume of 0.5 cc. was injected into each mouse. Upon death of the mice, organisms obtained from peritoneal washings and cultures of heart blood were examined for capsular swelling with type-specific serum and the heart blood was streaked on rabbit blood agar plates for subsequent study.

4. Strains of Pneumococcus.—D39/S: a fully encapsulated strain of pneumococcus Type II.

D39/R19: a rough strain obtained by growing the Type II S strain D39/S for 19 serial transfers in Type II antipneumococcal rabbit serum.

D39/Int53: a strain intermediate between the classical R and S forms with respect to SSSII formation, derived from the rough strain D39/R19 by selection.

R36NC: colonial variant of the rough strain R36 which was used in studies by Avery, MacLeod, and McCarty (7). R36 was derived originally from Type II strain D39S.

A/66: a fully encapsulated strain of pneumococcus Type III also used in previous studies (7).

5. Anti-R Serum.—Sterile pleural fluids containing R agglutinins were obtained from patients suffering from streptococcal pneumonia and tuberculosis respectively. The pleural fluids were clarified by centrifugation and heated at 60° for 30 minutes before being used in the tests.

EXPERIMENTAL

Isolation of Intermediate Variant D39/Int53.—Dawson (10) observed that in broth cultures seeded with different amounts of R and S pneumococci the same relative proportions of R and S forms were maintained for several transfers. In other words, when R and S pneumococci are grown together in broth culture, neither form appears to have a selective advantage over the other. This observation of Dawson's has been confirmed in the present study. However, when an anti-R serum is added to the medium, S pneumococci appear to have a selective advantage over R although it is possible that some R cells under these circumstances might be transformed to S cells because of the transforming principle present in the S organisms.

When a mixed culture containing a large seeding of R organisms and a comparatively small number of S organisms is grown in broth it is a matter of chance whether any S forms will be carried along when transfers are made using only a loopful of whole culture. On the other hand, if such a mixed culture is grown in the presence of anti-R serum, the R organisms are agglutinated in the bottom of the tube and unagglutinated organisms grow throughout the supernatant. Thus when a mixed culture is grown in the presence of anti-R serum and the supernatant only transferred, the unagglutinated organisms will be recovered even though they are present in comparatively small number in the original inoculum.

When the cells from 10 cc. of whole broth culture of the rough strain D39/R19 and 1×10^{-8} cc. of culture of the smooth strain D39/S were inoculated together into 4 cc. of 10 per cent anti-R serum broth, S pneumococci were demonstrated on plating the first subculture even though the ratio of R to S in the original inoculum was 10^9:1. The transfer was made by centrifuging the 24 hour mixed culture for 5 minutes at low speed to pack the R cells and transferring the entire supernatant to 4 cc. of 10 per cent anti-R serum broth. The original mixed culture was grown in a long narrow tube, 12.5 cm. by 8 mm. This afforded a tall column of medium through which non-agglutinated organisms could grow and permitted easy removal of the supernatant. Tubes of this sort are not a necessary prerequisite but make it easier to obtain the supernatant relatively free of R cells. The efficiency of this method of selection is indicated by the ease with which 1–3 S cells could be detected when inoculated along with 10^9 R cells.

By using this technique it was found that when the rough strain D39/R19 was grown in 10 per cent anti-R serum broth and all or only a loopful of supernatant was used in making transfers to serum broth, intermediate variants

could be selected readily in three subcultures. These variants, although forming colonies with a rough surface, were not agglutinated when grown in 10 per cent anti-R serum broth.

A similar, if not identical intermediate variant was isolated by chance from subcultures of a single colony of the rough strain D39/R19 by repeated transfer of a loopful of whole culture to fresh 10 per cent anti-R serum broth. At the 14th transfer a faintly turbid supernatant was present and in 53 subsequent transfers almost all of the organisms failed to be agglutinated by the anti-R serum and grew diffusely in the supernatant. This intermediate variant, designated D39/Int53, forms rough colonies on blood agar, is not agglutinated in anti-R serum broth, but is readily agglutinated to high titer by a true anti-SII serum. It appears to be an intermediate between the classical R and S forms of pneumococci. Classical encapsulated S forms, which grow in smooth colonies on blood agar, were not encountered at any time either when supernatants only or whole mixed cultures of D39/R19 were repeatedly transferred in anti-R serum broth. There is no evidence, therefore, that cultures of the rough strain D39/R19 contain any encapsulated Type II pneumococci.

Production of SSSII by the Intermediate Variant D39/Int53.—Failure of strain D39/Int53 to be agglutinated when cultivated in anti-R serum broth, even though it still forms a rough surfaced colony on blood agar, suggested that a component is present on the surface of organsism of this intermediate variant which is absent from classical R organisms. It seemed likely that the surface component is the Type II polysaccharide, though present in an amount insufficient to give the colonies of D39/Int53 the characteristic smooth, glistening appearance of encapsulated pneumococci. Immunological evidence indicates clearly that the intermediate variant produces SSSII identical with that formed by encapsulated pneumococci of the smooth strain D39/S but in markedly reduced amount.

Antisera prepared in rabbits by immunization with the intermediate variant D39/Int53 gave precipitin reactions with purified Type II capsular polysaccharide diluted to 1:5,000,000 and protected mice against infection with fully virulent Type II pneumococci. Moreover, such sera agglutinated fully encapsulated Type II pneumococci to a titer equal to the best obtained by immunization with the smooth strain itself as shown in Table I. The same sera agglutinated the intermediate variant to a much higher titer. Furthermore, a sample of serum prepared by immunization with the smooth strain D39/S, and repeatedly absorbed with R organisms until it no longer caused their agglutination, maintained its original titer against the smooth strain and also against the intermediate variant. This is also shown in Table I. On the other hand, when antiserum to the smooth strain was absorbed with the intermediate variant, its agglutinating capacity for both strains was removed, as recorded in Table I, and likewise absorption of an anti-intermediate serum

with smooth Type II encapsulated organisms removed the agglutinins for both intermediate and smooth strains. This shows that the specific soluble substance produced by both strains is identical.

The difference in the agglutinating titers of the various sera for the smooth and intermediate strains is of some significance with respect to the amount of SSSII present on the surface of the cells. Titers with the intermediate strain are invariably much higher than with S organisms. The amount of polysaccharide available for combination with antibody is greater in the case of the fully encapsulated smooth strain than with the intermediate variant and, accordingly, the agglutination titer with the smooth strain would be expected to be lower since each cell would be able to combine with more antibody than each cell of the intermediate variant.

TABLE I

Agglutination Reactions with Antisera to Smooth Type II Pneumococcus Strain D39/S, and to the Intermediate Variant D39/Int53

Antiserum	Agglutination titers	
	Suspension of smooth strain D39/S	Suspension of intermediate strain D39/Int53
Anti-D39/S (smooth strain)	256	1000
" absorbed with R pneumococci	256	1000
" " " intermediate variant D39/Int53	0	0
Anti-D39/Int53 (intermediate variant)	256	2000
" absorbed with smooth strain D39/S	0	0

Absorption of antiserum to the intermediate variant with the fully encapsulated strain D39/S was accomplished relatively easily. However, with the intermediate variant, D39/Int53, repeated absorptions of Type II antiserum with very large amounts of organisms were necessary to remove completely anti-SSSII. Probably this is because of the much smaller amount of SSSII produced by the intermediate variant as compared with the encapsulated strain since the amount of antibody in the two samples of serum was comparable as shown by the agglutination titers recorded in Table I.

Further evidence for the greatly decreased production of SSSII by the intermediate strain is shown by precipitin tests using an antiserum prepared against the smooth strain D39/S from which all anti-R antibodies had been removed by absorption, and culture supernatants of the respective smooth and intermediate variants. The cultures were grown in plain broth for 16 hours at 37°C. There was no obvious difference in the amount of growth of the two strains as indicated by turbidity. The organisms were removed by centrifugation and

dilutions of culture supernatants were added to R-absorbed Type II antiserum diluted in saline in the proportion of 2:3. The results of these precipitin reactions are recorded in Table II.

From the results shown in Table II it can be seen that although the culture supernatant of D39/S when diluted 1:10 gives a well marked precipitin reaction with R-absorbed Type II antiserum, and a trace of precipitation at 1:20, the supernatant of the intermediate variant shows only a trace of reaction when diluted 1:2.

Preparations of purified SSSII in a dilution of 1:2,500,000 gave a definite precipitin reaction with the R-absorbed Type II antiserum used in the tests recorded in Table II.

The question of the quantitative production of SSSII by the intermediate variant was not further explored except for a single attempt to isolate SSSII from the autolysate of a mass culture of D39/Int53 grown in the presence of 1

TABLE II

Precipitin Reactions of Culture Supernatants of Smooth and Intermediate Organisms with Anti-II S Serum Absorbed with R Organisms

Strain	Final dilutions of culture supernatant			
	1:2	1:10	1:20	1:200
D39/S (smooth strain)	++++(disc)	++	Trace	−
D39/Int53 (intermediate variant)	Trace	−	−	−

++ and ++++ indicate the relative intensities of the precipitin reactions.

per cent glucose employing intermittent neutralization. The amount of SSSII, as indicated by precipitin reactions, was so small as to make isolation from other cell constituents impracticable.

Failure to Demonstrate a Capsular Swelling Reaction with the Intermediate Variant.—The observations described above indicate that the intermediate variant produces SSSII although in an amount considerably less than that produced by encapsulated organisms. In addition, SSSII would appear to be disposed over the surface of the cells since they are no longer agglutinated by an anti-R serum but are agglutinated by Type II antiserum. It was of interest, therefore, to determine whether capsular swelling could be demonstrated with the intermediate variant.

D39/Int53 was grown under various conditions: in blood broth for 18 hours at 37°C., in broth containing 5 per cent normal horse serum for 4 to 6 hours, and in broth containing 5 per cent glucose for the same period. Pneumococci cultivated under these conditions and obtained from the peritoneal washings of mice that died following injection of 1 cc. of an 18 hour blood broth culture of the intermediate variant were tested for the capsular swelling reaction using

antisera prepared against both the smooth and intermediate variants. In no instance was capsular swelling apparent on repeated test, although it could be seen readily with the same antisera and smooth organisms grown under conditions similar for capsular development. It would appear that although the intermediate variant produced SSSII, and even though the polysaccharide is disposed on the surface of the cells, the amount is not great enough to give the capsular swelling reaction.

Virulence of the Intermediate Variant for Mice.—The virulence of the intermediate D39/Int53 was compared to that of the rough strain R36 by injecting mice intraperitoneally with organisms from 18 hour blood broth cultures. The results of this experiment are summarized in Table III. As shown in Table III, 1 cc. of an 18 hour broth culture of D39/Int53 killed all three mice within

TABLE III

Virulence of Intermediate Strain, D39/Int53, and an R Strain, R36, for Mice

Strain	Amount of culture injected	D/S*	Survival time‡	Culture of heart blood
	cc.		*hrs.*	
D39/Int53 (intermediate strain)	1.0	3/0	24	Intermediate variant only
" " "	0.5	0/3		
R36 (rough strain)	1.0	2/1	24	R organisms only
" " "	0.5	1/2	23	" " "

* Ratio of deaths to survivals.

‡ Survival time of mice that died.

Surviving mice discarded 7th day.

approximately 24 hours, whereas 0.5 cc. did not cause death. Compared with the encapsulated strain of Type II pneumococcus, D39/S, which kills mice regularly in an amount as small as 1×10^{-8} cc., the intermediate variant is comparatively avirulent. The rough strain, R36, killed 2 of 3 mice when 1 cc. of culture was injected and 1 of 3 mice when 0.5 cc. of culture was injected. It appears, therefore, that the intermediate variant, D39/Int53, is no more virulent for mice than the R strain, R36, despite the fact that it produces SSSII and that the polysaccharide appears to be a surface component. The reason may be that the intermediate variant does not produce the capsular polysaccharide in amounts sufficient to protect itself against phagocytosis.

Intratype Conversion of R Pneumococci to an Intermediate Variant.—The foregoing data demonstrate the existence of an intermediate variant of Type II pneumococcus which forms colonies similar in character to those of typical rough organisms, but nonetheless synthesizes the Type II polysaccharide, which appears to be disposed on the surface of the cells although in amount considerably less than produced by the classical S organisms. Since this

intermediate variant remained stable through repeated subcultures in nutrient broth, it seemed of interest to determine whether from the cells a "transforming principle" could be obtained which would be capable of converting typical R pneumococci into variants of the intermediate type.

The technique for preparing transforming extracts has been described under Methods. The R strain used for the transformation reactions, R36NC, is a variant of strain R36 previously used in transformation reactions (7). Strain R36NC does not grow diffusely when cultivated in anti-R serum broth but remains clumped in the bottom of the tubes. The intermediate variant, as noted above, grows diffusely in serum broth. The property of diffuse growth

TABLE IV

Transformation of R36NC by an Extract of the Intermediate Variant D39/Int53

Amount of transforming extract of D39/Int53	Turbidity of culture supernatants after 24 hrs.' incubation*		Identification of organisms in supernatant
	Original culture	First subculture‡	
cc.			
0.2	Trace	++++	Intermediate variant
0.2	"	++++	" "
0.02	"	++++	" "
0.02	"	+	Not studied
None	Clear	Clear	R36NC

* Trace to ++++ designates degree of turbidity of supernatant, indicating whether transformation has occurred.

‡ Subculture in 10 per cent serum broth without added transforming extract from D39/Int53.

in anti-R serum broth was used as an indication of whether conversion of R36NC to the intermediate type had occurred in the presence of an extract of intermediate cells, since colonies on blood agar of R36NC and the intermediate variant derived from it appear identical whether viewed with the naked eye or under a colony microscope. In order to prove that conversion to the intermediate type had occurred, reliance had to be placed on serological methods.

Although the means of identifying the converted cells are considerably more difficult than in the case of the transformation of R cells to smooth, encapsulated S forms, it was possible to demonstrate that under the conditions described, R36NC can be converted at will into cells of the intermediate variety. Table IV shows the results of a typical experiment.

No evidence was obtained at any time that strain R36NC was contaminated with intermediate mutants corresponding to D39/Int53 as in the case of the other R strain, D39/R19, from which D39/Int53 was selected. As illustrated in Table IV, however, R36NC could be readily transformed into a strain having the properties of the intermediate variant D39/Int53, originally ob-

tained by selection. Table V shows a comparison of the properties of the original intermediate mutant D39/Int53 and the transformed intermediate mutant R36NC/Int with those of the classical R and S forms of pneumococcus Type II.

In addition to the properties shown in Table V, antisera prepared in rabbits against the respective intermediate variants protected mice to an equal degree

TABLE V

Properties of Original Intermediate D39/Int53, Transformed Intermediate R36N/Int, and Classical S and R Forms of Pneumococcus Type II

Properties of pneumococcal strains	Strains of pneumococcus			
	Intermediate D39/Int53 obtained by selection	Intermediate R36NC/Int obtained by transformation	Smooth pneumococcus Type II, strain D39/S	Rough pneumococcus R36NC
Colony morphology on blood agar	Rough	Rough	Smooth	Rough
Growth in anti-R serum broth	Diffuse	Diffuse	Diffuse	Agglomerated
Reactions in Type II antipneumococcal serum (anti-D39S)				
Agglutination titer	1000	1000	256	2000
Quellung reaction	Negative	Negative	Positive	Negative
Reactions in antiserum to intermediate variant D39/int53				
Agglutination titer	2000	2000	256	8000
Quellung reaction	Negative	Negative	Positive	Negative
Reactions in antiserum to intermediate variant R36NC/Int				
Agglutination titer	2000	2000	256	8000
Quellung reaction	Negative	Negative	Positive	Negative
Character of organisms agglutinated in Type II antipneumococcal serum (anti-D39S)	Small, hard flakes; difficult to break up	Small, hard flakes; difficult to break up	Hard, disc-like mass	Fine, soft clumps; easily broken up

against infection by the fully virulent encapsulated Type II strain D39S. 0.001 cc. of either antiserum gave protection against approximately 1000 lethal doses of D39S injected intraperitoneally.

Although the properties described for the two intermediate variants indicate strong similarities, it should not be inferred that they are identical in all respects. For example, colonies of the transformed intermediate R36NC/Int resemble closely those of the R strain from which it was derived by transformation, and are different from the colonies of D39/Int53 in certain respects. These colonial differences indicate the presence of systems other than that involved in SSS formation which are not affected in the transformation reaction.

Conversion of Intermediate D39/Int53 into Fully Encapsulated Type II Pneumococci.—Attempts to convert the intermediate D39/Int53 to fully encapsulated Type II pneumococci *in vitro* were unsuccessful using either transforming extracts prepared from Type II S or heat-killed cells. It should be recalled that the intermediate variant is not agglutinated when grown in anti-R serum broth, and since growth in agglomerated fashion appears to be one of the prerequisites for transformation *in vitro* (11), it is possible that this accounts for the failure. Other methods for producing agglomeration of the growing organisms, such as cultivation in semisolid agar, were not tried.

Though unsuccessful *in vitro*, conversion of the intermediate D39/Int53 to Type II S was readily accomplished *in vivo* by the original Griffith technique (9) of injecting mice subcutaneously with a mixture containing small numbers

TABLE VI

Conversion of the Intermediate Variant D39/Int53 to Fully Encapsulated Pneumococcus Type II in Vivo

Heat-killed pneumococci*	Living pneumococci‡	No. of mice injected	No. of mice dying§	Heart blood culture
Type II S (D39S)	Intermediate D39/Int53	7	5	Pneumococcus Type II
" " "	—	5	0	—
—	Intermediate D39/Int53	7	0	—

* 0.4 cc. of a thick suspension of Type II pneumococcus heated at 65°C. for 30 minutes.

‡ 0.1 cc. of an 18 hour blood broth culture.

§ Average survival time of mice that died was 79 hours; surviving mice were discarded on the 8th day.

of living organisms of the strain to be converted together with large amounts of a heat-killed suspension of smooth organisms. Table VI shows the results of such an experiment. Five of the 7 mice injected with D39/Int53 together with a heat-killed vaccine of Type II S died. From the heart blood of all of these animals fully encapsulated Type II pneumococci were recovered, forming smooth colonies on agar and virulent for mice. Proof of the sterility of the heat-killed suspension of Type II S was obtained not only by injecting mice with 0.4 cc. amounts but also by inoculation into blood broth and on blood agar plates.

Failure to Transform Type II Intermediate D39/Int53 to Pneumococcus Type III.—As noted above, the intermediate D39/Int53 could be converted to Type II S *in vivo*, though not *in vitro* by the techniques employed. It was of interest to determine whether it could be transformed to a fully encapsulated heterologous type of pneumococcus. Repeated attempts both *in vitro* and *in vivo* to transform D39/Int53 to pneumococcus Type III were unsuccessful

using extracts of Type III strain A/66 *in vitro*, and heat-killed suspensions of Type III both *in vitro* and *in vivo*. In addition to anti-R serum in the *in vitro* experiments, in certain instances rabbit antiserum to intermediate D39/Int53, previously heated at 65°C. to inactivate desoxyribonuclease, was added in order to cause the intermediate strain to grow in clumps, but without success.

Conversion of Rough Strain R36NC to Pneumococcus Type II and Transformation to Pneumococcus Type III in a Single Step.—R36NC is convertible to an intermediate form R36NC/Int, and the intermediate can be converted to fully encapsulated homologous pneumococcus Type II, as shown above. In addition, R36NC can be converted to pneumococcus Type II in a single step by cultivation *in vitro* in the presence of an extract of Type II S. However, it is possible that one or more intermediate phases may appear during the course of the progression from R to S and may be necessary for R——S conversion to occur. Intermediates appearing in the course of "single step" R——S conversion have not been looked for.

The rough strain R36NC can also be transformed to pneumococcus Type III *in vitro*, using transforming extracts prepared from strain A/66.

DISCUSSION

The experiments described indicate that in the transformation of an R strain of pneumococcus to the fully encapsulated S form more than a single character may be involved. Mutants intermediate in respect to SSS production occur and conversion can be carried out in two stages, first from R to intermediate and then to the fully encapsulated S form. On the other hand, transformation from R to S may take place in a single step, apparently without the necessity of going through an intermediate phase, although with the usual methods of carrying out transformation reactions, intermediate variants would not ordinarily be detected despite the possibility that they may occur under these circumstances. In this regard it should be recalled that detection of transformation *in vitro* has been based primarily on the gross differences in the appearance of R and S colonies of pneumococcus, so that even though intermediates forming rough colonies were to appear, as in the present studies, they would be missed unless special care were used for their detection.

The SSSII synthesized by the intermediate variant is immunologically indistinguishable from that formed by encapsulated Type II pneumococci as shown by absorption studies. The difference in the strains would seem to reside in the amount of SSSII produced. It is possible that the S gene of the intermediate differs from that of encapsulated Type II organisms in the same respect that alleles for a particular character may differ from each other in higher organisms. On the other hand there may be variation in a separate gene system which exercises control over the amount of SSSII formed by the intermediate as compared with encapsulated pneumococci. An additional

possibility is that the difference between the intermediate and encapsulated cells rests in the permeability of the cell wall to SSSII. An analogy to the latter hypothesis is perhaps to be found in the case of secretors and non-secretors of the blood group–specific substances in man, the capacity to secrete the group-specific substances being transmitted as a Mendelian dominant (12).

It is of considerable interest that although the intermediate variant synthesizes SSSII which is disposed on the surface of the cells, it is not more virulent than R organisms which lack SSSII. It seems likely that the difference in virulence between the intermediate and fully encapsulated pneumococcus Type II is related to the quantity of SSSII synthesized by the respective forms, the intermediate producing an amount insufficient to protect itself against phagocytosis. If this is the explanation of the difference in mouse virulence of these variants, it may be possible in certain instances to account for differences in virulence of various strains of smooth pneumococci of the same serological type on the basis of the quantity of SSS synthesized. From this point of view, enhancement of a potentially virulent strain on repeated mouse passage, for example, would involve selection of variants producing the most SSS. There seems to be little question that virulence is not related solely to the quantity of SSS produced by the various types and strains of pneumococci. The results of the present study indicate, however, that this may be one of the important factors involved.

Virtually complete loss of virulence for mice has not affected to a noticeable extent the capacity of heat-killed suspensions of the intermediate variant to stimulate the production in rabbits of type-specific antibodies reacting *in vitro* with SSSII and protecting mice against infection with the fully virulent smooth organisms. Quantitative estimations of the amount of antibody produced in rabbits have not been made, but the results of precipitin, agglutination, and mouse protection tests indicate that the quantity is comparable to that ordinarily found in the serum of rabbits immunized with similar amounts of fully encapsulated Type II pneumococci. It may be suggested that studies of attenuated strains of bacteria which have been used as immunizing agents for specific prophylaxis might reveal differences in specific cellular components comparable to the reduction in SSSII production of the intermediate variant described. In the case of the intermediate variant the amount of SSSII is insufficient to permit the strain to exhibit significant virulence, but enough is present to act as a potent antigen in rabbits. It should also be possible by techniques analogous to those used in pneumococcal type transformation to "synthesize" bacteria or even viruses having an appropriate combination of avirulence and specific antigenicity.

Failure of the intermediate variant to be transformed to a heterologous type, pneumococcus Type III, may have been due to ignorance of the appropriate conditions. On the other hand, the intermediate already produces SSSII and

if transformation to Type III were to occur, the new strain would be forced to synthesize two capsular polysaccharides at the same time. It seems unlikely that the Type III–synthesizing pattern could be imposed unless that responsible for SSSII production were suppressed or lost through mutation.

In relation to the attempted transformation to pneumococcus Type III, it is of interest that although this transformation did not occur, neither was the Type II intermediate converted into a fully encapsulated strain of pneumococcus Type II by the Type III transforming principle. It would appear, therefore, that the component responsible for *increased* SSSII production in the fully encapsulated SII forms, as compared with the intermediates, is specific for SSSII, and not present in Type III cells.

SUMMARY

1. A variant intermediate between the classical R and S forms has been isolated by selective procedures from a rough strain of pneumococcus originally derived from Type II S.

2. The intermediate variant D39/Int53 is avirulent for mice, forms rough colonies, and does not possess a demonstrable capsule. However, it synthesizes SSSII which is immunologically indistinguishable from that produced by fully encapsulated pneumococcus Type II, though in much smaller amount. The polysaccharide is present as a surface component and as it exists in the cell is highly antigenic for rabbits.

3. An extract of the intermediate variant causes the transformation *in vitro* of an R strain into a variant resembling the intermediate in SSSII production but without any apparent alteration in the colonial characteristics of the R variant.

4. The intermediate variant is convertible *in vivo* into a fully encapsulated strain of pneumococcus Type II. Transformation of the intermediate to a heterologous type of pneumococcus (Type III) was unsuccessful.

5. A method is described for the preparation of transforming extracts of pneumococci utilizing the massive growth of the organisms obtained in the presence of a large concentration of glucose.

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