THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

I. The Conversion of R Forms of Pneumococcus into S Forms of the Homologous Type*

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In previous communications (1, 2) it was shown that avirulent, non-type-specific R forms of Pneumococcus may be converted into virulent, type-specific, S organisms either "*in vivo*" by animal passage, or "*in vitro*" by growth in anti-R serum. Conversion by both methods was invariably accompanied by the acquisition of all the characteristics of the S type, including maximal virulence. In every instance in which the change was effected by these procedures the R forms were converted to the same specific type from which they were originally derived.

Since the publication of the foregoing results there has appeared a most significant article by F. Griffith (3), in which he stated that:

1) R forms of Pneumococcus may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2) R forms of Pneumococcus may be similarly converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures.

3) R forms of Pneumococcus may be transformed into S forms of heterologous types by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of heterologous S cultures.

The present communication is concerned with the first two of the

* In this and succeeding instances 'homologous type' indicates that specific S type from which the R forms were originally derived.

above findings. The third finding, which involves the question of actual transformation of type, is the subject of the succeeding paper.

A. Conversion of R Forms of Pneumococcus into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Large Amounts of Living R Organisms

Methods

Ten-hour plain broth cultures of Pneumococcus were centrifuged and the bacteria were resuspended in plain broth in varying dilutions as outlined in the following experiments. Mice were inoculated subcutaneously in the right inguinal region, with 0.5 cc. of each of the various dilutions, care being taken that none of the material escaped along the track of the needle. All mice were autopsied in the following manner: The skin was washed with alcohol and a subcutaneous incision was made along the mid-line of the abdomen. A flap of skin was then reflected and cultures were made from the site of injectioon on blood agar plates and in blood broth. The inguinal gland, or a portion of tissue from this region, was carefully excised under sterile precautions and cultures were made from this material in blood broth. Except in earlier experiments contaminations rarely occurred. Occasionally mice developed ulcers at the site of injection and such animals were discarded. Cultures were made from the heart's blood in the usual manner.

EXPERIMENTAL

(*a*) *2 R culture, strain D/39/R.*

This culture was obtained from a typical Type II S Pneumococcus by growth in homologous immune serum. Its virulence was such that 0.5 cc. of culture occasionally killed white mice, but amounts of 0.25 cc. or less uniformly failed to do so. It produced only Rough colonies, did not agglutinate specifically in type sera, and did not produce the specific soluble substance upon which type-specificity depends (4). It could be changed to the S type by either the "*in vivo*" method of animal passage, or by the "*in vitro*" method of growth in media containing anti-R serum. Three to four mouse passages usually sufficed to bring about the R→ S change, while five to seven transfers in 10 per cent anti-R serum induced a similar transformation. Single-cell cultures derived from the mass culture have been shown to react in precisely the same manner.

A series of eight mice were injected subcutaneously in the right inguinal region with the bacteria from varying amounts of culture, a volume of 0.5 cc. being injected in each instance.

TABLE I

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
	cc.				
2	10	1 d. 40 hrs. 2 " " "	R and S " " "	S "	Type II " "
2	5	1 d. 18 hrs. 2 " 40 "	R only R and S	R only S	R Type II
2	2.5	1 d. 40 hrs. 2 " 72 "	" " " " " "	" "	" " " "
2	1	1 d. 30 hrs. 2 " 48 "	" " " " " "	" "	" " " "

d; died.

P. I. place of injection.

H. B. heart's blood.

Summary: No. mice injected, 8.

Died, 8. Reversion to the homologous S type 7.

No reversions, only R organisms recovered, 1.

In seven out of eight animals injected, type-specific organisms, possessing all the attributes of the S type, were recovered from the heart's blood. Cultures from the site of injection yielded a mixture of R and S colonies. The one animal yielding only R forms died in 18 hours, apparently before there was time for the transformation to be effected.

(*b*) *3 R culture, strain M/3/R.*

This culture was obtained from a typical Type III S Pneumococcus by growth in homologous immune serum. It possessed all the characteristics of the R form and could be converted to the S type, although not so readily as the 2 R culture above described. Twenty to thirty animal passages by the intraperitoneal route were necessary to restore type-specificity, while ten to fifteen transfers in 10 per cent anti-R serum were required to effect the R→ S change.

Ten mice were injected subcutaneously as follows:—

TABLE II

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
	cc.				
2	30	1 d. 18 hrs. 2 " 48 "	R only R and S	R only S	R Type III
2	15	1 d. 48 hrs. 2 " 64 "	R and S " " "	S S	" " " "
2	10	1 d. 18 hrs. 2 " 100 "	R only R and S	R only S	R Type III
2	5	1 d. 40 hrs. 2 s.	R and S —	S —	" " —
2	2.5	1 s. 2 s.	— —	— —	— —

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

Summary: No. mice injected, 10.
Died, 7. Reversion to the homologous S type, 5.
No reversion, only R organisms recovered, 2.
Survived. 3.

While this culture reverted to the homologous S type in five out of ten animals injected, larger doses were required than with the 2 R culture described. This finding is in accord with the results obtained by the other methods of inducing reversion.

(*c*) *1 R culture, strain 1/192/R.*

This R culture had been under artificial cultivation in this laboratory for many years. All previous efforts to effect reversion to the S type had been unsuccessful. Reimann (5) passed the same strain through 105 consecutive mice without altering its characteristics and 100 transfers in 10 per cent anti-R serum likewise induced no change.

Nine mice were injected subcutaneously, each with the bacteria from 50 cc. of R culture, as follows:

TABLE III

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
	cc.				
9	50	1 d. 18 hrs.	R only	R only	R
		2 " " "	" "	" "	"
		3 " " "	" "	" "	"
		4 " " "	" "	" "	"
		5 " 21 "	" "	" "	"
		6 " " "	" "	" "	"
		7 " 60 "	" "	" "	"
		8 " 80 "	" "	" "	"
		9 s.	—	—	—

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

Summary: No mice injected, 9.
Died, 8. Reversion to the homologous type, O.
No reversion, only R organisms recovered 8.
Survived, 1.

In spite of the large amounts of organisms employed,—the bacteria from 50 cc. of culture—this strain, in all animals, failed to revert to the S type. Smaller amounts of culture were used in other experiments with uniformly negative results. This finding offers further evidence of the existence of different degrees of constancy in the R form of Pneumococcus and confirms the results previously obtained.

Conversion of Single-Cell R Cultures into S Forms of the Homologous Type

In a previous paper (2) it was reported that single-cell cultures have always reacted in the same manner as the mass cultures from which they were obtained. To substantiate this finding single-cell R strains were selected from the above mass cultures, according to the method of Avery and Leland (6), and injected subcutaneously into white mice. In all cases essentially the same results were obtained as when the

mass cultures were used. The following protocol shows the results of a typical experiment:

Single-cell strain, 2 R culture, strain D/39/R.

TABLE IV

No mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
	cc.				
2	10	1 d. 24 hrs. 2 " 40 "	R only R and S	R only S	R Type II
2	5	1 d. 22 hrs. 2 " 24 "	R only " "	R only " "	R R
2	2.5	1 d. 54 hrs. 2 s.	R and S —	S —	Type II —
2	1	1 d. 40 hrs. 2 s.	R and S —	S —	Type II —

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

Summary: No. mice injected, 8.
Died, 6. Reversion to the homologous S type, 3.
No reversion, only R organisms recovered, 3.
Survived, 2.

In this experiment three mice apparently died prematurely of an R infection. Otherwise the results were essentially the same as those recorded in Table I in which the mass culture was employed.

Attempts to Cause Further "Degradation" of an R Culture by Prolonged Growth in Homologous Immune Serum

In the course of later work it became essential to have a 2 R culture which would not revert so readily to the S type. Accordingly the above 2 R strain was grown in 50 per cent Type II serum for twelve further transfers and the resulting culture injected into mice as follows:—

TABLE V

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
	cc.				
2	10	1 d. 21 hrs. 2 " 40 "	R only R and S	R only S	R Type II
2	5	1 d. 40 hrs. 2 " " "	R and S " " "	S "	Type II " "
2	2.5	1 d. 54 hrs. 2 s.	R and S —	S —	Type II —
2	1	1 d. 70 hrs. 2 s.	R and S —	S —	Type II —

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

Summary: No. mice injected, 8.
Died, 6. Reversion to the homologous S type, 5.
No reversion, only R organisms recovered, 1.
Survived, 2.

By comparison with the results in Table IV it is seen that, in spite of twelve further transfers in 50 per cent homologous immune serum, this strain reverted to the homologous S type as readily as the original culture.

It would appear that an R culture possesses the capacity to become "stabilized" at some phase of the "degradation" process. In subsequent experiments evidence will be offered to show that a corresponding condition of partial degradation may occur in S cultures. In other words, the terms R and S, as applied to cultures of Pneumococcus, have only a relative value.

The results of the preceding experiments may be summarized as follows:—R forms of Pneumococcus can be converted into S forms of the homologous type by the subcutaneous injection in white mice, of suitable amounts of R cultures. As in other methods, conversion was

invariably accompanied by the acquisition of all the attributes of the S type, including maximal virulence. Single-cell cultures reacted in the same manner as the mass cultures from which they were derived. Attempts to cause a further "degradation" of R forms by continued growth in homologous immune serum were unsuccessful.

B. Conversion of R Forms of Pneumococci into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Small Amounts of Living R Organisms Together with the Heat-Killed Bacteria from Large Amounts of the Homologous "S" Culture

Methods

It is of paramount importance to consider in detail the methods employed in the production of the vaccines[1] and the controls adopted to eliminate the possibility of the persistence of viable forms in the suspensions of heat-killed organisms. All vaccines were made from 1500 cc. of plain broth cultures grown in 3 liter flasks. In earlier experiments little attention was paid to the phase of growth at which the culture was killed, or to the amount of autolysis which might have taken place at the time of heating. In later experiments, however, these factors were found to be of considerable importance, and ten-hour plain broth cultures were uniformly used. Moderately heavy growth was found to be essential. After ten hours growth at 37°C. the flasks of culture were subjected to a *preliminary* heating at 60° for 10 minutes. This preliminary heating inhibited further autolysis and was found to have considerable influence on the efficacy of the vaccine. The cultures were concentrated by centrifuging and taken up in 1/100th of their original volume of plain broth. This concentrated material was transferred to glass ampules, sealed in a blow flame, and heated when totally immersed in water. The vaccines were subjected to definite temperatures for varying periods, as will later be described. Since the factors of time and heat materially altered the results obtained they were most carefully regulated. Fifteen minutes was the shortest period and 60°C. the lowest temperature to which the bacterial suspensions were exposed, and invariably this minimal exposure was found sufficient to kill all pneumococci. In many experiments, however, the vaccines were heated at much higher temperatures and for longer periods of time.

Mice were injected subcutaneously in the right inguinal region, the total volume introduced never exceeding 0.75 cc. Under these conditions the development of ulcers at the site of injection did not occur except in occasional instances. The

[1] For the sake of convenience the term vaccine is used to denote a suspension of heat-killed organisms.

animals were autopsied as described in the first part of this paper. Contaminations were rarely encountered. Cultures were invariably made, both from the site of injection and the heart's blood, on blood agar plates and in blood broth. The colonies were examined under a Zeiss "plate culture" microscope; but morphology alone was never considered a final criterion as to the nature of the organisms constituting the colony. Agglutination tests were done on all cultures. In cases of doubt a second mouse was inoculated and the organisms from the peritoneal contents were typed in the usual manner.

Controls on the Viability of the Vaccines:

The possibility of potentially viable organisms surviving in the concentrated vaccines demanded that more than ordinary control measures should be adopted to eliminate such a contingency. The "*in vitro*" and "*in vivo*" controls employed were as follows:

1. In Vitro Controls:

(a) Cultures were made from all vaccines in blood broth and on blood agar plates. In many experiments this was done in varying dilutions. In no instance was growth obtained.

(b) Many lots of S vaccine were used repeatedly in "*in vitro*" attempts to secure the R→ S change. Broth containing concentrated S vaccine was seeded with R forms and subcultured serially for twenty transfers. No growth of "S" organisms occurred and the final culture remained avirulent for mice.

(c) In one critical experiment cultures were made, both aerobically and anaerobically, in 5 per cent blood broth and blood-extract dextrose broth. The cultures were incubated two weeks, plates poured, and the material injected into mice. No growth occurred and the mice survived.

2. "In Vivo" Controls:

(a) Control mice were injected with the vaccine alone. At least four mice were always used and in many experiments the number of control animals was equal to the number of experimental animals. Varying amounts of vaccine up to and including the bacteria from 100 cc. of culture were injected. Both the subcutaneous and intraperitoneal routes were used. Without exception all animals survived. They were sacrificed at intervals up to three weeks and autopsied. The inguinal lymph gland, or a portion of subcutaneous tissue at the site of injection, was dissected out and cultures were made from this material in blood broth and on blood agar plates. In some cases this tissue was ground up and injected into other mice. Cultures were also invariably made from the heart's blood. In no instance were living pneumococci recovered.

(b) Control mice were injected with the vaccine together with other live organisms. The possibility of the existence in the vaccines of potentially viable organisms, which could not multiply by themselves, but which might, in some way, be stimulated to renewed growth by other live organisms, received careful consideration. Mice were injected with vaccines together with living cultures of Staphylococcus, Streptococcus, B. Influenzae, and Friedländer's bacillus. All

animals which succumbed were autopsied and all surviving animals were sacrificed at appropriate intervals and careful cultures were made. In no instance was a viable pneumococcus recovered.

As Griffith pointed out (3), in certain instances, the temperature at which the vaccines were heated exerted a definite influence on the effect which they produced. The following experiments are therefore divided into two groups, 1) those in which the vaccines were heated at 60°C, and 2) those in which the vaccines were heated at 100°C. In both groups the time of heating was fifteen minutes.

I. R Cultures Together with Homologous S Vaccines, Heated for 15′ at 60°C.

(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15′ at 60°C.

TABLE VI

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
				P.I.	H.B.	
	cc.	*cc.*				
4 Controls	90	Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R only	R only	R
			2 " " "	R and S	S	Type I
			3 " 5 "	S	S	" "
			4 " 7 "	S	S	" "
			5 s. k. 7 "	R only	—	—
			6 s. k. 7 "	R only	—	—

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

Summary: No. test mice injected, 6.
Died, 4. Reversion to the homologous S type, 3.
No reversion, only R organisms recovered, 1.
Survived, 2. R organisms recovered when sacrificed, 2.

(*b*) *2 R Culture (Strain D/39/R) + II S Vaccine, Heated for 15′ at 60°C.*

TABLE VII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
	cc.	*cc.*				
4 Controls	90	Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R and S	S	Type II
			2 " " "	" " "	S	" "
			3 " 2 "	" " "	S	" "
			4 " " "	" " "	S	" "
			5 " " "	" " "	S	" "
			6 s. ulcer at P.I.	—	—	—

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

Summary: No. test mice injected, 6.
Died, 5. Reversion to the homologous S type, 5.
Survived, 1. (Ulcer).

(c) 3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15′ at 60°C.

TABLE VIII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
	cc.	*cc.*				
4 Controls	90	Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 2 days	R and S	S	Type III
			2 " " "	" " "	"	" "
			3 " 2½ "	" " "	"	" "
			4 " 3 "	" " "	"	" "
			5 " 4 "	" " "	"	" "
			6 s. k. 11 "	R only	—	—

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

Summary: No. test mice injected, 6.
Died, 5. Reversion to the homologous S type, 5.
Survived, 1. R organisms recovered when sacrificed, 1.

The results of the three preceding experiments may be summarized as follows; R forms of Pneumococcus, when injected subcutaneously in white mice, together with S vaccines of the homologous type, were converted, in the majority of the animals, to the specific S type from which they were originally derived. In these experiments the vaccines were heated for 15′ at 60°C. All control mice survived. At the end of seven days the controls were sacrificed and cultures were made from both the site of injection and the heart's blood. Without exception all cultures were sterile. The 1 R culture (Strain 1/192/R) which had remained totally avirulent after all previous efforts to effect the R→S change, reverted to the S type in three out of six animals injected.

II. R Cultures Together with Homologous S Vaccines, Heated for 15' at 100°C.

(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15' at 100°C.

TABLE IX

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
				P.I.	H.B.	
	cc.	*cc.*				
4 Controls	90	Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R only	R only	R
			2 " 2 "	R only	R only	R
			3 s.	—	—	—
			4 s.	—	—	—
			5 s. k. 9 days	R only	—	—
			6 s.	—	—	—

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

Summary: No. test mice injected, 6.
Died, 2. Reversion to the homologous S type, 0.
No reversion, only R organisms recovered, 2.
Survived, 4. R organisms recovered when sacrificed, 1.

Since reversion to the S type failed to occur in any of the mice which received Type I S vaccine heated at 100°, while the change was frequently effected in those which received the vaccine heated at 60°C., this experiment was repeated. Entirely similar results were obtained. Apparently, as reported in Griffith's paper, Type I vaccine, heated at 100°C. fails to produce the change brought about by the vaccine heated at 60°C.

Attention is drawn to the fact, that, in some mice, living R forms

were found in the subcutaneous tissues nine days after injection. In other experiments they have been recovered as late as twenty days after inoculation. The ability of R forms to survive in the tissues, then, is not the only condition necessary to bring about conversion to the S type.

(*b*) *2 R Culture (Strain D/39/R) + II S Vaccine, Heated for 15' at 100°C.*

TABLE X

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
	cc.	*cc.*				
4 Controls	90	Nil.	All survived. Sacrificed 9 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R and S	S	Type II
			2 " " "	" " "	"	" "
			3 " 2 "	" " "	"	" "
			4 " " "	" " "	"	" "
			5 " " "	" " "	"	" "
			6 " " "	" " "	"	" "

d; died.
P. I. place of injection.
H. B. heart's blood.

Summary: No. test mice injected, 6.
Died, 6. Reversion to the homologous S type, 6.

(c) 3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15' at 100°C.

TABLE XI

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
	cc.	*cc.*				
4 Controls	90	Nil.	All survived. Sacrificed 9 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R and S	S	Type III
			2 " 2 "	" " "	S	" "
			3 " " "	" " "	S	" "
			4 " " "	" " "	S	" "
			5 s. k. 13 "	R only	—	—
			6 s. k. " "	—	—	—

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

Summary: No. test mice injected, 6.
Died, 4. Reversion to the homologous S type, 4.
Survived, 2. R organisms recovered when sacrificed, 1.

The preceding experiments show that vaccines prepared from Types II S and III S organisms are equally effective in producing reversion whether heated for 15 minutes at 60°C. or for 15 minutes at 100°C. Type I S vaccine, on the other hand, while effective in causing reversion of 1 R forms to the homologous S type when heated for 15 minutes at 60°C. did not possess this property when heated for 15 minutes at 100°C.

Two possible explanations may be advanced for the apparent differences in effect produced by Type I S vaccine heated at 60° and at 100°C. First, that property of the vaccine responsible for reversion might have been destroyed, in the case of Type I vaccine, by heating at 100°C. but not in the case of Type II and Type III vaccines similarly

treated. Second, the failure of Type I vaccine to effect reversion when heated at 100°C. might not have been due to the destruction of any property of the vaccine itself, but rather to the difficulty which had always been encountered in effecting the R→S change with this particular 1 R strain. It has been repeatedly shown that the 2 R and 3 R strains employed in these experiments can be much more readily converted to the S type. However, in view of the entirely similar results recorded by Griffith, and because of the findings to be reported in the subsequent paper, the former explanation is much the more probable.

Attempts to Effect the R→S Change by the Injection of Living R Organisms Together with the Heat-Killed Bacteria from Large Amounts of R Cultures

It was thought that the effect of the vaccines in producing reversion might be due to one of two causes:—First, the injection of such large amounts of heat-killed culture might overwhelm the general resistance of the animal and so allow the R forms to grow in an environment suitable for the development of S types. Second, the vaccines might act locally to protect the R forms from phagocytosis, and so enable them to survive and produce their own S substance. In either case it was thought that the vaccine of an R culture, if injected in sufficiently large amounts together with living R organisms, would similarly allow reversion to take place.

2 R Culture + 2 R Vaccine of the Same Strain Heated for 15' at 60°C. (Strain D/39/R).

TABLE XII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. cultures
				P.I.	H.B.	
	cc.	*cc.*				
2	200	0.25	1 d. 2 days	R only	R only	R
			2 s. k. 11 "	—	—	—
4	150	0.25	1 d. ½ days	R only	R only	R
			2 " 1 "	" "	R only (2 colonies)	R
			3 " 2½ "	" "	R only (few)	R
			4 s. k. 11 "	—	—	—
4	100	0.25	1 d. 1 day	R only	R only	R
			2 " 1½ "	" "	" " (8 colonies)	R
			3 " " "	" "	R only (6 colonies)	R
			4 " " "	" "	R only	R

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

3 R Culture + 3 R Vaccine of the Same Strain Heated for 15' at 60°C. (Strain M/3/R).

TABLE XIII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. cultures
				P.I.	H.B.	
	cc.	*cc.*				
2	200	0.25	1 d. 1 day 2 s. k. 11 "	R only —	R only —	R —
4	150	0.25	1, 2, 3, 4 } s. k. 11 days	—	—	—
4	100	0.25	1 d. ½ " 2 " 1 " 3, 4 } s. k. 11 "	R only " " —	R only R only (6 colonies) —	R R —

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

Summary: (Tables XII and XIII).
No. test mice injected, 20.
Died, 11. Reversion to the homologous S type, 0.
No reversion, only R organisms recovered, 11.
Survived, 9. R organisms recovered when sacrificed, 0.

R vaccines, even when heated for so short a period as 15 minutes at 60°, and in huge doses, representing the bacteria from 200 cc., 150 cc., and 100 cc. of broth culture, completely failed to cause the R cultures to revert to their own type. Similar negative results were obtained when vaccines of S Friedländer bacilli were inoculated together with the R forms of Pneumococcus. Francis (7), in this laboratory, has made similar observations while working with rabbits. He found that S vaccines were effective in causing R forms to revert to the S type;

while R vaccines and vaccines of Staphylococcus failed to produce the change.

"In Vitro" Attempts to Effect the R→S Change by Growth of R Forms with S Cultures and with S Vaccines

(1) In previous work experiments had been done to observe the effect of growing R and S cultures in symbiosis. R and S forms of the same strain were grown together for serial transfers in varying dilutions, as follows:—

	cc.						
Dilution of S Culture	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Dilution of R Culture	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}

0.1 cc. quantities of each of the above R and S dilutions were seeded together in blood broth and daily plates and subcultures made from the mixtures. It was obvious that in such experiments considerable variation in results would be obtained, but generally speaking, the cultures maintained the same relative proportions of R and S colonies for several transfers. Apparently the mere presence and growth of S cells in intimate association with R forms was not sufficient to cause the latter to revert to the S variety.

(2) R organisms were cultured in blood broth to which was added the bacteria from 100 cc. of homologous S culture, heated for 15 minutes at 60°C. Transfers were continued for fifteen subcultures without the appearance of S colonies. At the end of this time the virulence of the R culture was determined by mouse inoculation and found to be unchanged. Such experiments suggest that the R form is not able, under ordinary "*in vitro*" conditions, to utilize an S vaccine, as such, to build up its own polysaccharide.

(3) Moreover, under similar conditions of cultivation, when 10 per cent anti-R serum was added to the media, the change failed to occur; whereas, in the absence of the vaccine, reversion regularly took place after the appropriate number of transfers. Such a result can be explained by assuming that the vaccine, which contains, in addition to its type-specific antigen, the common group-specific protein (or R) antigen (8), absorbed the anti-R antibodies from the immune serum. In the absence of the anti-R antibodies the change failed to occur. This experiment, then, offers further proof of the rôle played by anti-R antibodies in effecting R→ S reversion "*in vitro*."

(4) The possibility that the vaccine might be effective in producing reversion only in the presence of tissues under anaerobic conditions received some consideration. R forms were grown, under vaseline seal, in blood broth to which was added lymph-tissue, muscle tissue and ground up spleen, in addition to large amounts of vaccine. Subcultures were made daily for six days. No S colonies appeared at any time during the process and the cultures remained avirulent.

Thus, under the conditions employed, all attempts to produce the R→S change *in vitro* by the use of vaccines have uniformly failed.

The possibility that the vaccines became "digested" in the subcutaneous tissues of the mice, and that the R forms were able to utilize the "digestion" products to build up their own S substance also received attention. Attempts to reproduce such "digestion" "*in vitro*" will be considered in the subsequent paper.

DISCUSSION

The conversion of relatively avirulent pneumococci into highly virulent organisms is obviously a matter of considerable biological and epidemiological significance. Recent observations made in this laboratory suggest that R Pneumococci are not infrequently found in the flora of the upper respiratory tract of normal individuals. Such observations indicate that this form of the organism appears not only under artificial conditions of cultivation in the test-tube but may be considered as an evidence of biological adaptation to environment on the part of the bacteria. Moreover, the avirulent R form is potentially capable of again developing into the virulent S type under favorable circumstances. The factors determining such development in the human are difficult of analysis but under experimental conditions certain observations can be made in animals.

Attention is drawn first to the "in vitro" method of producing the R→S change,—the growth of R organisms in anti-R serum. In this connection the existence of anti-R antibodies in the sera of normal individuals, as shown in a previous paper (2), is believed to be a point of considerable significance, and may play a rôle in the R→S reversion process in the human being.

Griffith's observations on the conversion "in vivo" of avirulent R pneumococci into virulent, type-specific, S, organisms have been completely confirmed. In attempting an analysis of the causes responsible for reversion by the technique adopted by him certain points must be considered. He suggests that the mass of culture forms a nidus in which the attenuated pneumococci are protected from the bactericidal action of the tissues. But, as he himself indicates, this can play only a small part in the reversion process. Large amounts of R vaccine, amounts larger than those of the S vaccines

employed, as well as vaccines of other organisms, should also suffice to protect the R forms from phagocytosis and from the bactericidal action of the tissues. Nevertheless, reversion has never been effected under those conditions. Moreover, the finding of living R organisms in the subcutaneous tissues as late as twenty days after injection proves that opportunity to survive is not the only condition necessary to bring about the R→S change in the animal body. Other factors must play a rôle in the reversion process. Is it possible that the S vaccine, disintegrating in the animal tissues, supplies a suitable pabulum from which the living R organisms are able to resynthesize their own specific soluble substance?

A point of considerable difficulty in such a hypothesis is the explanation of the differences in results obtained by the use of Type I S vaccine heated at 60° and at 100°C. It is necessary to assume that Type I S vaccine, when heated at the higher temperature, becomes altered in such a way that it, or its disintegration products, can no longer be utilized by the R forms. But it has been shown (4) that heating at 100° in no way alters the specific carbohydrate fraction of the Type I Pneumococcus. This hypothesis, therefore, is inadequate unless it can be shown that the portion of the vaccine effective in causing reversion in some fraction other than the specific soluble substance as such.

The fact that large amounts of living R cultures by themselves produce the same effect as small amounts of live R cultures when the latter are injected together with S vaccines, suggests that the causes responsible for reversion under these two conditions must be closely related. In this connection it is of importance to point out that all the R strains used in these experiments yielded traces of the specific soluble substance of their original S type. This has been shown in this laboratory by Julianelle (9) in the case of the 1 R strain which offered the greatest resistance to reversion. It is reasonable, then, to assume that large amounts of living R cultures may yield a sufficient amount of S substance, (or the closely related substance necessary for reversion) from which the R forms may resynthesize their own specific polysaccharide. And apparently, once the process is initiated, it is carried on indefinitely, so long as the environment is suitable. However, on this basis, it remains difficult to explain why large amounts

of R vaccines fail to provide the necessary material; while relatively small amounts of living R cultures are able to supply this factor. It may be that the necessary substance is not present in sufficient quantities in the R vaccines; while it is elaborated in an adequate amount by the growth of the living R forms.

The failure of "*in vitro*" attempts to effect reversion by the aid of vaccines deserves some consideration. It was not found possible to induce the R→S change by growing R forms in symbiosis with S organisms, or in media containing large amounts of S vaccines. Likewise, attempts to produce the change by growing R Pneumococci with S vaccines under partial anaerobiosis in the presence of animal tissues were unsuccessful. The failure to effect reversion by these procedures suggests that either the conditions, as provided, were not adequate, or that some factor must be provided by the animal body.

It has been suggested that the S vaccine may possess some "activating" or "co-ferment" property, which, working in conjunction with the synthesizing enzymes of the R form, enables the latter to build up its own S structure. If the effect of the S vaccine is due to such a property it apparently can exercise this function only in the presence of living tissues. Moreover, one is confronted with the difficulty of explaining how such a property becomes inactivated, in the case of Type I S vaccine, by heating at 100°C., and not in the cases of Type II S and Type III S vaccines similarly treated.

Another possibility is that the conditions created in the mouse by the injection of S vaccines may be the determining factors in inducing the R→S change. It has been pointed out previously that all the R cultures used in these experiments yielded traces of specific soluble substance of their original S type. Nevertheless the animals were able to withstand the injection of comparatively large amounts of living R organisms by themselves. The mouse must therefore possess some capacity to overcome infection by organisms producing minimal amounts of S substance. It is possible that the injection of S vaccines may destroy or inhibit this limited ability of the mouse. Under such conditions the R forms may elaborate S substance in greater quantities, and as a consequence develop into S organisms.

In this connection attention is directed to the work of Sia (10). Employing serum-leucocyte mixtures in a specially constructed apparatus,

he reported the following observation. "The presence of a small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixtures so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which ordinarily possess the power to destroy such pneumococci in relatively large numbers."

However, in any of the explanations considered, it is impossible to account for the different effect produced by Type I vaccine heated at 60°C and at 100°C. The exact causes responsible for reversion, under these experimental conditions, therefore remain unexplained. Whatever they may be, the fact remains that when R cultures are injected in large amounts by themselves, or in small amounts together with the heat-killed vaccines of S organisms, the characteristics of the R organisms are actually altered. Comparable phenomena may play a rôle of great importance in many infectious processes. A focus of infection may be a point at which relatively harmless organisms assume virulent characteristics; for the subcutaneous injection, in white mice, of large amounts of avirulent pneumococci produces conditions quite analogous to those existing in a focus of infection.

SUMMARY

R forms of Pneumococcus may be converted into S forms of the homologous Type. In addition to the methods previously reported,—(1) animal passage and (2) growth in anti-R sera,—conversion may be effected by the following procedures as employed by Griffith; (1) The subcutaneous injection, in white mice, of large amounts of living R organisms. (2) The subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures. There are "varying degrees of constancy of the R variant"; but by these means it has been possible to effect conversion of all R forms selected. Attempts to cause a further "degradation" of R organisms by continued growth in homologous immune serum have been unsuccessful.

Type II S and III S vaccines are equally effective in producing conversion when heated for 15′ at 60°C., or for 15′ at 100°C. Type I S vaccine, however, while effective in causing conversion when heated for 15′ at 60°C., apparently loses this property when heated for 15′ at 100°C.

R vaccines, and vaccines of other organisms, when injected together with live R cultures, have always failed to produce conversion.

The causes responsible for conversion under these experimental conditions are discussed and the possibility of the occurrence of a similar process under natural conditions in human beings is indicated.

CONCLUSIONS

1. R forms of Pneumococcus may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2. R forms of Pneumococcus may similarly be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms, together with the heat-killed bacteria from large amounts of S cultures.

3. By these methods Types II S and III S vaccines are equally effective in producing conversion when heated for 15′ at 60°C., or for 15′ at 100°C. Type I S vaccine is effective in producing conversion when heated for 15′ at 60°C., but not when heated for 15′ at 100°C.

4. R vaccines and the vaccines of other organisms are not effective in producing conversion.

5. All "*in vitro*" attempts to produce conversion by the use of vaccines have been unsuccessful.

6. The rôle which the phenomenon of conversion may play in infectious processes is indicated.

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