IMMUNOLOGICAL RELATIONSHIPS OF ENCAPSULATED AND CAPSULE-FREE STRAINS OF ENCAPSULATUS PNEUMONIÆ (FRIEDLÄNDER'S BACILLUS).

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In a preceding paper (1) evidence was presented that bacilli of the Friedländer group are separable into sharply defined and specific types. Of 30 strains employed in the study, three specific types and a heterogeneous group were demonstrated by agglutination, agglutinin adsorption, protection, thread and precipitin reactions. These types have been designated Type A, Type B, and Type C, while the remaining unclassified strains were placed in a tentative group, Group X. In the light of the present studies, the difficulties encountered in previous attempts to interpret the immunological reactions of Friedländer's bacillus appear to be due in large measure to the failure to distinguish in the cell the type-specific from the species-specific antigen; and the failure to recognize in immune serum the two distinct antibodies provoked by the respective antigens. The application of this concept to the Friedländer group was suggested by studies carried out in this laboratory on the immunological relationships of the cell constituents of Pneumococcus (2–7). Briefly, this concept involves two separable and distinct antigens—the one a soluble specific substance (now identified as a carbohydrate) which endows the organism with type specificity; the other, a protein substance which exhibits only the common and undifferentiated characters of the species.

Since the relation of the capsular material of Friedländer's bacillus to type specificity has been demonstrated (1, 8, 9), it seemed of importance to determine the immunological relationships of encapsulated and capsule-free strains of this organism. For this purpose, S and R varieties of the bacterial cell were obtained as will be de-

scribed later. It was observed that while S cells were encapsulated, the R forms no longer possessed capsules.

The nomenclature of S and R was first used by Arkwright (10) to describe biological variations in single strains of bacteria. His observations included the occurrence of two forms of colonies in old cultures of bacilli of the intestinal group. The S organisms grew in colonies with a smooth surface while the R strains presented colonies with a rough and irregular surface. The terms S and R have been universally adopted to describe similar changes in other species of bacteria. Observations on encapsulated organisms, however, fall mainly within the Pneumococcus group. With these organisms, it has been shown that accompanying the change from S to R, there is a loss of agglutinability by specific sera, loss of capsule, and attenuation of virulence (10–19).

Cultures of Friedländer's bacillus, also, have been separated by earlier workers (20–24) into S and R components, although such a designation was not in use at the time. The method of decapsulation as devised by Porges (25) must also be considered of the same order. More recently, Friel (13) and Hadley (26) have reported the occurrence of the two varieties in individual cultures of Friedländer's bacillus and have attributed to them the accepted characteristics of S and R forms.

In this study, the criteria for the conversion of S organisms to R forms have been: (1) loss of capsule and mucoid characteristics, (2) loss of agglutinability in type-specific sera, (3) attenuation of virulence, and (4) the development of colonies which present under the microscope a rough and irregular surface. In the case of R cells derived from Type C, loss of virulence was not an accompanying change since none of the Type C strains were virulent for mice.

The present report deals with the immunological reactions of encapsulated and capsule-free strains of Friedländer's bacilli and anti-S and anti-R sera, and the serological behavior of encapsulated bacilli after removal of capsules by the chemical method of Porges.

Methods.

Loss of Capsule Formation Induced by Growth in Vitro.—The non-encapsulated or "R" strains employed in this study were derived from pure S strains. An S strain representative of each of the three fixed types and one strain chosen from Group X were transplanted daily in broth to which had been added 10 per cent of homologous immune serum. Plates, streaked at the time of each transplant, were examined microscopically after 18–24 hours incubation, as recommended by Reimann (18). Within 6–10 transplants, plate cultures were obtained in which a

number of R colonies were observed. Single, typical R colonies were then transplanted into plain broth and the resultant R culture showed loss of capsule and attenuation of virulence. In each instance, the R culture failed to kill white mice, in doses of 0.5 cc., while the parent S strain from which the R had been derived regularly killed within 48 hours at a dilution of one ten-millionth cc.

Destruction of Capsule by Chemical Means.—The method devised by Porges was used. S strains were grown on agar slants and the organisms were washed off and suspended in salt solution. The suspensions were made acid with one-fourth volume N/4 HCl and heated at 80°C. for 15 minutes. The suspensions were then cooled immediately under tap water and neutralized with an equivalent quantity of N/4 NaOH. The exposure to heat was varied from 60°C. to 100°C. and from 10 minutes to 30 minutes without appreciable differences. No spontaneous clumping of the treated organisms was experienced when freshly prepared suspensions were used. After standing in the ice chest several days, however, preparations from two different strains became granular.

The method of immunization and the reaction of agglutination, precipitin and protection tests were conducted in the manner described in the preceding paper (1).

EXPERIMENTAL.

I. Immunological Reactions of "S" (Encapsulated) Strains in Immune Sera.

(*a*) *Anti-S Sera.*—It has already been shown in the preceding paper (1) that antisera prepared by immunization with encapsulated strains possess type-specific immune bodies, which agglutinate the encapsulated organisms and precipitate the soluble specific substance derived from them. In addition, such sera confer specific protection upon white mice against infection with strains of homologous types.

(*b*) *Anti-R Sera.*—Since the anti-R sera were prepared against strains of organisms which were capsule-free and which had lost the function of elaborating the type-specific soluble substance, it was to be anticipated that these sera would be lacking in type-specific antibodies. Definite proof of this, however, was obtained by determining the agglutination of S strains in anti-R sera. For purposes of comparison, the specific reactions of these same strains in anti-S sera of the homologous type were included. The results presented in Table I show conclusively that encapsulated strains are not agglutinated by sera prepared by immunization with the capsule-free (R) variants. This is true even though the organism employed for

immunization was derived from the S strain later used for agglutination.

TABLE I.

Agglutination of Friedländer S Strains by Anti-R Sera.

Antigen encapsulated strain	Anti-R sera									Anti-S sera			
	Type A			Type B			Group X			A	B	C	X
	*1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:5	1:5	1:5	1:5
Type A	−	−	−	−	−	−	−	−	−	++++	−	−	−
" B	−	−	−	−	−	−	−	−	−	−	++++	−	−
" C	−	−	−	−	−	−	−	−	−	−	−	++++	−
Group X	−	−	−	−	−	−	−	−	−	−	−	−	++++

* The figures in this row represent ultimate dilution of serum.

++++ indicates compact disk agglutination with clear supernatant; −, no reaction.

TABLE II.

Precipitation of the Soluble Specific Substance of Friedländer's Bacillus by Anti-R Sera.

Antiserum	Soluble specific substance of Friedländer's bacillus											
	Type A						Type B					
	*2	20	50	100	250	500	2	20	50	100	200	500
Type A (R)	−	−	−	−	−	−	−	−	−	−	−	−
" B "	−	−	−	−	−	−	−	−	−	−	−	−
Group X "	−	−	−	−	−	−	−	−	−	−	−	−
Type A (S)	−	+	++	++	+	−	−	−	−	−	−	−
" B "	−	−	−	−	−	−	−	++	+++	++++	+++	++
Normal	−	−	−	−	−	−	−	−	−	−	−	−

* The dilutions are expressed in thousands.

++++ indicates heavy, compact, disk precipitate; +++, marked disk precipitate; ++, thin, film-like scale; +, ground glass turbidity.

Further evidence of the lack of type-specific antibodies in anti-R sera was sought by the precipitin reaction. Solutions of the isolated soluble specific substances of Types A and B were tested against three anti-R sera. As demonstrated in Table II, these sera contained no pre-

cipitins for the type-specific substances just as in the preceding experiment they were shown to possess no agglutinins for the encapsulated cells from which these substances were derived.

It has been shown in the previous communication that anti-S sera contain antibodies which afford specific protection in mice against

TABLE III.

Protection Offered by Anti-R Sera against Infection by Type A, Friedländer's Bacillus.

Type A encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
	cc.		*cc.*		
.001	.2	D. 16 hrs.	.2	D. 20 hrs.	
.0001	.2	" 19 "	.2	" 20 "	
.00001	.2	" 39 "	.2	" 22 "	D. 24 hrs.
.000001	.2	S.	.2	" 65 "	" 39 "
.0000001					" 65 "

S. indicates survival; D., death, the numerals representing the number of hours before death occurred.

TABLE IV.

Protection Offered by Anti-R Sera against Infection by Type B, Friedländer's Bacillus.

Type B encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
	cc.		*cc.*		
.001	.2	D. 15 hrs.	.2	D. 15 hrs.	
.0001	.2	" 15 "	.2	" 15 "	
.00001	.2	" 39 "	.2	" 24 "	D. 16 hrs.
.000001	.2	" 22 "	.2	" 65 "	" 39 "
.0000001					" 39 "

infection with virulent encapsulated bacilli of homologous types. Accordingly, an analysis of anti-R sera was made to determine the presence of protective properties. The results of these determinations are given in Tables III and IV. An anti-R serum prepared by immunization with a degraded Type A organism affords no measura-

ble protection against infection with an encapsulated (S) strain of Type A or Type B. Similarly, an anti-R serum prepared by immunization with a capsule-free organism derived from Type B offers no protection against infection with virulent strains of either of these specific types.

In summary, then, the foregoing experiments show that antisera prepared by the immunization of rabbits with degraded, capsule-free strains of Friedländer's bacillus (R forms) are devoid of specific agglutinins, precipitins, and protective antibodies for the virulent, type-specific, encapsulated bacilli. Anti-R sera, therefore, exhibit none of the type-specific reactions which characterize anti-S sera.

II. Immunological Reactions of "R" (Non-Encapsulated) Strains in Immune Sera.

(*a*) *Anti-S Sera.*—The anti-S sera used for the determination of specific types (1) were prepared in such manner as to avoid or at least minimize the concurrent presence of the common specific antibody. As was pointed out in the preceding paper, this may be accomplished most successfully by using young cultures of encapsulated organisms and by avoiding prolonged immunization. It was considered of interest to determine the agglutinative action of the type-specific sera against R strains of different origin. The results of these tests are given in Table V. It is seen that an immune serum prepared against an encapsulated strain of Type A contains only a small amount of the species antibody. In addition, this antibody is shown to be equally operative against four R strains, each of which in turn had been derived from an S organism of a serologically different type. The three anti-S sera of Type B, Type C, and Group X contained only minute and, for practical purposes, negligible traces of the R or common species antibody. Thus, further confirmation is advanced for the concept that the R organisms, devoid of capsules, are no longer type-specific.

(*b*) *Anti-R Sera.*—Evidence has been presented that capsule-free strains of Friedländer's bacillus are lacking in the ability to engender type-specific antibodies. However, they stimulate the formation of antibodies which react not only with the particular strain used for immunization, but with all other R forms regardless of the type from

which they were derived. This fact is revealed in the cross-reactions of agglutination presented in Table VI in which all R strains are shown to be reciprocally agglutinated by each of the different anti-R sera.

The agglutination of the R strains in anti-R serum is characteristically different from the agglutination of S strains in anti-S serum of the homologous type. It will be recalled that with S strains, the reaction results in the formation of a compact, firm disk of agglutinated bacteria and occurs almost immediately in the more concentrated dilutions of the serum. The R organism, on the other hand, agglutinates very slowly, and in the higher concentrations of serum forms a fluffy precipitate; while in greater dilutions of the serum a fine, granular agglutination occurs which is difficult to read without a

TABLE V.

Agglutination of Friedländer R Strains by Anti-S Sera.

Antigen capsule-free strain	Anti-S sera											
	Type A			Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A	++	+	+	±	−	−	−	−	−	−	−	−
" B	++	+	+	±	−	−	−	−	−	−	−	−
" C	+	+	+	±	−	−	+	−	−	+	−	−
Group X	++	+	+	±	−	−	+	−	−	+	−	−

lens. The agglutinins in anti-R sera, which react with capsule-free strains, bear a marked similarity to the "fine flaking" agglutinins for the H form of *proteus* and typhoid bacilli described by Felix (27) and to the somatic agglutinins for the non-motile forms of hog-cholera bacillus reported by Orcutt (28). Furthermore, the agglutinin titre of anti-S sera is low (1:40–1:80) while the agglutinin titre of anti-R sera is high (1:2500).

Corroborative evidence of the serological identity of R strains was gained by the agglutinin adsorption test. Each anti-R serum was adsorbed with heat-killed suspensions of each R strain until all the agglutinins were removed for the adsorbing organisms. The adsorbed sera were then tested for the presence of agglutinins for other R strains. Repeated tests yielded constant results. To avoid un-

TABLE VI.

Cross-Agglutinations of R Strains of Friedländer's Bacillus by Anti-R Sera.

Anti-R sera	Antigen derived from	Final dilution of serum										Normal serum 1:5
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Type A	Type A	++++	++++	++++	++++	++++	++++	++++	++++	+++	+	−
	" B	++++	++++	++++	+++	++	+	+	±	−	−	−
	" C	++++	++++	++++	++++	+++	+++	++	+	−	−	−
	Group X	++++	++++	++++	+++	++	++	+	+	−	−	−
" B	Type A	++++	++++	++++	++++	++++	+++	++	+	+	−	−
	" B	++++	++++	++++	++++	++++	+++	+++	++	++	+	−
	" C	++++	++++	++++	++++	+++	+++	++	++	+	−	−
	Group X	++++	++++	++++	++++	++++	+++	+++	++	++	+	−
Group X	Type A	++++	++++	++++	++++	++++	+++	++	++	+	−	−
	" B	++++	++++	++++	++++	+++	++	++	+	−	−	−
	" C	++++	++++	++++	++++	+++	+++	++	++	+	−	−
	Group X	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	−

++++ indicates complete agglutination with flocculent sediment and clear supernatant; +++, almost complete, supernatant clouded; ++, marked agglutination; +, slight agglutination; −, no agglutination.

necessary repetition, a single typical protocol is given in Table VII. It is evident that the adsorption of an anti-R serum with any R strain removes from the serum agglutinins for the homologous organism as well as for heterologous strains. It is apparent, therefore, that immunologically all R cells are identical, as tested by the reactions of agglutination and agglutinin adsorption.

TABLE VII.

Agglutinin Adsorption.

Results of Agglutination with Anti-R Serum (Type B) after Adsorption by R Strains Derived from Homologous and Heterologous Types of Encapsulated Organisms.

Antigen capsule-free strain from	Anti-R serum (Type B) after adsorption with R strains derived from															
	Type A				Type B				Type C				Group X			
	*20	50	100	500	20	50	100	500	20	50	100	500	20	50	100	500
Type A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Group X	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* Figures represent ultimate dilution of serum.

III. Immunological Reactions of "S" (Encapsulated) Strains after Decapsulation by Porges' Method.

Appreciating the difficulties in the agglutination of Friedländer's bacillus, Porges devised a method of removing the capsule by weak acid in order to render strains more antigenic and better agglutinable. To test the effect of decapsulation on the serological behavior, suspensions of encapsulated bacilli representative of each of the specific types were subjected to the Porges technique. The cells were then tested for agglutinability in sera prepared by immunization with encapsulated and capsule-free organisms, respectively. For purposes of comparison, a strain of *Encapsulatus granulomatis* was included in the experiment.

(*a*) *Anti-S Sera.*—The results obtained with anti-S sera are summarized in Table VIII. It is evident that encapsulated organisms

which are heated in the presence of acid are not agglutinated appreciably in purely anti-S sera of types which are serologically different from that yielding the decapsulated cells. Agglutination in such sera depends upon the presence of species antibody. In antisera of the parent strains, however, there is a definite precipitin reaction which is referable to the presence of unhydrolyzed soluble specific substance. In this connection it will be recalled (1, 9) that the capsular material of Friedländer's bacillus is precipitated by anti-S serum of the homologous type. Since the method of Porges strips the bacillus of the capsule with apparently only partial hydrolysis, it is not surprising that precipitation of the soluble specific substance is observed.

TABLE VIII.

Agglutination by Anti-S Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Antigen derived from	Anti-S serum								
	Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A.....	+	−	−	+	+	−	+	+	−
" B.....	*+++	++	+	+	+	−	+	+	−
" C.....	+	+	−	*+++	++	+	++	+	−
Group X.....	+	+	−	+	+	−	*+++	++	+
Gran.....	+	+	−	+	±	−	++	+	−

* Typical "S" reaction.

(*b*) *Anti-R Sera.*—In anti-R sera, however, chemically decapsulated suspensions show a marked agglutination to a high dilution of serum and the reaction gives the characteristic appearance of R agglutination. The suspensions react equally well in all anti-R sera regardless of their type derivation. (Table IX.)

It is interesting to note that a closely related organism, *Encapsulatus granulomatis*, when subjected to the same conditions, is also agglutinated by anti-R sera. This explains in a measure the confusion experienced with allied encapsulated Gram-negative organisms. The species antigen of the Friedländer's bacillus is related to some extent to the species antigen of what have been considered allied organisms.

To recapitulate, encapsulated strains (Friedländer's bacillus) are transformed into "R" strains by heating in acid solution. The transformed cells react identically as "R" cells obtained by cultural methods. They agglutinate in anti-S sera depending upon the presence of species antibody, and in anti-R sera they behave as typical "R" strains.

TABLE IX.

Agglutination by Anti-R Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Anti-R sera	Antigen derived from	Dilution of serum				
		1:50	1:100	1:250	1:500	1:1000
Type A	Type A	+++	+++	++++	++++	+++
	" B	++++	++++	++++	+++	++
	" C	+	++	+++	++	+
	Group X	+++	++++	++++	++++	+++
	*Gran.	++	++	+	−	−
" B	Type A	+++	+++	++++	++++	+++
	" B	++++	+++	++	+	−
	" C	++	++++	++	+	−
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	++	+	−
Group X	Type A	++++	++++	++++	++++	+++
	" B	++++	++++	++	++	+
	" C	++	+++	++++	+++	++
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	+	−	−

* This organism was isolated from an infection of granuloma inguinale.

DISCUSSION.

"S" strains of Friedländer's bacillus are characterized biologically by mucoid growth in liquid media, by capsule formation, and by exalted virulence. As antigens they give rise in the serum to type-specific antibodies. Consequently, such an immune serum will agglutinate all organisms of the homologous type, afford passive protection in white mice against infection by strains of the same type, and precipitate the soluble specific substance derived from the type strains. The extent of species antibody in anti-S sera is negligible

provided immunization has not been intensive. The experience of former investigators, including our own, has been that in general prolonged immunization, even with capsule-bearing organisms, has yielded a serum so abundant in the species antibody as to obscure type-specific reactions. It is now recognized (7) in this connection that dissociation of the bacterial antigen occurring spontaneously *in vitro* and *in vivo* is a factor to be considered in immunization. That the dissociation observed in this study is due to a cleavage of the specific antigen complex in the animal body rather than a condition of cultural development is substantiated by certain evidence to be presented in a later paper.

"R" strains of Friedländer's bacillus on the other hand are characterized by loss of capsule formation, loss of the elaboration of soluble specific substance, and by attenuation of virulence. The sera of animals immunized to R strains contain only the species antibody. In other words anti-R sera agglutinate only capsule-free strains, irrespective of their type antecedent. Furthermore, anti-R sera fail to react with encapsulated organisms. They afford no passive protection against infections with virulent type-specific strains of Friedländer's bacilli, and they do not precipitate the soluble specific substance derived from organisms of homologous or heterologous type.

It is obvious, therefore, that the nature of antibody response in the animal is dependent upon the character of the bacterial cell which is utilized for immunization. Immunization with encapsulated strains engenders type-specific antibodies. On the other hand, a bacterial culture composed of both encapsulated and capsule-free cells, as is often the case, induces both the type-specific and species-specific antibodies. The predominance of the S or R component determines the predominance of the one or the other antibody. In either case confusing cross-agglutination reactions will be encountered, as they have been in the past, which are difficult of interpretation unless cognizance is made of the underlying principles. Immunization which is effected with cells devoid of capsules, as advocated by former workers (20, 21, 25); gives rise to only the species antibody which exhibits none of the type relationships. Similarly, in the agglutination reaction will be reflected the composition of a strain.

The studies on the cell constituents of Pneumococcus to which reference has already been made reveal a striking similarity in the immunological behavior of the two encapsulated races. There has been observed in both species the occurrence of encapsulated, virulent organisms which differ serologically and which undergo degradation to such an extent as to become capsule-free, avirulent, and serologically undifferentiated.

These principles find a remarkable analogy even among flagellated bacteria as is indicated by Smith and Reagh (29), Orcutt, and Felix. These authors have shown independently that certain differences are demonstrable in the serological behavior between flagellated and non-flagellated organisms of the same strain. These differences have been related to the presence of two antigens, the flagellar or ectoplasmic, and the somatic or endoplasmic antigen.

CONCLUSIONS.

1. "S" strains of Friedländer's bacillus produce capsules, soluble specific substance, and are of exalted virulence. "S" strains are type-specific and react with only the type-specific antibodies of the homologous types.

2. Immunization with "S" cells induces the formation of antibodies which agglutinate type specifically, precipitate the corresponding soluble specific substance, and protect white mice against infection caused by organisms of the same type.

3. "R" strains of Friedländer's bacillus produce no capsules, produce no soluble specific substance, and are not pathogenic. "R" strains are serologically undifferentiated from each other and react with only the species antibodies.

4. Immunization with "R" cells induces antibodies which do not agglutinate encapsulated organisms, do not precipitate soluble specific substance, and do not afford protection against infection by Friedländer's bacillus. Anti-R serum contains only the species antibody which reacts with any capsule-free organism regardless of its type origin.

5. Decapsulation of "S" cells by heat and acid chemically converts a type-reacting organism into a species-reacting organism.

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