RELATION OF VIRULENCE OF PNEUMOCOCCAL STRAINS FOR MICE TO THE QUANTITY OF CAPSULAR POLYSACCHARIDE FORMED IN VITRO*

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It has long been recognized that virulence of pneumococci depends upon the presence of the specific capsular polysaccharide (SSS) on the surface of the cells. SSS is non-toxic in the isolated state, but contributes to the virulence of pneumococci by acting as an antiphagocytic agent. Within a single capsular type of pneumococcus, however, different strains may exhibit wide variations in virulence for a given animal species. It has been conjectured that such differences in virulence are due to one or more factors distinct from the capsular polysaccharide. The present study demonstrates that variation in virulence for mice in the strains of the three capsular types investigated (types II, III, and VII) is associated with differences in the amount of SSS synthesized by different strains of the same type, and indicates that one of the factors concerned in mouse virulence of these strains is probably the genetic apparatus that controls the amount of SSS produced.

Previous studies (1) of a strain of pneumococcus type II intermediate between the classical R and S forms showed that the intermediate strain synthesizes a small amount of SSSII which is disposed on the surface of the cells, but nevertheless it did not appear to differ significantly in mouse virulence from an R strain derived from type II which does not produce SSSII in demonstrable amount. More detailed testing has shown, however, that the type II intermediate is slightly more virulent than the IIR strain.

In the same study (1) it was also observed that the capacity to produce different amounts of SSSII is hereditary and that transforming extracts can be prepared from strains showing different capacity for SSS synthesis which confer the same differential properties on R cells subjected to these transforming extracts.

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Materials and Methods

Strains of Pneumococcus.—

Type II: II-D39S: a fully encapsulated, highly virulent strain.

II–R36NC: an R strain selected from II-D39S following cultivation in the presence of type II antiserum. It produces no demonstrable SSSII, and does not revert spontaneously to the fully encapsulated form.

II-D39-Int53: A strain of type II pneumococcus that produces a small amount of capsular polysaccharide, which was selected from II-R culture by serial cultivation in anti-R serum. Strain II-D39-Int53, during prolonged cultivation, has shown no tendency to mutate to either the R form or to the fully encapsulated S form. These strains of type II pneumococcus were described previously (1).

Type III: III-A66: a fully encapsulated, highly virulent strain.

III-Int: a strain of type III obtained originally from a patient with pneumonia. With respect to SSS production, it is classed as an intermediate strain. On blood agar plates the colonies are smooth, but much smaller than those produced by fully encapsulated strains.

III-vir: a fully encapsulated virulent mutant selected from III-Int on mouse passage. The colonies of III-vir on blood agar are comparable in size to those of III-A66.

In addition to studies of these spontaneously occurring variants of type III, mouse virulence and SSSIII production were measured on strains obtained by transformation of the rough strain II-R36NC using transforming extracts prepared from strains III-Int and III-vir, respectively. The transformed strains are designated III-Int (transformed) and III-vir (transformed). The transformed strains showed the colonial characteristics of the strains from which the transforming extracts were prepared.

Type VII: VII-av: a relatively avirulent strain of pneumococcus type VII obtained from Miss Annabel Walter, New York City Department of Health. This strain was isolated originally in Denmark and labelled type VIIa because serological tests indicated that it was similar to, but not identical with type VII. Reciprocal absorption tests in our hands have shown, however, that the capsular polysaccharide produced by strain VII-av is identical with that of pneumococcus type VII. Strain VII-av differs from the commonly encountered strains of type VII only in producing less SSSVII.

VII-vir: a virulent strain of type VII pneumococcus selected from strain VII-av after five serial mouse passages.

Transformation Reactions.—The preparation of the transforming extracts and technique for carrying out the reactions were the same as described previously (1).

Quantitative Precipitin Reactions.—Types II, III, and VII horse antipneumococcal sera[1] were standardized by quantitative precipitin reactions against solutions of the homologous purified polysaccharides. Various amounts of capsular polysaccharide in saline solution were mixed with 1 ml. of antiserum and the total volume brought to 3 ml. by adding saline. The tubes were shaken and placed at 42°C. for 2 hours. They were then placed at 4°C. for 48 hours[2]. The supernatant fluids were examined by precipitin tests for antigen or antibody

[1] These antisera were kindly supplied by the Bureau of Laboratories, New York City Health Department.

[2] Heidelberger and Kendall (2) have shown that more antibody nitrogen is precipitated from horse antipneumococcal serum by pneumococcal polysaccharides when the reactions are carried out at 0°C. than at higher temperatures. The quantitative precipitin reactions in the present study were carried out at temperatures above 0°C. In the equation for the standardized sera, therefore, the amount of SSS is expressed in terms of less antibody N than would be precipitated at lower temperature. However, since the same conditions of temperature

excess. The precipitates were washed three times with cold saline by centrifugation, and nitrogen was determined by micro-Kjeldahl technique.

Quantitative estimation of SSS formed by the different strains of pneumococci was carried out as follows and the results expressed as micrograms of SSS per microgram of bacterial nitrogen. The strains were grown for 18 hours in neopeptone-meat infusion broth at 37°C. Five ml. aliquots of each culture were heated at 65°C. for 30 minutes and centrifuged. Bacterial nitrogen was determined on the thrice washed heat-killed cells. 0.5 to 2.0 ml. portions of the supernatant fluid were added to 1.0 ml. of the homologous standardized antiserum. The specifically precipitable nitrogen was determined as described above and the amount of SSS in the culture supernatant was calculated from the equation for the curve of the standardized antiserum as described by Heidelberger and Kendall (3). Finally, to determine *total* SSS produced, an unheated portion of the same culture was permitted to autolyze at room temperature for approximately 2 weeks. At the end of this time stained smears revealed amorphous debris only which was removed by centrifugation. 0.25 to 2.0 ml. amounts of the centrifuged autolysate were added to 1.0 ml. of the homologous standardized antisera and the specifically precipitable nitrogen was determined as described above. At the same time the amount of nitrogen precipitated by the autolysate from an equivalent amount of SSS-absorbed standardized antiserum was determined and subtracted from the value obtained with the unabsorbed serum. This corrected value was used to calculate total SSS in the autolysate by means of the equation for the standardized antiserum. The amount of non-anti SSS nitrogen precipitated from the unabsorbed standard type II, III, and VII antisera by the homologous autolysates varied from 8 to 33 per cent of the total.

The above procedures were found satisfactory where large amounts of SSS were produced as in the case of Type III, or where the difference in SSS production between avirulent and virulent strains was great as was true for the type II strains. However, with both virulent and avirulent type VII strains the amount of SSS produced was comparatively small and the ratio of SSS produced by the virulent strain as compared with the avirulent was not so striking as with the other types. It was felt to be desirable, therefore, in the case of the type VII strains, to carry out an additional set of determinations for SSS using type VII antiserum that had been absorbed with R pneumococci to remove non-anti SSSVII antibody.

A sample of type VII antiserum, which at a dilution of 1:16,000 caused marked agglutination reaction of R pneumococci, was absorbed repeatedly with large amounts of heat-killed R cells until the agglutination reaction with suspensions of R cells was almost completely abolished (slight agglutination at 1:4 dilution). This R-absorbed type VII antiserum was then standardized against a solution of SSSVII and used to determine the amount of SSSVII present in autolyzed cultures of the two variants of type VII.

Mouse Virulence Tests.—Mice were injected intraperitoneally with dilutions of cultures in rabbit blood broth which had been incubated for 16 to 18 hours at 37°C. The size of the inoculum was determined by counting the colonies in poured blood agar plates to which 10^{-7} ml. of culture had been added. Mice were observed for at least 5 days after infection.

EXPERIMENTAL

Virulence for Mice of Variants of Pneumococcus Types II, III, and VII.—In Table I are shown the results of virulence tests with the three variants of type II pneumococcus. The rough strain, II-R36NC, which produces no de-

were used both for the standardization of the antisera and measurements of SSS production by pneumococcal strains, there is no reason to suppose that the values found for SSS in the unknowns do not represent the actual amounts present.

tectable SSSII, killed eight of ten mice that were injected with 1.0 ml. of whole culture, but no deaths occurred in mice injected with smaller amounts. The intermediate strain, II-D39-Int53, which forms a small amount of SSSII, was slightly more virulent, three of ten mice dying when injected with 0.1 ml. In contradistinction, with the fully encapsulated strain, II-D39S, all twenty mice died that were injected with 10^{-7} or 10^{-8} ml. of culture. Autopsy of mice that died following injection of the R variant showed R organisms only. Similarly,

TABLE I

Virulence for Mice of Variants of Type II Pneumococcus

Infecting dose	Strain of pneumococcus				
	II-R36NC Rough strain derived from II-D39S		II-D39-Int53 Intermediate strain derived from II-D39S		II-D39S Fully encapsulated strain
	Died*	Autopsy‡	Died*	Autopsy‡	Died*
ml.					
1.0	8/10	R only	9/10	Intermediate only	
0.5	0/10		8/10	" "	
10^{-1}	0/10		3/10	" "	
10^{-2}	0/10		0/10		
10^{-7}					10/10
10^{-8}					10/10
Colony count of inoculum 10^{-7} ml....	39–81		30–50		88

* Indicates the number of mice dying over the total number infected.

‡ All mice that died following injection of II-R36NC and II-D39-Int53 were autopsied and the organisms from heart blood culture identified.

with the intermediate strain, at autopsy intermediate organisms only were recovered.

Table II shows the virulence data on the four strains of pneumococcus type III that were studied. The naturally occurring intermediate, III-Int, is somewhat less virulent, though not strikingly so, as compared with the fully encapsulated mutant, III-vir, selected from the intermediate strain on mouse passage. From the heart blood of six of forty-eight mice that died following injection of the intermediate III-Int, fully encapsulated strains forming large mucoid colonies were obtained.

The virulence of the intermediate and fully encapsulated strains prepared by transformation reactions with R strain, II-R36NC, using transforming extracts prepared from strains III-Int and III-vir, approximated that of the strains from which the transforming extracts were prepared, although the

transformed intermediate was less virulent than the naturally occurring intermediate strain. It should be noted, however, that mice which died following infection with the transformed intermediate showed intermediate forms only in their heart blood, whereas from six of forty-eight mice that died following injection of the naturally occurring intermediate, fully encapsulated

TABLE II

Virulence for Mice of Intermediate Type III and Fully Encapsulated Type III Strains, both Naturally Occurring and Prepared by Transformation Reactions

Infecting dose	III-Int Naturally occurring intermediate	III-vir Selected from III-Int by mouse passage; fully encapsulated	III-Int (transformed) Intermediate prepared by transformation	III-vir (transformed) Prepared by transformation; fully encapsulated
	Died*	Died*	Died*	Died*
ml.				
10^{-2}			9/10	
10^{-3}			6/10	
10^{-4}			6/10	
10^{-5}			3/20	
10^{-6}	21/25	5/5	1/20	18/20
10^{-7}	16/25	10/10	4/20	20/20
10^{-8}	12/25	10/10	1/20	17/20
No. of fully encapsulated mutant strains isolated at autopsy.....	6 strains from 48 mice autopsied	16 strains from 16 mice autopsied	None from 30 mice autopsied	22 strains from 22 mice autopsied
Colony count of inoculum 10^{-7} ml...	34–56	45–53	43–77	65–75

* Indicates the number of mice dying over the total number infected.

strains were recovered at autopsy, as noted above. The transformed intermediate mutant would appear, therefore, to be either more stable than the naturally occurring intermediate, or to contain such a small number of fully encapsulated mutants that intraperitoneal infection of mice does not result in their selection.

The avirulent type VII strain, VII-av, killed twelve of fourteen mice injected with 0.5 ml. of whole culture, and three of eleven mice when 0.1 ml. was injected, as shown in Table III. On mouse passage the virulence of the culture became markedly enhanced, so that after five serial mouse passages, all of fifteen mice died on injection of 10^{-7} ml. of culture and nine of fifteen

at 10^{-8} ml. Although a detailed study of the colonial characteristics of the avirulent and virulent strains was not made, no striking difference could be observed in their appearance either to the naked eye or under low power magnification.

Comparison of Specific Capsular Polysaccharide Production by Virulent and Avirulent Strains.—Estimation of the amount of specific capsular polysaccharide produced by the various strains of the three different pneumococcal types showed that the virulent strains produced more SSS than the avirulent or moderately virulent strains. SSS production was determined both in the supernatants of 16 to 18 hour broth cultures, and also in whole cultures that were permitted to autolyze at room temperature until formed bacterial elements were no longer present. These data are shown in Table IV. The amount of SSS produced per milliliter of culture is expressed in terms of bacterial nitrogen per milliliter, and the ratio of the amount of SSS synthesized by the avirulent or moderately virulent strains, as compared with the highly virulent strains, has been calculated.

TABLE III

Virulence for Mice of Avirulent and Virulent Strains of Pneumococcus Type VII

Infecting dose	VII-av strain	VII-vir Selected from VII-av by mouse passage
	Died*	Died*
ml.		
0.5	9/10	
10^{-1}	3/10	
10^{-2}	0/10	
10^{-6}		10/10
10^{-7}		15/15
10^{-8}		9/15
Colony count of inoculum 10^{-7} ml.....	69	45–47

* Indicates the number of mice dying over the total number infected.

In the supernatant of broth cultures of the virulent mutant of pneumococcus type II, II-D39S, the amount of SSSII, as measured by quantitative precipitin reactions using unabsorbed type II antiserum, was 7.8 times as great as that found for the avirulent intermediate mutant II-D39-Int53. A ratio of 4.3 was found when SSSII production was measured in autolysates of the two type II strains (Table IV).

Similar results were obtained in comparing the amount of SSSIII formed by moderately and highly virulent strains of type III pneumococcus, both in naturally occurring strains and in strains produced by transformation of an R culture derived from type II, using transforming extracts prepared from the naturally occurring type III strains of moderate and high virulence. As shown in Table IV, both the supernates and autolyzed cultures of the highly

TABLE IV

Relation of Virulence of Strains of Pneumococcus for Mice to Amount of SSS Formed in Vitro

Strains of pneumococcus	Virulence for mice	SSS in culture supernate		SSS in autolysate of whole culture	
		$\frac{\mu g. \text{ SSS/ml.}}{\mu g. \text{ Bact N/ml.}}$	Ratio of virulent/avirulent	$\frac{\mu g. \text{ SSS/ml.}}{\mu g. \text{ Bact N/ml.}}$	Ratio of virulent/avirulent
Type II					
II-D39-Int53	Avirulent	0.28	7.8	0.79	4.3
II-D39S	Highly virulent	2.17		3.38	
Type III					
III-Int (Naturally	Moderate virulence	1.99	2.6	2.09	2.7
III-vir occurring)	Highly virulent	5.15		5.60	
III-Int (transformed)	Moderate virulence	1.88	2.1	2.10	2.4
III-vir (transformed)	Highly virulent	4.04		5.11	
Type VII					
VII-av	Avirulent	0.34	1.6	1.65	1.5
VII-vir	Highly virulent	0.53		2.43	
VII-av	Avirulent	0.62	1.8	0.56*	3.4
VII-vir	Highly virulent	1.11		1.88*	

* Precipitin reactions carried out with type VII antiserum previously absorbed with R pneumococci to remove non-anti SSS antibody.

virulent strains contained more SSSIII per microgram of bacterial N than the strains of moderate virulence. The results in the case of the type III strains confirm the previous observation (1) that the amount of SSS produced by strains of pneumococcus is an hereditary characteristic, and that transforming extracts prepared from strains showing differences in the amount of SSS produced, confer the same properties on transformed strains of R pneumococci.

Measurement of SSSVII in culture supernates and in whole autolyzed cultures showed that the virulent strain of type VII pneumococcus produced more SSSVII than the avirulent strain (Table IV). The amount of SSSVII formed by both strains was small and the difference between them was not great. The

determinations were repeated using different lots of cultures of the two strains than had been used in the first tests. SSSVII in the culture supernates was measured by means of unabsorbed type VII antiserum, whereas that in the autolysates of the whole cultures was determined by precipitin reactions with type VII antiserum, from which non-anti SSS antibody had been removed by absorption with R pneumococci before standardization against SSSVII. In the supernates of both cultures more SSSVII was present as compared with the previous lots of cultures but the ratio of virulent/avirulent remained practically the same. The autolysate of the virulent culture contained 3.4 times as much SSSVII as that of the avirulent in the tests using R-absorbed type VII antiserum.

DISCUSSION

The observations recorded in this paper indicate that for the strains of pneumococcus types II, III, and VII that were studied, virulence for mice bears a relation to the amount of SSS produced *in vitro*. Virulent strains formed more SSS than moderately virulent or avirulent strains. It is of interest that in the highly virulent strain of type VII, the amount of SSSVII formed was less than the amounts of SSSII and SSSIII produced respectively by strains of types II and III of comparably high virulence. Furthermore, the highly virulent strain of type II formed considerably less capsular polysaccharide than the comparably virulent strains of type III. If the function of the capsular polysaccharides of pneumococcus with respect to virulence is purely antiphagocytic, as is commonly believed, it follows that SSSVII is a more potent antiphagocytic substance than SSSII, and that SSSIII is the weakest of all, since more of it appears to be required for *maximal* virulence. It seems likely that the differences in production of SSS observed *in vitro* are present also *in vivo*, since the capsule of virulent type III in mouse peritoneal exudates is very large, that of virulent type II is intermediate in size, while the capsule of virulent type VII is smallest of the three.

On the basis of the present studies with transformed strains of pneumococcus type III, and the previous observations (1) on strains of type II, it is apparent that the amount of SSS synthesized by transformed pneumococci is similar to the quantity produced by the strains from which the transforming extracts were prepared, and that differences in this capacity are hereditary. Comparable observations of a qualitative nature have also been reported by Taylor (4). It seems likely, therefore, that an important factor concerned in virulence of pneumococcus, at least in so far as virulence for mice is concerned, is the genetic apparatus which controls the quantity of polysaccharide synthesized by the cells. In an earlier paper (1) it was suggested among other possibilities, that the genes controlling differences in polysaccharide synthesis are allelic in nature. On the basis of extensive studies with mutants of pneu-

mococcus type III, Taylor (4) has also suggested that "mutated and normal SIII transforming principles are related to each other as are the genes of an allelic series."

SUMMARY

The amount of capsular polysaccharide formed *in vitro* by strains of pneumococcus types II, III, and VII of different virulence for mice has been measured by quantitative precipitin reactions. The quantity produced by strains of high virulence was greater for all three types than that formed by moderately virulent or avirulent strains.

It is suggested that an important factor concerned in variations of virulence of pneumococci for mice is the genetic apparatus that controls the amount of SSS synthesized by the cells.

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