THE COMPOSITE NATURE OF A PURE CULTURE OF A VIRULENT PNEUMOCOCCUS.

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(Received for publication, February 5, 1925.)

In studying the various factors which operate to cause the rise and fall of experimentally induced epidemics[1] it was conceived that one of them might be explained by the hypothesis that the infecting strain is made up of the descendants of many individuals of differing potentialities. Each line of descendants would have characters more or less constant or varying within narrow limits, but they would differ more or less from one another in these respects. Acccording to this hypothesis, the virulence of a strain, that is to say of a composite of individuals mixed in derivation, would depend upon the relative numbers of virulent and avirulent organisms composing it at the time of the experiment. Gross changes in virulence would be explained by a disturbance of the equilibrium of virulent and non-virulent organisms: should virulent organisms be present in large numbers, the strain would be stamped as virulent, but if influences should operate repressing the virulent organisms and allowing its avirulent members to come into ascendency, the virulence of the whole strain would be depressed. The experiments of Benians[2] on the dysentery bacillus, and those of Arkwright[3] on the typhoid-dysentery-enteritidis group show that so called pure cultures are not composed of organisms possessing identical properties.

The experimental epidemics above referred to were incited among mice by the feeding of an organism belonging to the group of *Bacillus typhi murium*. Intraperitoneal inoculations of this organism into

[1] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.

[2] Benians, T. H. C., *J. Path. and Bact.*, 1919–20, xxiii, 171.

[3] Arkwright, J. A., *J. Path. and Bact.*, 1921, xxiv, 36.

mice did not yield results that were clear-cut in their bearing on the hypothesis now under consideration. A strain of Pneumococcus Type I, of known virulence, was therefore selected for the experimental tests.

Attempts at Mechanical Analysis of a Bacterial Strain.

A culture of Type I pneumococcus, original Neufeld strain which had been passed through 189 mice, was obtained from the Hospital of The Rockefeller Institute for Medical Research. This strain was plated on blood agar and ten colonies picked. Each new strain was grown under standard conditions and tested for virulence. No difference in virulence was observed. Intraperitoneal injection into 16 gm. mice of 0.5 cc. of a dilution of 10^{-6} of a 14 hour growth in beef infusion killed in 26 hours.

As was expected, the observations failed to reveal avirulent forms. In the next experiment the strain was analyzed with the help of a Barber pipette. From a 6 hour growth in beef infusion broth, pH 7.8, individual diplococci were picked and deposited in a drop of broth on a cover-glass, taken up into a new sterile capillary tube, and deposited on a blood agar slant. Each strain resulting was subjected to two further analyses by the Barber method to insure origin from a single parent diplococcus. In these procedures only two out of every five diplococci that were picked grew when deposited on the blood agar.

Seven "pure line" or "derivative" strains were grown under standard conditions and tested for virulence. No great difference in virulence for mice was detected. All were virulent for mice, but one, Derivative Strain C, was slightly more virulent than the others. This strain, as will be shown later, possessed a much greater degree of virulence for rabbits.

Obviously a large number of individuals might have to be isolated from a virulent strain before avirulent forms would be chanced upon. It was decided, therefore, to attempt to disturb the supposititious equilibrium between virulent-avirulent individuals by different methods.

Analysis by Cultural Methods.

Three methods were used: (*a*) growth in immune serum, (*b*) growth in diluted bile, and (*c*) growth in slightly acid broth.

(*a*) *Growth in Immune Serum.*—It had already been shown by Stryker[4] that virulent strains of pneumococci become avirulent when grown in broth containing homologous antiserum. The following experiment was performed.

0.5 cc. of a 14 hour culture of Pneumococcus Type I (Pn. I-190[5]) was transplanted into a tube containing 4.0 cc. of beef infusion broth, pH 7.8, and 0.5 cc. of Type I antiserum free from preservative. The organisms grew in this mixture equally as well as in the control series of tubes containing 0.5 cc. of Type II antiserum and in another series containing normal horse serum. Transplants of 0.5 cc. were made from tube to tube, so that in one series the pneumococcus was growing in the presence of 10 per cent of antiserum and in the other 10 per cent of normal horse serum. A control of antiserum in broth was set up each time to test the sterility of the materials used.

Throughout the entire series the culture in Type II antiserum-broth and in normal horse serum grew diffusely. On the other hand the pneumococci growing in Type I antiserum became agglutinated and settled to the bottom of the culture tube. On shaking they remained flocculent. At the fourth transfer the growth was less granular on shaking; but at the sixth it could be shaken to a turbidity that was not granular to the unaided eye. But the lens revealed clumping.[6]

After the fourth transfer all tubes were shaken vigorously and a loopful from each was distributed over a blood agar plate. The colonies on the plates from the series in which normal serum and that in which Type II serum had been placed presented the usual appearance of colonies of pneumococcus. But in addition to the colonies presenting the usual characteristics there were, on the plates seeded with the cultures in Type I antipneumococcus serum, colonies of entirely different appearance. These were small, flat, and compact, greyish white in color, with an

[4] Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.

[5] The figure 190 indicates that the strain had now been passed through 190 mice.

[6] At the time the observations were made no explanation for this change seemed apparent. The work of Avery and Heidelberger on the S and P factors of pneumococci now permits of one. It is possible that during the first four transplants the pneumococcus continued to elaborate the specific soluble substance, S, though probably in gradually decreasing amounts. The presence of the S factor permitted of a marked agglutination. When the S factor disappeared, the P factor remained, to be acted upon by the anti-P factors of the serum, with result in flocculation but one of less marked degree than when the S factor was present.

irregular surface. When picked with a platinum needle, the colonies were resistant and could be pushed about on the surface of the agar. With a platinum loop they could be removed *in toto*.

The new strains grew more luxuriantly in beef infusion broth at a pH of 7.8 than at 7.4. The slightly granular character of the growth was suggestive of a streptococcus, possibly a contaminant. But the uninoculated control of serum in broth remained sterile. The new strains fermented inulin, produced methemoglobin, and were bile-soluble. A 14 hour culture in pneumococcus broth was injected intraperitoneally into mice weighing from 14 to 18 gm., with the results shown in Table I.

TABLE I.

Amount of culture injected.	Result.
cc.	
3.0	Death in 24 hrs.
2.0	Survived.
1.0	"
0.5	"
0.2	"
0.1	"
0.01	"
0.001	"
0.0001	"

The culture obtained from the mouse dying after the injection of 3 cc. was passed through ten mice in series and tested for virulence on March 18, 1921. An 8 hour culture in pneumococcus broth was injected intraperitoneally into 16 gm. mice with the results shown in Table II.

One of these strains was kept in stock and labeled avirulent Y.

(*b*) *Growth in Diluted Bile.*—

The stock culture of Pneumococcus I-190$_7$ was inoculated heavily into 5 cc. of pneumococcus broth. After 3 hours, when there was slight turbidity of the medium, 0.01 cc. of sterile bile was added. 2 hours later the medium had increased in turbidity and 0.5 cc. of the culture was transferred to 5 cc. of pneumococcus broth containing 0.01 cc. of bile. A series was started from this point with a gradual increase in the amount of bile in the medium until on the 42nd transfer

5 cc. of the medium contained 1.0 cc. of bile. A transplant was made into pneumococcus broth and a loopful distributed on a series of blood agar plates.

Colonies of virulent and avirulent granular strains were obtained, the latter predominating. A study of one of these strains labeled avirulent Z showed it to be practically identical with the avirulent Y strain already described.

It is probably not necessary to go through so long a series of transplants in the bile medium to disturb the relation of virulent and avirulent individuals in a strain of pneumococcus. Shorter series were not carried out, but an effort was made to analyze a virulent strain by exposing it to concentrated bile.

TABLE II.

Amount injected.	Result.
cc.	
0.1	Survived.
0.01	"
0.001	"
0.0001	"
0.00001	"

TABLE III.

Amount injected.	Result.
cc.	
0.5	Survived.
0.1	"
0.01	"
0.001	"
0.0001	"
0.00001	"

An 18 hour culture in pneumococcus broth of Pneumococcus I-189-1a was found to be lethal in a dilution of 10^{-6}. To 5 cc. of such a culture in pneumococcus broth there was added 0.5 cc. of sterile bile and the mixture was allowed to stand in the water bath for 10 minutes. 5 cc. of isotonic NaCl solution was added and centrifugation done at high speed for 20 minutes. The residue was then transplanted to rabbit blood broth and incubated overnight.

Blood agar plates from this culture showed all but two colonies out of more than a thousand to be typical for pneumococci of the original strain. The small atypical colonies possessed the same characteristics described for the Y and Z avirulent strains. Control cultures of the bile remained sterile.

The new strains Y and Z did not grow well in beef infusion broth at pH 6.8 and 7.4 but grew well in pH 7.8 broth. They fermented inulin and produced methemoglobin.

An 8 hour culture in pneumococcus broth was used for a virulence test with the results shown in Table III.

The ready isolation of the Y-Z type from a virulent culture of Pneumococcus I-189-1a proved to be a chance occurrence. The experiment was repeated five times before positive results were again obtained. In the positive experiment, a 26 hour culture was employed. The significance of the finding will be discussed in the summary.

(c) Growth in Slightly Acid Broth.—

The stock Pneumococcus I-190 was passed through four transplants in beef infusion broth at the reaction of pH 6.8. To 5 cc. of the fourth transplant was added 1 cc. of bile and after 10 minutes in the water bath the mixture was centrifuged. The residue was transferred to rabbit blood broth and after 18 hours incubation plated on blood agar.

Colonies of the Y-Z type were obtained in considerable numbers. Cultures from such colonies proved avirulent for mice. A strain was kept in stock, labeled avirulent X.

Experiments with Strains Grown from Single Cells.

The results just described may be taken to indicate that the virulent strain of Pneumococcus I-190 is composed of descendants of two differing individuals, a virulent one, the descendants of which predominate, and an avirulent as represented by Substrains X, Y, and Z which are recovered with difficulty, as when the mixed strain is submitted to certain influences, such as growth in immune serum or in bile or slightly acid broth. To determine whether avirulent individuals are always present or whether they result from a change of virulent to avirulent individuals, experiments were carried out with cultures obtained from single diplococci by the Barber method.

Two derivative strains, A and C, from Pneumococcus I-190 and one derivative strain each from the avirulent Y and Z were employed. Strains A and C were of such virulence that six organisms, injected intraperitoneally, killed 16 gm. mice in 32 hours. Strains Y and Z remained avirulent throughout the observations upon them.

Recovery of Avirulent Strains from Derivative Virulent Strains.

The virulent Strains A and C were subjected to bile for 10 minutes, as previously described in experiments with Pneumococcus I-189-1a, centrifuged, cultured, and plated. No avirulent colonies were found.

After growth for six transplants in 10 per cent Type I antiserum, plating revealed numerous colonies of avirulent strains, in every way identical with X, Y, and Z strains.

Failure of Avirulent Strains to Revert.

Derivative Strains Y and Z were transplanted twelve times in rabbit blood pneumococcus broth and again plated and tested for virulence. The strains bred true, remaining avirulent and retaining the other characteristics distinguishing them from the parent strain. Reference has been made to the fact that avirulent Strain Y did not become virulent, even after passage through ten mice.

Effect of Long Continued Growth in Immune Serum.

The following virulence tests were made of strains grown in immune serum.

Strain I-190 and Derivative Strains A, B, C, and L derived from it were grown in 10 per cent Type I antipneumococcus serum in pneumococcus broth for twelve transplants. A similar series of transfers was made in the same concentration of normal horse serum in broth.

The two series, after twelve transplants in serum broth, were cultured in rabbit blood broth. After two transplants the strains in the immune serum series no longer grew in granular form but the growth in each case became as diffuse as that of the strains in diluted normal horse serum. 0.1 cc. of each culture was transferred to 5 cc. of pneumococcus broth (pH 7.8) and after 8 hours the amounts indicated in Table IV were injected intraperitoneally into mice.

The strains grown for twelve transplants in diluted immune serum were now grown for twelve transplants in rabbit blood broth.

These tests for virulence showed no return of pathogenic power in the strains that were avirulent to begin with. Growth in blood broth to which dextrose, sterile extract of tomato, leucocytes, and leucocytic extract had been added did not increase the virulence.

Multiplication in Increasing Concentrations of Bile.

The parent strain Pneumococcus I-190, three pure line virulent strains derived from it, A, B, C, and the pure line, avirulent Strain Z were transplanted daily in beef infusion broth pH 7.8 containing gradually increasing amounts of bile.

In the first 11 transplants 0.01 cc. of bile was added to 5 cc. of the medium. On the 12th and succeeding transplants 0.02 cc. was added and at the 20th transplant 0.07 cc. All cultures grew equally well to this point. On the 21st transfer, 0.08 cc. was added and after 24 hours only cultures of the parent strain and of Strain Z gave evidence of multiplication, though at 48 hours there was turbidity evidencing it in Cultures A and C. On the 22nd transfer 0.09 cc. of bile was added and in this concentration Strains A, B, and C failed entirely to grow, even in 48 hours.

Strains I-190 and Z were carried forward until 0.5 cc. of bile was added on the 31st transfer. At this point the I-190 culture became granular. Growth was obtained when 1.0 cc. of bile had been added to the medium and even when 1.25 cc. was added to the 41st transfer. The culture of Strain I-190 had now become completely avirulent.

TABLE IV.

Results of Virulence Test of Strains Grown in Immune Serum.

Amount injected.	Strains.			
	Pneumococcus I-190.	A	B	C
cc.				
0.1	Death in 36 hrs.	Survived.	Survived.	Survived.
0.01	Survived.	"	"	"
0.001	"	"	"	"
0.0001	"	"	"	"
0.00001	"	"	"	"

Strains Grown in Dilute Normal Horse Serum.

0.00001	Death in 25 hrs.	Death in 23 hrs.	Death in 23 hrs.	Death in 26 hrs.

The fact that the virulent derivative strains soon cease to multiply in gradually increasing concentrations of bile while the parent strain, like the avirulent substrain (Z) originally derived from it, continues to grow even in twelve times this concentration is further evidence of the composite nature of the parent strain. The failure of avirulent substrains to develop in the course of cultivation of the virulent substrains is to be laid to the working of the same law of chance which was responsible for the development of such a substrain in two of six experiments, as already stated. It is possible that although there was a change from the virulent to avirulent forms, no sufficient number of the latter were present at any time to establish growth under the

extremely unfavorable conditions. There is a suggestion too that avirulent strains may vary in solubility in bile and their ability to grow in dilute solutions of this lytic agent.

Multiplication in Acid Broth.

The parent strain[7] and pure line Strain C, both of which are constantly virulent, were transplanted successively in beef infusion broth with a slightly acid reaction (pH 6.8).

After eleven transfers they were transferred into blood broth (pH 7.8) and incubated for 15 hours. 0.1 cc. in each instance was transplanted to 5 cc. of beef infusion broth, pH 7.8, and after 8 hours the equally turbid cultures were tested for virulence by intraperitoneal injection into mice, with the results recorded in Table V.

TABLE V.

Amount injected.	Pneumococcus I-190.	Derivative Strain C.
cc.		
0.1	Death in 30 hrs.	Survived.
0.01	" " 36 "	"
0.001	" " 66 "	"
0.0001	Survived.	"
0.00001	"	"

Unfortunately the viable organisms in each dilution were not enumerated by plating but cultures plated on the tenth transfer showed approximately the same number of colonies.

Apparently the parent composite strain is more resistant to unfavorable conditions than the virulent derivative strain.

Virulence of Derivative Strains for Rabbits.

Some observations on the virulence for rabbits of Substrains A, B, and C derived from single diplococci were made in the course of the production of single type antisera.

[7] The parent strain in another series failed to survive more than four transplants in pH 6.8 broth. In the series now under consideration there was good growth in the eleventh transplant.

TABLE VI.

Protection Test of Sera C and Z against Pneumococcus I-190 and Strain C.

Serum.	Culture.	Result.	Serum.	Culture.	Result.
0.2 cc. Serum C.	0.1 cc. Pneumococcus I.	Survived.	0.2 cc. Serum Z.	0.1 cc. Pneumococcus I.	Death in 20 hrs.
0.2 " " "	0.01 " " "	"	0.2 " " "	0.01 " " "	" " 20 "
0.2 " " "	0.001 " " "	"	0.2 " " "	0.001 " " "	" " 35 "
0.2 " " "	0.0001 " " "	"	0.2 " " "	0.0001 " " "	" " 39 "
0.2 " " "	0.00001 " " "	"	0.2 " " "	0.00001 " " "	" " 35 "
0.2 " " "	0.1 " Strain C.	"	0.2 " " "	0.1 " Strain C.	" " 11 "
0.2 " " "	0.01 " " "	"	0.2 " " "	0.01 " " "	" " 20 "
0.2 " " "	0.001 " " "	Death in 96 hrs.*	0.2 " " "	0.001 " " "	" " 22 "
0.2 " " "	0.0001 " " "	Survived.	0.2 " " "	0.0001 " " "	" " 35 "
0.2 " " "	0.00001 " " "	"	0.2 " " "	0.00001 " " "	" " 35 "
0.2 " Type I.	0.1 " " "	"	None.	0.000001 " " "	" " 39 "
0.2 " " "	0.01 " " "	"			
0.2 " " "	0.001 " " "	"			
0.2 " " "	0.0001 " " "	"			
0.2 " " "	0.00001 " " "	"			

* Blood from heart yielded Gram-negative bacilli, no pneumococci.

1.0 cc. of a heat-killed culture of a 14 hour growth of each culture in pneumococcus broth was injected intravenously into rabbits. After 7 days 0.0015 cc. of a broth culture of living organisms was injected. The rabbits receiving Strain A and Strain B survived, but the two receiving Strain C died and pneumococci were cultivated from the heart's blood at autopsy. Another series of four rabbits weighing from 1,400 to 1,600 gm. received 1 cc. of heat-killed culture of Strain C and after 7 days were injected intravenously with 0.001 cc. of a live culture. All four rabbits succumbed to this injection. Another series receiving 0.0005 cc. of a broth culture survived and subsequently received larger doses. These observations show that Derivative Strain C is at least one and one-half times and possibly three times as virulent for rabbits as two other substrains derived by the same method of mechanical analysis.

Immunological Reactions of Virulent and Avirulent Substrains.

Single type serum prepared in rabbits against Derivative Strains C and Z agglutinated both of them and the parent strain as well. Strain Z is granular and is agglutinated with normal horse serum. The immune sera exhibited great difference in their capacity to protect mice against intraperitoneal injection of living cultures.

0.2 cc. of the serum was drawn into a syringe and the broth suspension, diluted according to the standard method so that the dose was in a constant volume of 0.5 cc., was then drawn up and the mixture injected intraperitoneally into mice weighing from 15 to 17 gm.

The results are shown in Table VI.

It may be concluded that while serum prepared by the intravenous injection of Strain Z contained agglutinins for Strain C and the parent strain, it has no protective power against living pneumococci injected intraperitoneally. Adsorption experiments were not made.

DISCUSSION AND SUMMARY.

The experiments reported in this paper indicate that a pure culture of a virulent Type I pneumococcus as transferred under ordinary conditions is made up of individuals which are not identical in virulence for animals,—in bile solubility, or in their power to multiply under unfavorable conditions.

By mechanical analysis alone no avirulent strains were obtained from the virulent strain, but one, Strain C, was separated which proved to be much more virulent for rabbits than its fellows.

The virulent, colony-pure, Type I pneumococcus was allowed to multiply for several transplants in a series of cultures in (*a*) pneumococcus antiserum broth, (*b*) bile broth, and (*c*) slightly acid broth. When cultures from each series were plated on blood agar there appeared amid the ordinary pneumococcus colonies some that were totally different therefrom. Cultures from these new colonies were macroscopically bile-soluble, fermented inulin, and produced methemoglobin. Their occurrence under these different sets of conditions as well as careful controls excluded the possibility of a derivation by contamination.

The new strains were avirulent and did not become virulent on repeated passage through mice. Antisera procured by the injection of them into rabbits were found to contain agglutinins for Type I pneumococci, but no protective antibodies.

The avirulent strains are evidently pneumococci. It is possible that they may have been present in the parent strain and the unfavorable conditions of growth (immune serum, bile, slight acidity) might have allowed them to multiply while repressing the virulent forms. The individuals composing the pneumococcus culture are not all equally bile-soluble. By means of differential bile solubility, the avirulent forms were obtained within 12 hours from the parent strain though only twice in six experiments. It is possible that the avirulent individuals might not have been present in the 26 hour stock culture which yielded one of the positive results and that the organisms surviving the lysis might have retained sufficient bile after washing with isotonic salt solution for this to be responsible for the development of the variant. The fact that the identical experiment with the virulent Derivative Strain C revealed no avirulent forms renders it likely that the majority of the avirulent forms originated from the virulent individuals under the unfavorable conditions. The correctness of this interpretation is proven by the finding of these avirulent forms in virulent single cell derivative strains grown under unfavorable conditions. By contrast, strains of these avirulent forms descended from single diplococci did not revert to virulence. Apparently there had occurred a genuine bacterial mutation.

These results in general are in accord with those of Griffith[8] who has

[8] Griffith, F., *Rep. Pub. Health and Med. Subi., Ministry of Health, No. 18*, 1923, 1.

made a more careful study of the properties of variants of pneumococci. He has recorded the occasional reversion of a variant when the single colony culture has been made after one serum passage. There is no reference in his article to any experiments with strains derived by the isolation of single diplococci.

Further evidence of the composite nature of the parent strain as ordinarily grown is presented by the difference in the effect of long continued growth in immune serum on the pure line strains and on the parent strain itself. The pure line strains, equally as virulent for mice as the parent, and one of them, Strain C, more virulent for rabbits, became less virulent than the parent strain after twelve transplants in immune serum. Growth in normal horse serum did not affect the virulence. The single cell derivative, moreover, was found to be the less resistant to bile when grown in increasing concentrations of this lytic substance. The virulent Derivative Strain C became avirulent in eleven transplants in acid broth pH 6.8 while the parent strain, though reduced in virulence, was still lethal in doses of 0.001 cc. after the same number of transplants in this medium.

CONCLUSIONS.

A virulent strain of Type I pneumococcus which had been passed through 190 mice has been found to be composed of individuals possessing different characteristics.

A strain isolated by mechanical analysis of a virulent composite strain possessed greater virulence for rabbits than the composite strain or other strains similarly isolated from the same source and was less resistant to unfavorable media than the composite strain.

An avirulent strain was isolated from the composite virulent strain by plating cultures which had grown on repeated transfer in (*a*) immune serum broth, (*b*) bile broth, and (*c*) slightly acid broth.

The virulent single cell derivative strain obtained by mechanical analysis gave rise to the avirulent strain when grown in immune serum, bile, or acid medium. Heterologous immune and normal sera did not favor the change from virulent to avirulent variants.

An avirulent strain was separated from the virulent composite strain by subjecting the latter to the action of bile for 10 minutes, centrifuging, washing, transplanting the residue into blood broth, and plating after 12 hours.

The avirulent strains, however procured, were all of a single sort. They formed characteristic colonies, showed no tendency to revert to the parent type, and did not become virulent on repeated passage through mice.

Serum from rabbits immunized with organisms of the avirulent sort possessed agglutinins but no protective antibodies for the parent strain.