STUDIES ON VIRULENCE

IV. Influence on Virulence of Pneumococci of Growth on Various Media*

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During a series of investigations of some of the factors influencing virulence of microorganisms grown *in vitro*, the effect of the material used for medium was naturally of prime importance. As indicated in an earlier publication (1), there is much evidence in support of the view that virulence is correlated with the ability of a microorganism to grow in or on living animal tissue. Indeed, the best known method for increasing this microbic characteristic is animal passage. Furthermore, there is a well recognized predilection of a given microorganism for a certain tissue of the host, and also a variation in the influence of different organ extracts on growth *in vitro*. Consequently it seemed worth while to study the virulence of microorganisms grown on extracts of different organs of the animal body.

Extracts from many of the organs of the body have been studied as media for the growth of various organisms; but few investigators have reported on the effect of these media on virulence. Thus, in more recent literature, Graham-Smith (2), in 1920, reported, in his study of conditions of growth, that staphylococcus and *B. coli* show a markedly better growth on ox pancreas extract than on meat extract. Proca (3), in 1924, made simple extracts from both spleen and liver of the calf, and found them satisfactory for growth of some ten organisms. In 1927, Hach (4) found that macerated brain substance (human or ox), although causing slight retardation in growth in about 700 strains studied, did not alter virulence of his strains of *B. anthracis*, *B. typhi murium*, *B. bipolaris septicus*, or *Staph. aureus*, even in periods ranging from 1 to 4 years. With a beef liver extract, Quiroga (5) (1928) has been able to maintain pneumococci at a high degree of virulence.

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Duran-Reynals (6) (1929), studying the effect of various organ extracts on vaccine virus, reported that as judged by the extent of lesions, testicle, kidney, and skin, and probably liver, brain, and placenta enhance the activity of the virus in rabbits. Pana (7) (1930) found that adrenalin or the suprarenal gland had no effect on the growth of *B. coli*, but that extracts of hypophysis, muscle, thymus, testicle, and thyroid increased the growth in this order. Applying the results of Duran-Reynals, Pijoan (8) (1931) tested the effect of testicle, kidney, and spleen extracts on twenty bacterial species, and as judged by the extent of the lesion produced, reported definite enhancement by testicular extract, slight enhancement by kidney, but lesions less than ordinary with spleen, at least when the organism used was staphylococcus.

Methods of Study

In our previous work (9) in which an automatic device was used to make transfers every 2 to 4 hours, it was found that some media, not suitable for maintenance of virulence under ordinary transfer methods, would maintain this characteristic in this apparatus. Thus milk as a medium was observed to maintain or even to increase the virulence of some strains of pneumococci grown in the automatic transfer apparatus. Since conditions of growth supplied by the automatic transfer device differ from those of ordinary methods of transfer and perhaps more nearly simulate those found in the animal body, the possibility is suggested that the influence of the medium on the virulence of the organism might be better established in this apparatus.

It might be mentioned here that recently two other workers have described apparatus for continuous growth of organisms. A device similar to the one used in our experiments, described by Weiner (10), in 1927, was used by Friedlander and Meyer (11), and Friedlander and Hertert (12) in a study of the virulence of *B. aertrycke*. No increase of the virulence of this organism was found in 3 or 6 hour periods of transfer, for either mice or guinea pigs; but the authors suggested the possibility that this result may have been obtained because the organism was of maximum virulence at the beginning of the experiment. Also an apparatus, which was essentially a specially constructed, slow-running filter was suggested by Haddon (13) (1928) for continuous growth; but results of the use of this apparatus have not been found in the literature.

The present study is a report of the effect on virulence of growth of pneumococci in an automatic transfer apparatus on media made from different organ extracts.

The various media were made by the usual bacteriological method; that is, the organs from freshly killed animals were comminuted in a meat grinder, and infused overnight in the ice box in distilled water at the rate of 1 liter of water to 2 pounds of ground organs. In the morning, the infusion was boiled for from 15 to 30 minutes, and while hot the meat particles were strained out. The water extract was then titrated to pH 7.6, and sodium chloride added to make an additional 0.5 per cent. The neutralized medium in 5 liter Florence flasks was then sterilized in the autoclave at 17 pounds pressure for 1 hour. With one exception no peptone or other ingredients were added to the media reported in this study. As in earlier work, the virulence of pneumococci was estimated from the death of mice injected with dilutions of culture made in logarithmic series. The number of organisms per cubic centimeter was estimated by plating three dilutions of each culture on rabbit blood agar plates.

Calf Lung Medium

This experiment is one of five in which calf lung medium was tested as to its suitability for maintenance of virulence of the pneumococcus.

TABLE I

Calf Lung Medium

2 hour interval of transfer

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
168	40,000,000	4
432	150,000,000	15
600	120,000,000	12
696	48,000,000	4

A Neufeld Type I pneumococcus, of virulence such that one organism was fatal for a white mouse, was used to inoculate the lung medium in a machine regulated so that transfer was made every 2 hours. From preliminary experiments it was found that this medium was satisfactory for maintenance of a high degree of virulence over a considerable period of time. For that reason, the experiment recorded in Table I includes tests for virulence begun only after the 168th transfer. Briefly, it was found that, although the number of organisms in the culture receptacle varied considerably from time to time, the original virulence remained unchanged even up to the 696th transfer, or the equivalent of almost 2 years of daily reinoculation.

Calf Heart Medium

This experiment is similar to the previous one except that the medium was made from heart, instead of lung, of the calf. As in the case of lung, preliminary experiments indicated that heart medium supplied the necessary constituents for maintenance of virulence over short periods of time. Hence, in the experiment in Table II, the first estimation was made only after 72 transfers at 2 hour intervals. The average number of organisms per cubic centimeter in the growth receptable was higher in heart than was observed in lung medium. Since virulence tests in this experiment were run in dilutions no higher than 1–10,000,000, the end-point may not have been reached. It

TABLE II

Calf Heart Medium

2 hour interval of transfer

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
72	120,000,000	120
192	50,000,000	50
360	240,000,000	24
432	230,000,000	23
504	250,000,000	25

is, however, quite evident that even after 504 transfers, the organism retained a high, and in all probability its maximum, virulence.

Calf Spleen Medium

Under ordinary methods of transfer spleen infusion proved to be unsatisfactory for maintenance of growth of pneumococci. However, the organisms were found to multiply on this medium when inoculated in the apparatus which made periodic transfers of culture at short intervals. To demonstrate the effect on virulence, two experiments are reported (Table III), the one on undiluted spleen medium and the second on spleen medium diluted with equal parts of physiological salt solution. When undiluted medium was used, a 4 hour period of transfer was found necessary to permit growth. In the diluted medium, however, a 2 hour interval was sufficient. The outstanding

differences between the growth on this organ extract and that on heart or lung extract were first, depression of the rate of multiplication of the organism, and second, rapid decrease in virulence. These differences are particularly well brought out in undiluted spleen medium. However, even with diluted medium, although virulence remained maximum through 168 transfers, there was subsequently a gradual decrease until 1 cc. of the culture containing 16 million microorganisms, was necessary to produce a fatal infection. The difference between the diluted and undiluted media would indicate that there may be some substance in the spleen which causes a decrease of virulence at least of

TABLE III

Calf Spleen Medium

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
Undiluted medium—4 hour interval of transfer		
42	25,000,000	2,500
78	20,000,000	200,000
120	10,000,000	10,000,000
Diluted medium—2 hour interval of transfer		
168	11,500,000	11
240	280,000,000	280
336	100,000,000	10,000
600	16,000,000	16,000,000

Type I pneumococcus. Whereas the diluted medium produced better growth and maintained virulence over a relatively longer period, the undiluted caused a rapid attenuation. This finding is thus seen to parallel the results of the work of Duran-Reynals and of Pijoan that spleen extracts depress the activity of vaccine virus and the virulence of staphylococcus.

Horse Skeletal Muscle Medium

In previous work, calf skeletal muscle proved to be an unsatisfactory medium for maintenance of virulent pneumococci; an infusion without peptone caused a very rapid decrease. Addition of peptone

or dextrose somewhat improved the quality of the medium, but in no experiment was it possible to make a medium from this extract which was capable of maintaining virulence. In the present experiment, skeletal muscle of the horse was used to determine the difference between the tissue from this animal and the one from the calf. As in most of the experiments with calf skeletal muscle, the medium in this experiment contained 1 per cent peptone. As can be seen from Table IV, this extract not only supported a rapid growth but also maintained a high degree of virulence over the period represented by 258 transfers. It would appear thus that tissues of the same type from two animal species contain, or lack perhaps, substances which cause the difference in activity of these extracts. For both

TABLE IV

Horse Skeletal Muscle Medium

2 hour interval of transfer

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
54	44,000,000	4
102	140,000,000	14
144	240,000,000	24
210	200,000,000	20
258	124,000,000	12

support growth equally; yet growth on the one, calf skeletal muscle, results in a decrease of virulence, while growth on the other, horse muscle, causes no alteration of this property.

Normal and Immune Horse Sera as Media

Serum from normal animals has been found by many investigators to maintain virulence of various microorganisms over at least a short period of time. Because of the observations of Stryker (14), confirmed by other investigators, that antipneumococcus horse serum supports the growth of pneumococcus but with a resultant rapid decrease in virulence, this experiment was planned to ascertain whether growth *in vitro* in the automatic transfer device would give similar results. This experiment consequently is a report of the difference in results obtained by the growth of pneumococci on normal horse serum and on

the serum of the same horse after immunization against Type I pneumococcus. The results in Table V show that the serum of the horse before immunization was a suitable medium for at least 90 transfers. On the other hand, the serum from the same horse after immunization caused a drop of virulence approximately 250 thousand-fold, and in 36 transfers 40 million-fold. In other words, the experiment confirms the observations of Stryker that the pneumococcus, although multiplying at a normal rate in immune serum, had lost its pathogenicity. This was found true even when the immune serum was highly diluted with normal horse serum. For in one experiment, in which immune

TABLE V

Normal and Immune Horse Serum

4 hour interval of transfer

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
Before immunization		
18	200,000,000	200
60	85,000,000	8
90	93,000,000	14
After immunization		
18	25,000,000	250,000
36	40,000,000	40,000,000
54	120,000,000	120,000,000

serum was diluted with 500 volumes of normal horse serum, the virulence decreased in 60 transfers over 100 thousand-fold.

Such striking differences between the effect of immune horse serum and that of normal serum led to an analysis of immune serum with the object of finding the fraction which caused this decrease in virulence. It was observed that the supernatant serum, after eliminating a water-insoluble protein fraction obtained by dilution of immune serum with ten volumes of water (15), supported growth and when transferred at 2 hour intervals maintained maximum virulence of pneumococci for at least 60 transfers. On the other hand, a salt solution of the water-insoluble fraction to which 0.1 per cent peptone was added supported

growth of pneumococcus, but, as in the case of the whole immune serum, caused a sudden drop in virulence. The water-insoluble fraction of the antipneumococcus serum was found to contain all the so called antibodies of pneumococcus. It would appear therefore, that the pneumococcus antibody is in the fraction of immune serum which causes the decrease in virulence.

Rabbit and Guinea Pig Media

In addition to the study of organ tissue extracts, two experiments are included on the extracts of two animal species, rabbit and guinea

TABLE VI, *a*

Whole Rabbit Medium

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
2 hour interval		
36	240,000,000	24
84	100,000,000	10
180	8,000,000	8,000
240	50,000,000	50,000
4 hour interval		
60	60,000,000	6,000
102	50,000,000	5,000
150	20,000,000	2,000
198	20,000,000	2,000,000

pig. These were chosen because of their difference in susceptibility to pneumococcus infection: the rabbit being highly susceptible, the guinea pig less so. The technic in preparing the media here used was similar to that given above, the only difference being that the entire animal including skin and bones, except the intestinal tract, was ground and infused. Table VI, *a*, represents the results obtained in the case of the rabbit medium with 2 and 4 hour intervals of transfer. With the 2 hour interval the virulence remained high for 84 transfers, then decreased gradually to an avirulent state. The longer period of transfer showed a lower rate of multiplication and a more rapid decrease in virulence than the 2 hour period.

Several experiments have been run with guinea pig medium, and in all, the results have been found to be similar to those represented in Table VI, *b*. No experiment was carried out, however, for a time longer than that represented by 144 transfers. The striking difference between growth on medium from whole rabbit, an animal that is susceptible to the pneumococcus infection, and that on medium from guinea pig, an animal not so susceptible to pneumococcus, is somewhat surprising. For guinea pig medium apparently furnishes substances or conditions suitable for the maintenance of the virulence of the pneumococcus. It is interesting to note, however, that whereas the tissues of one species of animal, or any one tissue of that species prove

TABLE VI, *b*

Whole Guinea Pig Medium

4 hour interval

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
84	90,000,000	90
102	60,000,000	6
144	50,000,000	50
84	60,000,000	6
102	80,000,000	8
144	240,000,000	24

satisfactory in maintaining the pathogenicity of a microorganism, the tissues of another species should produce opposite results. If these results were not due to unrecognized variables and hence to an actual difference, further investigation will be needed to tell whether or not this difference is bound up with the broader aspects of varying susceptibility of animal species.

Lung Medium for the Restoration of Virulence

In a previous study it has been shown that in an avirulent culture obtained from a single cell, grown on milk medium with 2 hours as the interval of transfer, the virulence was increased to a maximum state. In an endeavor to find out whether or not any one organ extract was superior to another for the enhancement of virulence *in*

vitro, media made from those organ extracts which have been shown to furnish conditions suitable for maintenance of virulence were inoculated with avirulent organisms. Briefly, it was found that whole guinea pig medium, calf heart medium, and horse skeletal muscle infusion all failed to enhance the virulence of a relatively avirulent pneumococcus. With lung medium, however, avirulent pneumococci were restored to maximum virulence.

TABLE VII

Influence of Lung Medium on Avirulent Culture

Culture No.	Interval	No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
	hrs.			
5	2	168	110,000,000	110,000
		372	80,000,000	80
		444	30,000,000	30
		600	20,000,000	2
5	4	84	70,000,000	7,000
		180	70,000,000	7,000
		342	130,000,000	13
		378	250,000,000	2
5	8	42	160,000,000	1,600
		108	140,000,000	1,400
		150	180,000,000	180
		189	80,000,000	8
6	4	84	90,000,000	90,000
		216	3,500,000	35,000
		342	200,000,000	2,000
		378	180,000,000	18
		414	350,000,000	35

Two of the avirulent strains studied are included in this report. Culture 5 in Table VII was attenuated to such degree that 1 cc. of the 8 hour culture was necessary to produce a fatal infection in white mice. From the table it is seen that, with 2 hour intervals of transfer on lung medium, after 372 transfers 80 organisms caused the death of a mouse, and after 600 transfers the pneumococci were of maximum virulence. With a 4 hour period of transfer, the same effect was obtained after 378

transfers, and with 8 hour intervals after 189 transfers. Culture 6 was a strain of Pneumococcus Type I obtained from a normal throat, and was of such low virulence that 1 cc. failed to kill a mouse but 2 cc. caused a fatal infection. Here again the culture after 378 transfers was raised to maximum virulence.

So far this lung medium has been used on seven different avirulent strains of pneumococcus, including one Type II and one Type III, and in each instance, as judged by ability to kill white mice, there was an increase in the virulence of the organism. No attempts have been made to increase the virulence on lung medium of an avirulent strain developed from a single cell. Hence, it is impossible at present to state whether this process is simply a matter of selection of virulent organisms or whether it is an inherent change in the cell itself. However, it should be borne in mind that the avirulent organisms multiply in this medium seemingly at the same rate as the virulent ones, and thus that the process would appear to be one not of killing the avirulent organisms, but of changing them in such manner that they are capable of producing disease in the experimental animal.

DISCUSSION

The work represented in this study is preliminary, and any final deductions in regard to specific tissue activity and virulence are not warranted. However, the results obtained on lung media in relationship to the predilection of the pneumococcus, and the localization of this organism in human pneumonia, are suggestive of a relationship between specific substance and virulence. This relationship gains in likelihood from the fact that, whereas other tissue extracts which have been tried permit at most only the maintenance of virulence, lung extract alone of the various extracts tested supplies conditions favorable to an increase of this characteristic.

That the conditions furnished by the automatic transfer device play a definite rôle in obtaining the results here represented, is clearly shown by a comparison of the results and those obtained by the growth of pneumococci on the various tissue extracts with ordinary transfer methods. Thus during our work in the last 5 or 6 years, media made from different organs have been used to maintain the virulence of pneumococci for the mouse protection test. It was readily found that,

using the customary method of transferring, heart muscle, skeletal muscle of the calf or horse, or lung media alike were all unsatisfactory in maintaining virulence. More than that, when the extracts from different organs, with and without peptone, were used to grow the pneumococcus from repeated mouse passages, no one extract was noticeably superior to the others for maintenance of virulence. Lung medium especially has been tried with just as indefinite results as any other tissue. It would thus appear that conditions which allow a rapid multiplication of the organism in the presence of young viable cells without repression in the so called lag period, really produce significant differences in the maintenance and increase of virulence outside of the animal body.

It should also be pointed out that during this study agglutinability of pneumococci against immune sera was tested almost as a routine procedure. In support of the theory of microbic dissociation, it was found that when the organism decreased in virulence, it became agglutinable in a higher dilution with a given immune serum than in the original virulent state. Conversely, as an avirulent organism became virulent, as in the case of the experiment with lung medium, agglutinability decreased until it reached a constant, that is, a constant dilution of serum with which agglutination took place.

CONCLUSIONS

From the study of different tissue extracts as media for the growth of pneumococci used in an automatic transfer device, certain inferences are warranted:

1. Media made from calf lung or heart, or from horse skeletal muscle maintain virulence over a long period of time. Conversely, media made from calf spleen lead to a decrease in virulence.

2. Lung medium causes an increase in virulence of seven strains of pneumococci.

3. Virulence is maintained in normal horse serum; but, it rapidly decreases in immune serum, or in pneumococcus antibody solution, a finding which confirms the work of Stryker. Immune serum freed from protective antibody gives results similar to normal serum.

4. Rabbit medium made from the entire animal apparently is less suitable for the maintenance of virulence of pneumococci than medium made in the same way from guinea pig.

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