DETERMINING THE VIRULENCE OF POSITIVE DIPHTHERIA CULTURES

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SINCE THE discovery and extended use of the Schick test for susceptibility to diphtheria and the use of the toxin-antitoxin mixture for prophylactic immunization, consideration of the virulence of the diphtheria organisms isolated from the patient seems to have been neglected. The latter question is very important from the point of view of the Board of Health that is holding a carrier under observation or restraining a child from attending school. It is also important from the viewpoint of a contagious disease hospital waiting to discharge a convalescent diphtheria patient. In both instances, it is essential to know whether the diphtheria organisms cultured from the patient are capable of producing the disease in another person if transmitted.

Tests for virulence may be divided into the following groups: (1) those involving special staining reactions, as discussed by Neisser,[1] and Westbrook, Wilson and McDaniel[2] (now universally disproved); (2) those calling for animal inoculation with pure cultures with the subsequent death of the animal, as recommended by Loeffler[3]; (3) those using intracutaneous inoculations of small amounts of a pure culture, determining the result by the character of the skin reaction, as described by Romer,[4] Neisser,[5] Zingher and Soletsky[6]; and (4) those using "field" cultures, either to kill the animal, as suggested by Wayson,[7] or in smaller amounts to give a skin reaction as described by Force and Beattie[8] and Havens and Powell.[9]

Disadvantages may be demonstrated in each of the above methods. In any method involving the use of pure cultures, the primary difficulty is that of isolation, as shown by Okell and Baxter,[10] Zinsser,[11] Eagleton and Baxter[12] and others. Furthermore, while the percentage of avirulent colonies that might be fished from a positive culture is low, yet the possibility is not to be overlooked.

ISOLATION AND TIME ELEMENT

In clinical diagnosis the time element makes a test involving isolation one to be avoided. If all the steps are accurately carried out, 4 to 9 days are necessary, even if one allows no time for replating or a second isolation.

In the intracutaneous test with "field" cultures the disadvantage of isolation is eliminated, and the time required is greatly decreased, but at the same time the difficulty of reading results is correspondingly increased, through masking of the true reaction by the presence of pyogenic infection.

Since it is universally accepted that virulent organisms cannot be distinguished from avirulent by morphological or cultural methods, the differentiation must necessarily be based on toxin production. The only method that is at present recognized for determining the presence of this toxin is by animal inoculation, a virulent culture giving a typical picture on autopsy of the animal or a typical skin reaction. Comparison is always made with a control animal previously immunized with diphtheria antitoxin. It is also generally accepted that the susceptibility of guinea pigs to diphtheria toxin is approximately the same as that of man, although probably in a

slightly less degree.[13] Hence, the pathogenicity of a suspected culture of organisms for guinea pigs has been accepted as an index of its pathogenicity for man.

The satisfactory virulence test should combine, in simple technic, the best points of the tests indicated above, but should at the same time be free from their faults. In other words, a test making possible the use of a "field" culture, without first isolating the suspected organisms, would be worth trying, provided the effect of the contaminating organisms on the experimental animal could be sufficiently reduced. Such a test has been developed, and is here described, together with its results.

METHOD OF PERFORMING THE TEST

Inasmuch as it is the diphtheria toxin which is the cause of the characteristic positive reactions in the injected animals, whether the injections are made subcutaneously or intradermally, it seemed that the most logical and pertinent method of attack might be summarized in the expression, *cherchez la toxine*. With this end in view, a method was chosen and modified with the purpose of furnishing the diphtheria organisms present the most favorable environment for toxin production, as far as this could be accomplished in the presence of the usual throat and nose flora. By employing for injection the Mandler filtrate from broth cultures, the disturbing effect of the contaminating organisms was greatly reduced. Any reaction which resulted would be largely or entirely determined by the amount of diphtheria toxin present in the filtrate, and the control furnished by the immunized animal would serve as a check for the specificity of the reaction.

PREPARATION OF THE FILTRATE

The selection of a medium that will furnish the optimum conditions of growth is of paramount importance. Three factors are essential: (1) Beef infusion must be the base; (2) the pH must be 7.8; (3) a suitable grade of peptone must be used. The last factor is especially important, for it is necessary to use a peptone that has a high buffering value to offset the acid production of the diphtheria bacilli, as well as of the associated organisms. Wilcox[14] found that Witte's peptone had an inhibitory or destructive influence on the toxigenic power of Park No. 8, and recommended the use of Parke-Davis peptone. Bronfenbrenner[15] has made an extensive study of the buffering values of different peptones in connection with their use for fermentation. "Since diphtheria toxin is produced best at pH 7.8," he states, "it will be necessary to use as a buffer a peptone whose pH lies in this range. Both Witte and Difco are naturally in this range, but either Aminoid or Armour's when properly adjusted with NaOH has a better buffering power between pH 7.0 and pH 8.0 than the two former."[16] In this study Armour's peptone (2 per cent) was used. By using 10 c.c. of this medium in a 150 c.c. Erlenmeyer flask inoculated with a loopful of culture, enough toxin is produced in 24 hours' incubation to give a specific reaction in from 1 to 4 days on intracutaneous injection into a guinea pig.

The details of the preparation of the filtrate are as follows: A loopful of organisms from a morphologically positive culture is inoculated into one of the broth flasks described above, and incubated for 24 hours at 37° C. The time element is of utmost importance because of acid production by contaminating organisms, as well as by the diphtheria bacilli, which will practically detoxicate the culture on the second day. The culture is then filtered through a tested Mandler filter, and the filtrate is used in the reaction.

Two guinea pigs are prepared for the test by shaving the entire abdomen. One of these, to serve as a control, is injected intracardially with about 300 units of antitoxin a few minutes before receiving the filtrate inoculation. Four cultures may be tested on each animal used, the injection being made at intervals of about

3 cm. and consisting of 0.2 c.c. of the Mandler filtrate, given intradermally.

Results are read at 24-hour intervals for 4 successive readings and interpreted as follows in degree of positivity:

0 No reaction
+ Reddening around point of inoculation at least 6 mm. in diameter, with no induration
++ Marked induration of the skin around the point of inoculation with redness
+++ Increasing induration and redness with beginning of crust formation
++++ Crust formation with cracking of indurated area

EXPERIMENT I

That a comparison might be made between the number of living organisms and the amount of toxin produced by these organisms necessary to give a reaction when injected intradermally, the following experiment was undertaken. A subculture of Park No. 8 strain of diphtheria was kindly furnished by Dr. Benjamin White of the Massachusetts Vaccine Laboratory. A fresh 18-hour broth culture of diphtheria bacilli was counted and 0.2 c.c. of varying dilutions (giving correspondingly varying bacterial counts) was injected intradermally into each of two pigs, one normal, the other protected with antitoxin. The results of the experiment showed that 100,000 live organisms were needed to give a good positive reaction.

A control experiment substituting Staphylococcus aureus for the diphtheria bacillus showed a definite pustule formation after injecting 1,000,000 organisms, with no induration, and no crusting.

EXPERIMENT II

A second experiment was made to determine the number of diphtheria organisms necessary to produce enough toxin, when grown in Armour's peptone broth for 24 hours, to give a skin reaction when injected intradermally. The different culture dilutions were made by inoculating counted diphtheria bacilli into flasks containing 10 c.c. of broth, and incubating these subcultures for 24 hours at 37° C. After 24 hours each culture was filtered through a Mandler filter and 0.2 c.c. of each filtrate was inoculated intradermally into each of two pigs, one normal, the other protected by antitoxin. The results are given in Table I.

CONCLUSIONS OF EXPERIMENTS I AND II

From this experiment it was discovered that 10 diphtheria bacilli are sufficient to produce enough toxin in 24 hours, so that 0.2 c.c. of the filtrate will give a positive skin test. This finding compares favorably with the results shown in Experiment I where 100,000 live organisms were necessary to give a positive skin reaction, when inoculated directly into the skin.

A similar test using the filtrate of a 24-hour growth of Staphylococcus aureus gave no reaction.

From these 2 experiments it may be concluded that filtrates will give a positive skin reaction if diphtheria toxin be present and that the Staphylococcus aureus (a common contaminating organism) does not produce any substance, toxin or otherwise, that will stimulate the reaction produced by diphtheria toxin.

TABLE I

Number of Live Diphtheria Organisms Necessary to Produce Enough Toxin to Give a Skin Reaction

Number of Organisms		Results 1st day	2d day	3d day
100,000	Normal pig	+	++	++++
	Immune pig	0	0	0
10,000	Normal pig	+	++	++++
	Immune pig	0	0	0
1,000	Normal pig	+	++	++++
	Immune pig	0	0	0
100	Normal pig	+	+	++++
	Immune pig	0	0	0
10	Normal pig	+	+	++++
	Immune pig	0	0	0
1	Normal pig	0	0	0
	Immune pig	0	0	0

EXPERIMENT III

To determine the ability of diphtheria organisms to grow and produce toxin in the presence of contaminating organisms, pure cultures, of diphtheria of known virulence, were inoculated into 10 c.c. of broth, with an approximately equal amount of organisms from a negative throat culture. These mixed cultures were incubated as before for 24 hours, filtered, and 0.2 c.c. of the filtrate injected intradermally into a guinea pig. No differences were noted from the presence of contaminating organisms in normal or immunized animals.

EXPERIMENT IV

A fourth experiment was made to determine the amount of toxin, expressed in M.L.D., necessary to give a skin reaction when injected intradermally. For this text, toxin was kindly furnished by Dr. White, having a M.L.D. dose of 0.003 c.c. Expressed in another way, 0.2 c.c. of a 1:70 dilution of the toxin would contain approximately 1 M.L.D. In the experiment 0.2 c.c. of varying dilutions of the diphtheria toxin were inoculated intradermally. The results are shown in Table II.

This table shows that approximately $\frac{\text{M.L.D.}}{12}$ will give a positive skin test, indicating the sensitivity of the test.

EXPERIMENT V

In attempting to determine whether a greater or less amount of toxin would be obtained by lengthening the incubation period, 5 cultures were taken from clinically sick cases of diphtheria and incubated for 3 or 4 days. The filtrates from these cultures gave negative or weakly positive results upon animal inoculation. The explanation of these results probably lies in the production of acid by contaminating organisms, which not only inhibits toxin production but also destroys toxin already produced during the first 24 hours. However, it must be noted here that no prior or subsequent virulence tests were carried out with any of these cultures in the usual manner, but in view of the fact that the cases were clinically sick with diphtheria, it seems only reasonable to asume that the cultures were virulent.

APPLICATION OF THE TEST

Tests were made on cultures from patients harboring diphtheria bacilli. The patients were divided into 3 groups, viz.: those with clinical diphtheria, convalescent carriers, and healthy carriers. This distinction is of necessity an empirical one for there is no sharp dividing line between the groups. The first group comprised all those cases actually sick with diphtheria, whether faucial, nasal, laryngeal or wound. The criteria for making the diagnosis are those of the clinical picture of diphtheria. Such cases usually get well and are discharged with negative cultures in 21 days. If, however, their cultures persist positive longer than this period, without clinical symptoms, they then may be considered as convalescent carriers. The organisms may or may not be virulent. Healthy carriers are contacts who harbor diphtheria bacilli, yet have never had the disease. They are themselves immune, but the organisms may or may not be virulent. In all instances the Mandler filtrate was injected intradermally (as described above), and in selected cases where isolation of pure culture was possible a control test was made, making subcutaneous inoculations. All tests were controlled by similar injections into immune pigs and all these tests were negative.

In all, 81 cases were tested. An analysis of the results found, shows that all cultures giving a positive virulence test by the subcutaneous test also gave a positive test by the intradermal method, using the Mandler filtrate from positive "field" cultures. Three cultures that gave negative tests by the subcutaneous route gave positive intradermal tests. (Of these, 2 were clinical diphtheria cases and 1 was a healthy carrier.) Six cultures that

TABLE II

Amount of Diphtheria Toxin Necessary to Give a Skin Reaction

0.2 c.c. of Dilution of Toxin		Results 1st day	2d day	3d day
*1:80	Normal pig	+	dead	
	Immune pig	0	0	0
*1:160	Normal pig	+	dead	
	Immune pig	0	0	0
1:200	Normal pig	±	++++	++++
	Immune pig	0	0	0
1:400	Normal pig	±	++++	++++
	Immune pig	0	0	0
1:800	Normal pig	±	++++	++++
	Immune pig	0	0	0
1:1600	Normal pig	±	++	+++
	Immune pig	0	0	0
1:3200	Normal pig	±	±	+
	Immune pig	0	0	0
1:6400	Normal pig	±	±	0
	Immune pig	0	0	0

* Several injections on one pig.

could not be isolated in pure culture gave a positive intradermal test. (One of these was a convalescent carrier and 5 were healthy carriers.) Of 45 cultures giving negative tests by the "filtrate" method, 26 gave a negative test by the pure culture method, 6 could not be isolated and 13 were not done.

It is recognized that the data obtained is incomplete, in that all of the 81 cultures were not controlled by the pure culture method. However, the results obtained seem to warrant a preliminary report. Further study is now being done, checking especially the cases carrying organisms that are virulent.

SUMMARY

A virulence test has been developed which is comparatively free from the objections applicable to some of the tests in general use. Its advantages are:

1. The final report is ready in from 2 to 5 days after the receipt of the field culture. This economy of time is due to the fact that,

(a) It is not necessary to first isolate the suspected bacteria from contaminating organisms.

(b) The animals react more rapidly without loss of specificity of reaction.

2. Economy in the use of animals. Four or even 6 tests can be carried out simultaneously on 1 normal and 1 immune guinea pig. As a rule the animals survive, and can be used later for other purposes.

3. The probability of obtaining false negatives is less than in methods requiring pure cultures, since the possibility of losing virulent organisms during the process of isolation is obviated.

It is superior to tests involving the subcutaneous or intradermal injection of mixed cultures in that, by filtering the 24-hour broth culture, the effect of the contaminating organisms is made negligible. The test is based solely on the ability of the organisms to produce diphtheria toxin, and errors due to pyogenic infection are avoided.

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