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AGGLUTINOGEN DERIVED FROM WHOOP-
ING COUGH ORGANISMS

Earl W. Flosdorf, Lansdowne, Pa.

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This invention relates to new products useful for the determination of susceptibility to, or presence of, whooping cough and to induce in non-immune persons the development of agglutinins and hence has indicated usefulness as a test reagent for and a vaccine against whooping cough.

Whooping cough is caused by an organism known as *Haemophilus pertussis* or by related organism known as *Bacillus parapertussis*. It is thought that some cases are caused by an organism known as *Brucella bronchiseptica*. Many attempts have been made to develop a satisfactory test for susceptibility to whooping cough, or for the diagnosis of the disease. Various proposals, for example, skin tests involving injection of killed organisms or washing or extracts of Phase I H. pertussis have been advanced, but so far as I am aware, these attempts to provide a satisfactory test have been unsuccessful because no testing solution or suspension has heretofore been known which permits statistical discrimination between immune and non-immune persons. With injections of the various products that have previously been developed, there has always been a tendency to cause a positive reaction whether or not the person into whom the injection is made is or is not immune to whooping cough. The reason for this has not heretofore been known, but I believe that it is due to the fact that the toxins developed by H. pertussis do not necessarily cause the production, in man, of an anti-toxin, so that when the organisms or extracts or washings therefrom are injected, the toxin present elicits a positive reaction whether or not the person in whom the injection is made is immune or not, particularly where immunity is due to vaccination or the like. I believe that immunity to whooping cough is not the result of anti-toxins, but the result of agglutinins which react with the surface of the organisms causing whooping cough so that the organisms are readily disposed of. The agglutinin is the bacterial antibody which is formed in the blood to combat the disease. Even if the killed organisms or extracts or washings therefrom contain an agglutinin which might cause a positive reaction in an immune person and no reaction in a susceptible person, this effect tends to be masked by or confused with the positive reaction induced at least in susceptible persons by the toxins present, so that reliable discrimination between susceptible persons and immune persons has been impossible.

In accordance with the present invention, 55

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there are provided suitable skin testing media which permit reasonably accurate determination of susceptibility to whooping cough. The invention includes processes by which these compositions may be produced advantageously as well as the compositions themselves.

In accordance with the present invention, a reagent comprising agglutinin from organisms causing whooping cough, and particularly Phase I H. pertussis, substantially free from toxins, is provided for use as a skin testing reagent and as an agent for causing the development of agglutinins in the blood, i. e., immunization.

The agglutinin composition, which is substantially free from toxins, is antigenic, that is, it not only reacts with agglutinins present, but also causes the formation of agglutinins. As recovery from whooping cough apparently results from interaction between agglutinins and the agglutinin of the surface of the organism, the formation of agglutinin, caused by injection of the agglutinin of the invention, is helpful in establishing immunity.

Because the agglutinin reagent is essentially free from toxins or toxic constituents, untoward reactions, other than the agglutinin-agglutinin reaction, are avoided when the material is injected as in use in a skin-test or for immunization or for other purposes, and in this respect the reagent is radically different from the previously proposed preparations for the determination of susceptibility to whooping cough or the determination of the presence of the disease. No reaction results from intradermal injection into non-immune persons.

The agglutinin reagent of the invention appears to be the isolated antigenic part of the surface of the organisms which react with agglutinins in the blood; and the isolated reagent appears to retain this property of reaction with the agglutinin.

In preparing the agglutinin reagent in purified form, it is necessary to follow procedures which avoid deterioration of any of the constituents which, being of biological origin, are labile. The organisms used are advantageously grown on a Bordet-Gengou culture medium, although other suitable culture media may be used. The strain used should either be freshly isolated from active cases of whooping cough or regenerated from the form resulting from the desiccation from a frozen state of organisms freshly isolated from active cases of whooping cough. The organisms used should be a virulent strain, usually Phase I H. pertussis.

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The organisms as harvested from the culture medium are subjected to disintegration in any suitable way which is adapted to disintegrate the organisms without causing deterioration of the constituents. One advantageous way of accomplishing the disintegration is through the use of intense sonic vibration, as described, for example, in United States Patent 2,230,997. This treatment not only kills the organisms, but also disintegrates them without denaturation of the heat labile constituents. Another method which may be used for the disintegration of the organisms involves the use of a ball mill operating on the desiccated organisms at very low temperatures, for example, those obtained with the use of dry ice in methyl Cellosolve, the organisms being dried from the frozen state prior to the grinding. This method of drying and disintegrating the organisms is known.

After the organisms have been disintegrated by one of the suggested methods, or other suitable methods which avoid deterioration of the proteins, the disintegrated material, in the form of a suspension (as produced where the sonic method is used, or after conversion to a suspension as by extraction with water or saline or buffer solutions in the case of the dried, disintegrated organisms resulting from the low temperature ball mill disintegration) is centrifuged or otherwise cleared of insoluble material and undisintegrated organisms.

The supernatant or filtrate, which is a clear liquid, and which contains the agglutinin along with other materials, is then treated with electrolyte to adjust its pH value to that at which a thermolabile toxin, apparently typical of whooping cough organisms, and extremely toxic, present in the clear liquid, is precipitated. The normal pH of the clear filtrate or supernatant is generally between 6.0 and 8.0, and adjustment of the pH to about 4.5, as by the addition of a small quantity of acid or acid salt, generally results in precipitation of the thermolabile toxin. In some cases, the volume of the original solution, i. e., filtrate or supernatant, is too large to permit adequate precipitation of the thermolabile toxin, and in such cases it may be desirable to desiccate the original solution by removing the water from it in the frozen state in the known way and then to restore it with a smaller quantity of water.

After the removal of this toxin, other constituents present, for example, material dissolved from the culture medium, may be precipitated by a further adjustment of the pH of the solution, for example, by the addition of small quantities of acid or acid salts, and in some cases of alkali. Most of these other constituents may be precipitated by reducing the pH to 3.9, and then to 3.0, with formation of precipitates which are removed by centrifuging, etc., in the known way. Instead of accomplishing this precipitation by adjustment of the pH, it may be accomplished by the addition of other reagents, such as 25% ammonium sulfate, which will precipitate the constituents but still leave the agglutinin in solution.

After the removal of the major portion of the impurities as described, the pH of the solution is brought to the neutral point, by the addition of alkali or alkaline salts or buffer salts. If tests, to be described, show that the purification is not yet sufficient, further purification may be accomplished by the precipitation of the agglutinin itself as by the addition of a more concentrated ammonium sulfate solution or the addition of ethanol. 50% ammonium sulfate, or 68% ethanol,

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is adequate to precipitate the agglutinin and yet leave certain other materials in solution. One of the materials which will remain in the solution under these conditions is a stable toxin sometimes found in the extract. The quantity of this toxin may vary considerably and in some cases there may be no significant quantity present. If there is any present, however, it is highly desirable to remove it.

If purification by precipitation of the agglutinin is used, the precipitate is separated, dissolved in water and dialyzed free of the salt used for the precipitation. Further purification may be accomplished by a second precipitating step, using, for example, picric acid. Such a step has the additional advantage that the picric acid acts as a sterilizing agent. The resulting precipitate is again dissolved in water and the picric acid removed by dialysis leaving the purified agglutinin in a sterile condition.

In the foregoing description of the purification of the reagent, precipitation of various materials by adjustment of the pH to certain specified values has been given. It is to be understood that one or more of these precipitations may be omitted, or that the extracts may contain materials which are precipitated at different pH values and it may be desirable to vary somewhat the procedure described. Furthermore, in some cases it is possible to purify the agglutinin by its precipitation by appropriate adjustment of the pH, and such a procedure is not excluded from the scope of the invention. Thus with certain strains of *B. parapertussis*, the agglutinin itself maybe precipitated from the extract by adjustment of the pH to around 4 to 4.5, providing that the solution has been dialyzed free from salt before the adjustment, and this method of purification may be used in such cases.

In carrying out the operations described, certain precautions must be followed if any reasonable yield of agglutinin is to be obtained. Thus it is advisable to wash all of the precipitates formed very thoroughly with a wash liquid adjusted to the conditions of the mother liquid from which the precipitate is formed. Thus in those steps in which the agglutinin remains in solution and impurities are precipitated out, washing of the precipitate is necessary to avoid loss of agglutinin by adsorption by the precipitate. Where the agglutinin is precipitated, washing of the precipitate with a solution comparable to that from which it is precipitated is desirable to remove adhering contaminants.

The resulting product is a solution of purified agglutinin substantially free from toxic constituents. It is fairly stable, but for proper preservation, it is advantageous to dry it by removal of the water from the material while it is maintained in a frozen state, in the known way. Advantageously, it will be subdivided into suitable quantities for dosage, and desiccated in final containers in the subdivided quantities. Storage of the material in this desiccated state maintains the potency for long periods of time, and even under relatively adverse conditions of storage.

The purified agglutinin must be subjected to a suitable assay, to determine both its potency and the content of toxic constituents, including both the thermolabile and the stable toxin characteristic of whooping cough organisms. Various methods of assay may be used. The best method is perhaps the use of the product in actual skin-tests in either animals or humans, usually both. Animals to be used, for example, rabbits, are im-

munized. When such immunized animals are treated with the agglutinin solution, by intradermal injection, a positive reaction is obtained if the animal is immune and the solution contains sufficient agglutinin, and a negative reaction if the animal is susceptible. After such preliminary tests, it is usually advisable to also conduct a test on human beings.

Such tests will establish both the potency and the purity of the agglutinin reagent. Normal rabbits or human beings, that is, rabbits not immunized against organisms which cause whooping cough in man, or human beings susceptible to whooping cough, when treated with an appropriate, usually small, quantity of the agglutinin, will show no reaction. If the animal or person is immune, a positive reaction will be obtained. Thus upon intradermal injection, a wheal, generally circular in shape and about 10 mm. in diameter, will form. The wheal is generally edematous, with an elevation of the normal skin layer of about $\frac{1}{2}$ to 2 mm. The reaction generally appears within four hours and usually lasts one or two days, and may even last longer.

In human beings, those who have neither had the disease nor been vaccinated or otherwise become immune, show no reaction in a half hour; and in any case in which there is no appreciable redness in one half hour or any wheal which extends beyond the area of the skin into which the actual injection was made the result is regarded as negative, i. e., as showing susceptibility. With such persons, after four hours there is usually no substantial indication that any test has been made.

In persons who are either in the later stages of the disease or who are convalescent or who have acquired immunity either by recovery from the disease or successful vaccination or otherwise, a reaction usually begins to appear in about one half hour, although sometimes it is delayed for a few hours. It usually consists of erythema or edema which extends beyond the area of the actual test, and sometimes grows in size up to twenty-four hours and persists for two or even three days. Usually any reaction of this nature is regarded as positive, and indicates immunity to the disease or indicates the actual presence of the disease in the case of those who are in its later stages or recovering from it. For final decision regarding susceptibility vs. immunity, readings at two times are advisable; if the test is negative the entire time susceptibility is indicated; if positive at either or the entire time, immunity is indicated.

Because of the atypical nature of many cases of whooping cough, its diagnosis is frequently very difficult, and for this reason there has been a great demand for some test which will permit determination of the presence of the disease, as well as a test for immunity or susceptibility to the disease, and this is provided by the agglutinin reagent of the present invention.

The new agglutinin of the invention is substantially free from all toxic constituents, in contrast with the vaccines heretofore proposed or used, which are substantially free of the thermolabile toxin only. The reagent may therefore be advantageously used for immunization, by intra- or sub-cutaneous injection or in other ways. Immunization may be carried out simultaneously with testing for immunity. Thus by repeating the skin test at intervals, progressive immuniza-

tion is induced, and it is possible to observe when the immunization has proceeded sufficiently far. The reagent may also be administered in other known ways. For example, it may be precipitated with alum or other reagent and given simultaneously with other reagents used, for example, for immunization against diphtheria or other diseases.

I claim:

1. A reagent for determination of susceptibility to or for immunizing against whooping cough which comprises agglutinin derived from whooping cough organisms substantially free from toxins of the organisms, said agglutinin causing a positive skin reaction in individuals immune to whooping cough and a negative skin reaction in individuals susceptible to whooping cough, and having the property of causing the formation of anti-bodies capable of reacting with organisms causing whooping cough.

2. A non-toxic antigenic reagent derived from whooping cough organisms and causing a positive skin reaction in individuals immune to whooping cough and a negative skin reaction in individuals susceptible to whooping cough and having the property of causing the formation of anti-bodies capable of reacting with organisms causing whooping cough, comprising the purified antigenic portion of the surface of the organisms substantially free from the toxins of the organisms.

3. The process of preparing a reagent useful for determining susceptibility to whooping cough by a skin-test technique which comprises disintegrating organisms which cause whooping cough without denaturation of the proteins and other constituents, forming an aqueous extract thereof, separating the agglutinin from the toxins in the extract by precipitation, and purifying the agglutinin.

4. A non-toxic reagent comprising an antigen derived from whooping cough organisms and causing a positive skin reaction in individuals immune to whooping-cough and a negative skin reaction in individuals susceptible to whooping-cough, said antigen having the property of causing the formation of antibodies specifically related to recovery from whooping-cough and which are capable of reacting specifically with the organisms causing whooping-cough.

5. The process of preparing a reagent useful for determining susceptibility to whooping cough by a skin-test technique which comprises disintegrating organisms which cause whooping cough without denaturation of the proteins and other constituents, forming an aqueous extract thereof, adding an electrolyte to said extract to change the pH thereof to a value at which toxins are precipitated from the agglutinin, separating the agglutinin from the precipitated toxins, and purifying the agglutinin.

6. The process of preparing a reagent useful for determining susceptibility to whooping cough by a skin-test technique which comprises disintegrating organisms which cause whooping cough without denaturation of the proteins and other constituents, forming an aqueous extract thereof, adding ammonium sulphate to the extract to precipitate toxins, separating the agglutinin from the precipitated toxins, and purifying the agglutinin.

EARL W. FLOSDORF.