Studies on Pertussis with Special Reference to its Immunological Prevention

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Studies on Pertussis with Special Reference to its Immunological Prevention

Report of the Work of
Research Committee on Pertussis,
April, 1951—March, 1954

by

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Japan Society for Promotion of Science
Tokyo, Japan

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PREFACE

As effective preventive measures, the pertussis vaccine has widely been used for years in America to protect the children from the scourge of whooping cough. In Iapan the practice of pertussis vaccination was made compulsory in the effect of the Vaccination Law newly promulgated in 1949 under the leadership of U. S. Military Government. The standard pertussis vaccine under this law was supposed to be prepared following the American prescription. However, the standard pertussis vaccine, thus prepared and circulated, was found to be practically ineffective, and expensive, and further to cause severe side reaction. To cope with such situation the Research Committee on Pertussis comprising of 21 prominent pertussis research workers throughout the country including myself as listed in the roster, was organised in 1951, for the chief purpose to improve the pertussis vaccine, with Grants in Aid from the Ministry of Education.

In America the application of human sera for prevention and treatment of whooping cough become so advanced, that the γ-globulin of hyperimmune human sera in powder form was available in the market, while in Japan the human sera was practically not utilized for the same purpose, and so exploitation of usage of human sera in prevention as well as treatment of pertussis was demanded. Such being the case, studies on prevention and treatment of whooping cough with human as well as animal sera, and also the combined therapy with human sera and antibiotics were taken up as the second objective of the Committee.

To further the investigations as mentioned above, the basic studies on *H. pertussis* as well as *H. parapertussis* and also *H. bronchisepticus* were absolutely indispensable. And so they should be also vigorously tackled by the Committee.

For these reasons reiterated above, such research scope was set up as seen in the table of contents of this monograph. This research program had strenuously been pursued by the members of the Committee since its establishment in April 1951, and until March 1954, when the Committee was disbanded quite many significant findings of practical importance had been made, which were considered worthy to be published in English for the reference of the oversea researchers. And further, Grants in Aid for English publication of the summarised report of the work of the Committee was subsidized by the Ministry of Education. Thus, publication of the present English report in monograph form was realised.

This monograph was edited by Drs. S. Someya, Y. Kaneko, A. Yamamoto,

H. Fukumi and Tadaki Matsumura, summarizing the three years' quite voluminous work accomplished by the members of the Committee since April 1951 till March 1954, and translated into English by Mr. Ikuo Sugahara. The members of the Committee are all deeply indebted to them for their painstaking task. We would like also to express our sincere appreciation of the generous Grants in Aid subsidized by the Ministry of Education which enabled us to accomplish our work and further to publish the present English report of it.

Keizo Nobechi, Chairman,
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Nagoya University
Medical School, Nagoya,
January, 1955

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Studies on Pertussis Group Organisms

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Studies on pertussis group microorganisms

As stated in the Preface, the present Committee was created for the chief purposes to improve the standard pertussis vaccine for compulsory general application under the effect of the present Vaccination Law promulgated in 1949, and also to disclose the practical value of human or animal sera to treat whooping cough.

In order to accomplish those two objectives, it was indispensable to elucidate the characteristics of *H. pertussis*, especially its antigenic structure and variation formula.

Contents

I. ANTIGENIC STRUCTURE AND VARIATION

In 1921, Arkwright[1] reported on the variation of colon-typhoid-dysentery group bacilli from "S" or smooth moist form to "R" or rough granular form, and also in the same year, de Kruif[2] reported that the "S" form culture of *Bact. lepisepticum* is virulent in rabbits while that of "R" form is not.

Prior to the report of Arkwright, Bordet[3] had accomplished a comparative study on the antigenicity of blood-agar culture and plain-agar culture of *H. pertussis* and he stated in his report as follows:

> "*Bacillus pertussis* develops readily in the medium that is rich in defibrinated blood, as already described by Gengou and myself in our first article on whooping cough. It may be taught *B. pertussis* to grow on ordinary agar, in which instance it gives a thick and rather coherent layer. The two varieties of organisms obtained in this manner, although coming from a single original colony, give rise on immunizing animals to two different sera. We may consider the serum of a rabbit that has been immunized against an organism grown on ordinary agar. It is found that the serum agglutinates these organisms energetically, but has no clumping effect on a cluture of whooping cough bacillus grown on the other medium containing defibrinated blood. On the other hand, if we test the serum of a rabbit that has been immunized against the organisms grown on blood media, we find that it agglutinates both races of bacteria. A careful study of this phenomenon brings out the fact that two definite agglutinins affecting different antigens are present in different proportions. One of these antigens which is present in large amounts in the organism which has developed on blood is not to be found in the organism grown on agar".

Emphasis should be laid on the fact that he was already aware of the presence of different antigenic structures and variation in whooping cough bacilli, and anticipated K and O antigens, which will be discussed later, before the discovery of S-R variation of microorganisms by Arkwright.

Later, the same problem was further investigated by Leslie and Gardner[4], who confirmed the presence of four phases in *H. pertussis*. It was stated that the phase I is the culture newly isolated from the patient. It produces toxin and confers prophylactic immunity on mice, while it changes into the culture of phases II, III and IV by subcultures gradually losing its toxigenic and immunogenic properties. The antigenic structure and variation of *H. pertussis* were investigated also by Shibley[5], Dawson[6], Toomy[7] and others, but the work accomplished by Leslie and Gardner was the most complete and supplied the basis for all modern studies on the same subject.

Later in 1939, Lawson[8] proved the presence of capsule in *H. pertussis*. This finding, together with the recent report on K and O antigen of enterobacteriaceae by Kauffmann et al, necessitated rescrutinization of the antigenic structure of *H. pertussis*.

Anderson,[9] in 1952, published a table of the antigenic structures of pertussis group microorganisms, *H. pertussis*, *H. parapertussis* and *H. bronchisepticus*, classifying them on the basis of K and O antigens.

Contemporaneously with Anderson, but detailed studies on the antigenic structure and variation of pertussis group microorganisms were intensively carried out in our country by the members of this Committee which was established in 1951, namely by Kasuga[10], Someya[11], Hosoya[12], Kojima[13], Hirano[14] and others.

1. *Antigenic Classification of Pertussis Group Microorganisms*

Kasuga cultured about 250 freshly isolated and old laboratory strains of *H. pertussis* on Bordet et Gengou (B. G.) medium and on ordinary agar plates for 3 days at 37°C, and the colonies formed were roughly classified by the colonial appearance (binocular microscope, magnification 20). Then, detailed classification was done by the findings of toxigenicity, hemagglutination, acid agglutination, capsule staining, virulence in mice etc. Several representative strains were selected from each of the groups thus classified. Immune sera, were prepared by using rabbits inoculating them with living and heat-killed (at 100°C for 2 hours) organisms of each group. Absorption tests with the immune serum thus prepared and the organisms of the respective group were carried out for confirmation of the reliability of classification. Standard techniques of agglutination and absorption tests were agreed and followed by all members.

The immune serum for absorption test was diluted with saline 2 to 5 times. This diluted serum was added to 1 ml of the bacterial suspension (50 mg) in an amount sufficient to absorb all the antibody contained therein. The mixture was incubated for 2 hours at 37°C and centrifuged after leaving overnight at 2° to 8°C. When absorbing with 2 kinds of organisms, the same procedure was repeated. Agglutination titer of the absorbed serum was determined by taking a 10 to 20 times dilution of the original serum as the starting point.

For agglutination test, the immune serum was first put into serial two-fold dilutions. Each dilution of the immune serum was added to 1 ml of the saline suspension of organisms in a concentration of 7.5 billion per ml. The mixture was incubated for 2 hours at 37°C, left overnight at room temperature, and the reaction was observed macroscopically. In the case of heat-killed organisms, the bacterial suspension was shaken well and left standing for 20 to 30 minutes and then the homogeneous suspension in the upper part of the tube was used for the test.

Growth characteristics of H. pertussis on B. G. medium

With smooth form colonies, inspite of coming from the same strain, the freshness and the quantity of the blood used in the preparation of B. G. medium may bring about some change in the appearance of the colonies. It is difficult, accordingly, to attempt their classification only by the difference of colonial appearance, but the differentiation of antigenic structure by absorption test will enable the classification of several phases. There is no difficulty, however, in distinguishing the rough form colonies from the smooth ones. And with rough form colonies, the differentiation of phases can be done only by their morphological appearance on B. G. medium. Thus, Kasuga classified *H. pertussis* into the following forms:

Phase I (Smooth): Most of the cultures of freshly isolated strains are of phase I. The colonies are small, dome-like to hemispheric in shape, smooth, wet and glistening, no internal structure, transparent, soft and viscous, but not stringy. Upon suspending in saline solution, they readily yield a homogeneous suspension.

Phase III (Smooth): Old laboratory cultures are mostly of this phase. This phase can also be found, however, among the cultures of freshly isolated strains. Nearly all the cultures of phase I successively subcultured on B. G. medium become the cultures of phase III, after passing the intermediate phase. When compared with the colonies of phase I, the colonies of phase III are much larger and flat, not so much smooth, wet, glistening nor transparent. On touching with loop, they are much harder and present raw gum like viscosity. When suspending in saline solution, they need to be smashed thoroughly on the wall of the tube in order to obtain a homogeneous suspension.

Intermediate phase (Smooth): When phase I cultures are subcultured on B. G. medium for 5 to 10 generations, they change into this phase. The colonies resemble very much to those of phase I in appearance, but they are not so much wet, transparent nor glistening. Following the advance in variation, they approach the characteristics of the colonies of phase III.

Phase R 1 (Rough): The colonies of this phase appear suddenly while transplanting stock cultures. They are the same in size as those of phase III, elevated at the center, irregularly round in shape, no glistening the surface being rough, opaque and appear to be quite dry. When felt with loop, they are fragile and have no viscosity. Upon suspending in saline solution, granular clumps are formed by spontaneous agglutination.

Phase R 2 (Rough): The colonies of this phase are seen on rare occasions when transplanting cultures of phase III. They are flat, large in size with

irregular periphery, navel-like eruption in the center. Their surface is wet but poor in smoothness and glistening. They are opaque in color without any interior structure, fragile when felt with loop but much more viscous than those of phase R 1. Upon suspending in saline solution, minute particles are formed by spotaneous agglutination.

Phase R 3 (Rough): Morphologically, the colonies of this phase are the same as those of phase R 2 except that their inside presenting pepper-and-salt pattern in the likeness of a whirl.

The cultures of smooth form, phases I, III and intermediate, do not form colonies on plain agar plates, provided that the amount inoculated is not large. While, the cultures of rough form, phases R 1, R 2 and R 3 develop considerably large colonies on agar plates.

Morphology and capsule formation of H. pertussis in varying phases

Morphological studies carried out by Kasuga[10] disclosed the following: Upon staining by the Lawson's modification of Smith stain, the organisms of phase I are found covered with extremely thick capsules. Likewise, the organisms of intermediate phase are observed to have capsules but of varying thickness depending on the culture used. The organisms of phase III and R do not develop capsules. All cultures of *H. pertussis*, regardless of their phases, are Gram negative. The organisms of phases I, III and intermediate are minute coccobacilli and the morphological differentiation of one phase from an other is extremely difficult. The organisms of phase R 1 are the mixture of large coccobacilli and small ones but much larger than those of phase I. The organisms of phases R 2 and R 3 are short bacilli with round ends, but much larger than those of phase R 1. In short, the organisms of smooth phase can be easily differentiated from those of rough phase by staining, while the differentiation of individual smooth phase merely by staining is impossible.

Antigenic structure of H. pertussis in varying phases

The antigenic structure of *H. pertussis* in different phases was investigated by Kasuga availing the findings of agglutination and absorption tests. He proposed a table of antigenic structures of *H. pertussis*, Table 1, which included also the antigenic findings of *H. parapertussis* and *H. bronchisepticus*. Kasuga anticipated the presence of K and O antigens in *H. pertussis*, and found that K antigen is contained in the capsule and consists of the heat-labile L antigen and the heat-stable S antigen. He also proved that the organisms of phase I, through the intermediate phase gradually losing capsule, change into the organisms of phase III when subcultured on artificial medium or spontaneously in the infected host. He proved, moreover, that the intermediate phase stands

between phase I and phase III, and the organisms of this phase are O agglutinable bacilli, having K antigen decreased of its amount in varying degree, and that those of the rough phases have lost their capsule completely, being deficient of K-antigen, while their O-antigen has undergone the change into ϕ.

Regarding the organisms of rough phases, he suggested their antigenic structures to be R 1 (ϕ1), R 2 (ϕ2, ϕ3) and R 3 (ϕ3, ϕ2), and indicated the presence of weak cross agglutination among them, but the antigenic structures of R phases are intentionally excluded from the table.

2. *Resistance of the Antigens of H. pertussis in Varying Phases*

Factor sera against L, S and O antigens of *H. pertussis* were prepared. Namely, a culture of phase I *H. pertussis* organisms freshly isolated from a patient was suspended in saline solution. After adding formalin to a concentration of 0.2%, the suspension was inoculated 6 times intravenously into a rabbit with intervals of 4 days. The total number of the bacilli inoculated was 100 billion. The animal was bled 1 week after completion of the inoculation. The immune serum obtained was absorbed by the organisms of phase III in order to prepare L-S factor serum specific to phase I organisms. Then, the serum was further absorbed by the organisms of phase I heated at 100°C for 2 hours to prepare L factor serum. Likewise, S-factor serum was prepared by immunizing a rabbit with the suspension of phase I organisms heated at 100°C for 2 hours and by absorbing with phase III organisms. The serum of a rabbit immunized with phase III organisms served as O factor serum. Availing the factor sera thus prepared, the reactivity and the absorptivity of various antigens as well as their resistance against the influence of heating and chemicals were investigated by Someya and Kasuga.

The influence of heating on the reactivity of phase 1 organisms against L-S factor serum and phase III organisms against O factor serum was investigated by Kasuga and the findings are shown in Table 2. Namely, the reactivity of L antigen disappears by heating at 70°C for 30 minutes and S antigen at 120°C for 30 minutes, while that of O antigen, though decreases gradually, still remains active even after heating at 120°C for 2 hours.

Someya observed the influence of heating on the reactivity of phase I organisms against L, S and O factor sera. The findings are shown in Tables 3 and 4. As shown in those tables, L agglutination, though weak, remains even after heating at 69°C for 1 hour. But it completely disappears by heating at 70°C for 1 hour, whereas O agglutination begins to appear. Namely, it suggests that L antigen has been inhibiting the development of O agglutination, but upon its disappearance by heating, the agglutination by O antigen begins to appear. Moreover, he pointed out, it seems that K (L-S) antigen

is freed at this moment from the bacterial cell. The bacterial suspension becomes slightly viscous by heating at 65°C to 70°C and as the result of which the reading of agglutination reaction becomes ambiguous. By the heating at 100°C, however, such difficulty in reading the reaction disappears all positive agglutinations becoming quite distinct. In short, the reactivity of L antigen is lost by heating at 70°C for 30 minutes, whereas, O antigen is a heat-resistant antigen still maintaining the reactivity even after heating at 120°C, and it is quite evident that the inhibitory influence of K antigen on O agglutination disappears by heating of the bacterial suspension.

It is to be added, however, careful scrutinization of the details of experimental conditions is necessary, for the investigation of the resistance of these antigens against physical and chemical actions is a delicate task.

3. *Characteristics of H. pertussis in Varying Phases*

Mutual relationships of the hemolysis, acid agglutination, catalase activity, carbohydrate fermentation, hemagglutination, virulence and the toxicity of *H. pertussis* were investigated by Kasuga on a total of 36 strains consisting of varying phases described above. Carbohydrates employed were glucose, galactose, dextrin, sorbit, maltose, mannit, adonit, saccharose, lactose, rhamnose, inosit and arabinose. The findings are shown in Table 5. Namely, *H. pertussis* organisms of phases I, III and intermediate show hemolysis, while those of phases R 1, R 2, R 3 do not. Regarding acid agglutination, the optimal pH for the organisms of phase I, in which K antigen is most abundant, is 4.2, and this optimal pH changes towards acid side as the amount of K antigen decreases. In addition, the wide range of the optimal pH is the characteristics of the organisms of intermediate phase. Catalase activity is positive in all cases except the cultures of phases R 2 and R 3 and the fermentation of carbohydrates is completely negative. Hemagglutination, though somewhat different depending on the kind of the blood used, is negative with the cultures of phase III and R phases, while the cultures of phase I and intermediate phase show the agglutination of varying degrees. As for the virulence and toxicity, they are found to decrease following the advance in the change from phase I to R phase when observed by Gundel's reaction and by the intracerebral or intraperitoneal lethal dose for mice.

4. *Clinical Course and the Phase of H. pertussis Isolated*

Strains of *H. pertussis* freshly isolated from patients on varying clinical stages of whooping cough were examined of their phases, basing on the colonial findings as well as on the slide agglutination tests made with L, S and O factor sera, in order to know the relationship existing between the

bacterial phase and the clinical course. And such findings were obtained as follows:

Clinical course and bacterial phase

As shown in Table 6, in the initial stage of the disease, the strains isolated are mostly of the phase I. However, with the course of the disease days, the detection rate of the organisms of intermediate phase and the phase III increases.

Phases of the strains isolated from the same patient on different clinical courses

The patients from whom only the organisms of phase I were isolated throughout entire course of the disease were 12 (13%) out of the 39 examined. In 17 cases, the organisms isolated in the beginning were of phase I, and later they were mixed with the organisms of intermediate phase, or they become the mixture of intermediate phase and phase III. In the remaining 10 cases (26%), the organisms isolated were the mixture of the phase I and the intermediate phase. Namely, there was a distinct tendency that, in most cases, the organisms isolated in an early stage of the disease were of phase I, but following the advance of the clinical course the phase of isolated organisms changed into intermediate phase and phase III.

Variation of H. pertussis and clinical course

Strains of the phase I organisms isolated in the above were successively transplanted on B. G. medium in order to observe the relation between days of illness and variation. The strains isolated in the later stage of the disease presented a tendency to change into intermediate phase much quicker than those isolated in an early stage. The strains isolated from the patients who yielded only phase I organisms throughout the entire course of the disease were quite stable on B. G. medium regardless to the time of isolation. Out of 5 such strains used in the present study, 3 strains were isolated as late as 13 to 20 days of ilness, but they maintained the characteristics of phase I even after subcultures on B. G. medium for 30 generations.

It is disclosed from the above mentioned findings that, not to speak of the organisms of phase I, the organisms of intermediate phase and phase III are also recovered in a fairly high rate from the lesions of children suffering from whooping cough and, moreover, that the detection rate of the organisms of intermediate phase also increases following the advance of the course of disease.

The organisms of intermediate phase and phase III, are considered to have

changed from the organisms of phase I within the patient's body, however, there may be an infection caused directly by the organisms of intermediate phase or phase III. For an instance, in some of the above patients, all the organisms isolated as early as 3 to 4 days after onset of the disease were of intermediate phase suggesting the possibility of an infection developed by the organisms of such a phase.

5. *Maintenance of the Phase I of H. pertussis*

Separately, Hosoya[12] proved that the bacterial toxin of staphylococcus has an aggressin-like activity on the *in vivo* proliferation of various pathogenic microorganisms, so that, investigations were made on the availability of this bacterial toxin for the *in vivo* maintenance of the bacterial phase of *H. pertussis*.

An amount of crude staphylococcus toxin was added to each of the bacterial suspension of *H. pertussis* phase I and phase III. Mice were inoculated intratesticularly with this suspension each in a dose of 0.025 ml and the fate of inoculated organisms was followed. The results were as follows:

a. When a small amount of the saline suspension of *H. pertussis* phase I added with staphylococcus toxin was inoculated into the testicle of mice, the organisms recovered as late as 25 to 26 days still maintained phase I specificity without any variation into phase III. While in the case of inoculation done only with the saline suspension of phase I organisms, the isolation of *H. pertussis* became negative by the time of 25 to 26 days after inoculation.

b. Likewise, in the case of intratesticular inoculation done with the suspension of phase III organisms alone, the detection of *H. pertussis* in the testicle was negative by the end of 30 days. But when the suspension was added with staphylococcus toxin, the isolation of *H. pertussis* was positive even after 30 days still maintaining the characteristics of phase III organisms.

c. As the intratesticular inoculation of mice maintains all the characteristics of phase I organisms for a relatively long period of time, it is considered to be an efficient measure for the maintenance of phase I organisms.

Summaries and Discussions

The above are the findings of the studies accomplished by Kasuga, Someya, Hosoya et al on the problems concerning (1) the antigenic classification of pertussis group organisms, (2) the resistance of *H. pertussis* antigens, (3) the characteristics of varying phases of *H. pertussis*, (4) relationship between the clinical course and the phase of *H. pertussis* isolated, (5) maintenance of the phase of *H. pertussis in vivo*.

H. pertussis phase I has been disclosed to possess K antigen which consists