

THE PREPARATION OF ANTI-TYPHOID SERUM IN THE HORSE FOR THERAPEUTIC USE IN MAN

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INTRODUCTION

THE object of this paper is to describe the immunizing procedures that have proved to be the most suitable up to the present time for preparing a potent serum from the horse for the treatment of typhoid fever. The principles on which the methods are based have their origin in the studies of Felix and his collaborators on the antigenic structure of *Salmonella typhi*. The technical details that follow will, we hope, assist those who are interested in the preparation of the serum as a therapeutic agent to obtain products which maintain a consistently high level of potency. This objective is not readily attained, but is of paramount importance, because the clinical use of products that fall below the permissible limits of potency would tend to bring discredit upon the serum as a specific remedy owing to insufficient dosage. The adoption of standards of potency that are prescribed by an official authority is an obvious desideratum and would guard against this risk, but in the meantime we shall be pleased to help by providing the requisite cultures and serological reagents.¹

¹ Application for these should be made to one of us (A. F.).

A SURVEY OF THE RESULTS OBTAINED IN THE HORSE FROM THE USE
OF VARIOUS IMMUNIZING MATERIALS

The production in the horse of anti-typhoid serum designed for therapeutic use in man is by no means easy, because experimental evidence shows that the efficacy of the serum depends upon the presence in it of effective amounts of two essential protective substances, namely, the "O" and the "Vi" antibody (Felix & Pitt, 1934; Felix, 1935). Immune serum containing O antibody of high titre can be obtained from the horse without difficulty; whereas the production of Vi antibody of high titre is beset with difficulties.

It is known from work already published (Felix *et al.* 1934; Felix & Bhatnagar, 1935) that the Vi antigen of the typhoid bacillus is highly susceptible to heat and to such apparently unimportant treatment as exposure to 0.5% phenol or 0.2% formalin. Sera from rabbits immunized with suspensions of Vi strains heated to 58° C. for 1½ hr. or sterilized by 0.5% phenol either contain no Vi antibody or only negligible amounts of it. On the other hand, the Vi antibody produced in response to immunization with formolized Vi antigen exhibits a peculiar "functional deficiency" which is characterized by a reduced power of promoting phagocytosis and of protecting against infection with virulent strains of the typhoid bacillus; these effects render formolized suspensions or extracts unsuitable for use in the preparation of therapeutic sera of high potency.

The first two horses to be immunized received a course consisting of doses of heat-killed bacilli followed by doses of living bacilli of the highly virulent strain Ty 2. The second immunizing course consisted of a series of doses of an extract derived from this strain, which had been sterilized by the addition of 0.2% formalin. After a third immunizing course, which consisted of a series of doses of killed bacilli followed by a number of doses of living bacilli of the strain Ty 2, one of the horses became ill with a continued fever, and was found to be passing fully virulent typhoid bacilli in the urine. When the illness had lasted for nearly a month recovery seemed to be unlikely and the animal was destroyed. Cultures from the kidneys yielded pure *S. typhi*. An account of the clinical and post-mortem observations has been given by one of us (Petrie, 1936).

The observation by Felix & Pitt (1935) that some rough avirulent variants of *S. typhi* contain the Vi antigen and stimulate the formation of Vi antibody which is not deficient in protective and opsonizing activities, made it possible to substitute such a strain (Ty 441, R 5); the response in the horse proved to be adequate. Our experience since February 1935 has shown that the substitution of this strain for the highly virulent smooth strain Ty 2 has greatly conduced to the safety of the animals that are being immunized; for the case of typhoidal bacilluria referred to above, which occurred in December 1934, has remained an isolated one.

Although the risks inherent in the original method were greatly diminished they were not entirely eliminated, and the necessity of using freshly prepared suspensions of living bacilli, even when these were of low virulence, caused technical difficulties and complicated the immunizing procedures. Numerous attempts have therefore been made during the past 2 or 3 years to discover some method of preparing a sterile antigen capable of replacing the "natural" Vi antigen in living bacilli. All these attempts have failed; a brief description of them follows.

(a) *Alcohol-treated vaccine*

It was stated in a previous paper (Felix & Pitt, 1936) that the agglutinogenic properties of the Vi antigen of *S. typhi* are not impaired by treatment with alcohol although the Vi agglutinability of alcohol-treated suspensions is much reduced and for practical purposes almost annulled. Vi immune sera of high titre are readily obtained by immunizing rabbits with alcohol-treated suspensions of smooth strains, containing both the Vi and the O antigen, or of rough strains, containing the Vi antigen alone. The Vi antibody elaborated by these suspensions has been found to be in all respects identical with that resulting from immunization with the "natural" Vi antigen from living Vi strains. Thus the potency in protective action of both varieties of immune serum invariably runs parallel with the titre of Vi agglutination: a correlation which serves as a criterion of the functional efficacy of the Vi antibody. Alcohol-treated vaccines soon became the antigen of choice for the preparation of potent Vi immune sera in the rabbit and it was natural to hope that this simple method could also be employed for the production of potent Vi sera from the horse.

This expectation, however, did not prove to be well founded. A considerable number of horses have been immunized by giving single or multiple courses of alcohol-treated suspensions of the strain Ty 2, which is particularly rich in Vi antigen, but in no instance have we succeeded in obtaining an immune serum with a sufficiently high content of Vi antibody. The O titres of most of these sera were high, varying from 1 : 40,000 to 1 : 100,000, as estimated by agglutination against the strain O 901. The Vi titres, however, remained far below the accepted standard titre, which for a natural, unconcentrated serum is 1 : 1200, as estimated by agglutination against the strain "Watson". The serum from horses immunized with the alcohol-killed vaccine usually had a titre of Vi antibody not more than 1 : 200; only three horses out of a total of eighteen immunized with an alcohol-killed vaccine elaborated a serum with a Vi titre of 1 : 400 or 1 : 500.

This result could not be improved upon by the use of a vaccine containing the Vi antigen alone, instead of the Vi + O vaccine derived from the strain Ty 2. Consequently the hope that Vi antibody of high titre could be produced by immunizing horses with alcohol-killed vaccines had to be abandoned.

(b) Phenol-treated vaccine

The peculiar susceptibility to the action of phenol of the Vi antigen of the typhoid bacillus was further investigated and it was found that the loss of its agglutinogenic activity is not a permanent one brought about by destruction of its capacity to induce the formation of circulating Vi antibody. Thus, when suspensions of Vi strains were sterilized by treatment with 0.5% phenol during 48 hours at room-temperature and the phenol was subsequently removed and replaced by fresh saline solution, such phenol-treated vaccines invariably stimulated the production of circulating Vi antibody in the rabbit and this antibody was found not to be deficient in protective power. The inactivation by phenol of the agglutinogenic function of the Vi substance is apparently due to some kind of reversible reaction between this antigen and phenol.

Two horses were immunized with a phenol-killed suspension which was subsequently washed in saline. One of the horses received a suspension which contained Vi and O antigen and the other a suspension with Vi antigen alone. Both horses failed to produce Vi antibody.

(c) The antigenic fraction prepared by Topley and Raistrick

Prof. Topley kindly supplied us with a considerable quantity of a purified antigen from an O + Vi strain, prepared according to the method of Raistrick & Topley (1934) and described by Topley *et al.* (1937). Our thanks are also due to Prof. Topley for the supply of serum from a rabbit which had been immunized with the purified antigen and had an O agglutination titre of 1:2000 and a Vi agglutination titre of 1:900. Protection tests with this serum carried out in parallel with our standard serum showed that the Vi antibody which was produced in response to the injection of the purified antigen was not deficient in protective action.

Three horses were immunized with this antigen. Two of them received one course of intravenous injections and one had two immunizing courses; the total amounts of the antigenic substance injected were 19, 38 and 64 mg. respectively. According to Prof. Topley's data these quantities of the antigenic substance correspond approximately to the antigen present in $2,000,000 \times 10^6$, $4,000,000 \times 10^6$ and $7,500,000 \times 10^6$ bacilli. The three horses produced only negligible amounts of Vi antibody although the serum from each of them contained O antibody of high titre.

(d) The antigenic fraction prepared by Henderson and Morgan

Reference may be made to the paper recently published by our colleagues Henderson & Morgan (1938), who investigated the properties of the antigenic fractions extracted from various strains of the typhoid bacillus according to the method previously described by Morgan (1937). Henderson & Morgan found that the serum from rabbits immunized with their extracted antigens

contained Vi antibody which in mouse-protection tests exhibited a functional deficiency similar to that which results from the use of formolized Vi antigen. These authors, likewise, were unable to produce Vi serum of high titre in the horse, whereas rabbit sera with a high Vi-agglutinin content were easily produced by immunization with fractions obtained by Morgan's method from strains that possess the Vi antigen.

(e) *A summary of the experimental results*

From the account given in this section the conclusion would appear to be well established that the horse and the rabbit differ greatly in their ability to respond to immunization with the Vi antigen. The "natural" Vi antigen, as contained in the living bacterial cells, stimulates the production of Vi antibody of high titre in both animal species, whereas the modified Vi antigen, which is present in the intact bacterial cells after treatment with various chemicals or in the antigenic fractions of Topley and of Morgan, is capable of inducing the formation of abundant Vi agglutinins in the rabbit but not in the horse. A comparable difference in immunity response between the two species of experimental animal is not known to exist in respect of the H and O antigens of the typhoid bacillus.

The underlying factor that accounts for these results is entirely obscure and we shall refrain from speculating on its nature, but the immediate bearing of the established facts on our particular problem, namely, the preparation of potent anti-typhoid serum in the horse, is obvious. The "natural" Vi antigen from living bacilli cannot, at the present time, be replaced by any sterile vaccine or antigenic fraction. Although hope of success in future attempts has by no means been abandoned, the position remains at present much as it was stated three years ago (Felix & Pitt, 1935). The O antibody can be produced by injecting a dead vaccine or a bacterial fraction containing the O antigen, whereas the living bacilli remain the indispensable source of the "natural" Vi antigen required for the elaboration of the Vi antibody.

THE ANTIGENIC MATERIALS NOW EMPLOYED IN THE ROUTINE
PROCEDURE OF IMMUNIZING HORSES

(a) *The O antigen.* This is given in the form of a vaccine of the strain Ty 2 which has been killed by treatment with alcohol. The growth on plain agar from one Roux bottle is washed off with 20 c.c. of saline into a flask containing glass beads, and 60 c.c. of 96% alcohol is added. After storage for 2 days at room temperature in the dark the suspension is centrifuged and the deposit washed four times by centrifuging it with fresh saline. It is important not to add any preservative whatsoever. The quantity of vaccine required for an immunizing course for each horse is kept in sealed ampoules in the cold room; each ampoule contains one dose. The unused residue of any ampoule that has been opened is discarded. The vaccine for each series of injections should be freshly prepared.

The alcohol-treated vaccine has been selected in preference to all other sources of the O antigen, not because it is superior to them in regard to the production of the O antibody, but because it alone, amongst all the various types of vaccine and antigenic fraction so far tested, produces in the horse a definite, though small, amount of Vi antibody of full functional efficacy. It has been already mentioned that the serum from horses immunized with the alcohol-killed vaccine from the strain Ty 2 usually has a Vi-agglutination titre not more than 1 : 200. In view of the relatively low Vi-agglutinin titres resulting from the immunization with the living bacilli, it is of considerable advantage to be able to produce invariably even a small amount of Vi antibody during the course of the preceding injections of the killed vaccine.

The strain Ty 2 is preferred to any other smooth virulent strain of *S. typhi* because cultures of this strain are known to contain maximum amounts of Vi antigen and to be superior to other strains in maintaining this character uniformly, so long as the cultures are adequately treated.

(b) *The Vi antigen.* The strain that is now used for the production of Vi antibody is the variant strain "Ty 441, 6 S". This variant is particularly rich in Vi antigen but contains no O and very little H antigen and thus the system of cells which is concerned with antibody production is free to concentrate upon the formation of the Vi antibody.

The origin of the variant, which was derived from the rough strain Ty 441, R 5 used in earlier work (Felix & Pitt, 1935) is as follows: One of the authors (G. F. P.) had kept the parent strain for 10 months at 37° C. without subculture in a sample of horse serum which contained H, O and Vi antibodies; his colleague (A. F.) isolated from this culture a variant, "6 S", which is still antigenically "rough", that is, devoid of O antigen, but which otherwise resembles the "smooth" type in broth and on agar; suspensions are stable in 5% saline and after heating at 100° C. It would be unwise to assume that the immune substances in the serum culture played a significant part in the origin of this variant.

The virulence to mice of the variant Ty 441, 6 S is of a low degree, similar to that of its typically "rough" parent strain, and large doses of a living vaccine of this strain can be given to the horse without causing unduly severe reactions. Freshly prepared saline suspensions of the culture are injected into the horse without undue delay in order to ensure that the bacilli are in the living state and that the Vi antigen is in its "natural" unaltered condition.

THE SELECTION OF SUITABLE HORSES

Horses are preferred which weigh 10–12 cwt., are between 15 and 20 years of age, and are in good condition, of good physique and of placid temperament. The doses, without exception, are given intravenously. A trial bleeding of 2 l. or more carried out by means of an ordinary bleeding cannula will give some idea of the reaction of the animal to the intravenous technique. If a

group of horses is tested in this way the nervous animals can be set aside for other purposes.

There is no constant relationship between the titres of the natural Vi and O antibodies in the normal serum and the subsequent immunity response. We have therefore abandoned the estimation of the titres of the natural antibodies as an aid in the selection of the horses although we continue to test the normal serum in order to secure further information.

THE IMMUNIZING PROCEDURE

A group of horses is chosen as already indicated and, in accordance with the provisions of the Therapeutic Substances Regulations, they are protected against the risk of spontaneous tetanus infection; a dose of 50 c.c. of tetanus toxoid with the addition of 2% alum is injected intramuscularly. This dose is amply sufficient to protect them during the comparatively brief period of the typhoid immunization. When, after a few days' rest, they have again become fit to receive a dose, a course of the alcohol-killed vaccine is begun. The initial dose should not be greater than 4000 million bacilli because otherwise delay may be caused by the resultant toxic reaction; even this dose may cause a considerable reaction; nevertheless it is worth waiting until the animal becomes normal because the subsequent immunity response may prove to be good. The later doses, represented as multiples of one million bacilli, are as follows: 8000, 20,000, 40,000, 80,000, 160,000, 240,000 and 300,000. The doses are diluted in saline to bring the volume to 50–100 c.c. and should be given, if practicable, on alternate days. The scheme of dosage is conditional on the reaction of the individual horse to a particular dose. Thus it may be advisable to give only a portion of a dose or the full dose may be repeated once or even twice. The inoculum should be administered very slowly in order to obviate the risk of a severe allergic reaction.

A sample bleeding is taken 4 days after the completion of a course of injections, and the horses whose serum is found to contain the highest O-agglutinin titre—1:80,000 being regarded as the minimum—are immunized without any delay with the living "rough" Vi-containing variant, Ty 441, 6 S, since the O titre gradually falls during the immunizing course with the living bacilli. Those horses whose serum gives an O titre of less than 1:40,000 after the first course of doses of alcohol-killed vaccine are rejected.

The suspensions used for the series of doses of living bacilli are freshly prepared in saline from 24-hour growths on digest agar in slopes or Roux bottles and are injected according to the following scheme of dosage, the numbers indicating millions of bacilli: 4000, 8000, 20,000, 40,000, 80,000, 120,000, 160,000 and finally 200,000. The precautions outlined above for the doses of dead vaccine apply equally to this immunizing course. If a sample bleeding is taken not less than 3 days after the last dose and if it gives, when tested, an adequate titre of the O and the Vi antibody a bleeding of 8 l. is

taken on the fifth day and the horse bled out from the carotid artery under an anaesthetic on the following day. This plan has been found to be the best since there is a strong tendency for the agglutinin titres to fall during subsequent immunizing courses, even after a prolonged period of rest. When it is intended not to bleed out but to take two full bleedings from a horse the fourth and sixth days will, as a rule, be found to be the most suitable.

If the response from the first course of killed or living bacilli is deficient and a short rest period ensues, a small dose of 4000 or 8000 million bacilli should be given a day or two before proceeding with the larger doses.

The titre of the antibodies in the natural serum before it is subjected to a process of concentration should be not less than 1 : 1200 for the Vi antibody and 1 : 50,000 for the O antibody as estimated by agglutination tests.

There appears to be no correlation between the responses of individual horses to the Vi and the O antigen. The natural serum of one horse, however, was exceptional in giving a titre of 1 : 100,000 for the O antibody and 1 : 3000 for the Vi antibody, a total antibody content which is virtually equivalent to that of the average batch of concentrated serum. Several horses have yielded serum with a Vi titre of 1 : 1800. As a rule, however, it is necessary to select sera obtained from a number of horses and to pool an amount of each of them in such proportions that the content of O and Vi antibody in the mixture is not less than the limits mentioned.

The details of the tests for the quantitative estimation of the Vi and the O antibody in therapeutic anti-typhoid serum will be described in a subsequent paper. It is a matter of great importance to take the utmost care in estimating the content of each of the two antibodies in the natural serum from each separate bleeding. This enables an exact calculation to be made of the quantities of serum from the different bleedings that can be pooled for the preparation of the concentrated product.

THE REACTIONS PRODUCED IN THE HORSE BY THE IMMUNIZING MATERIALS

The first two horses to be immunized received an initial dose of 20,000 million heat-killed bacilli of the virulent strain Ty 2. The intravenous injection of this dose was followed in both horses by a severe toxic reaction, the effects of which lasted for nearly a week. The temperature was raised, the pulse rate quickened and tremor, marked injection of the mucous membrane of the nose and mouth, some purging, and loss of appetite were present. But in later immunizations the injections are tolerated better and the febrile reaction has disappeared as a rule by the next morning, except when doses of living bacilli are given. The reactions from these resemble, but are, in general, more severe than those which follow doses of killed bacilli; this is indicated by the relatively high mean temperature and pulse rate. Thus the evening temperature and pulse rate of seven horses after receiving doses of killed bacilli is 102.0° F. and 49, the mean of 150 observations, as compared with 104.0° F.

and 58 after doses of living bacilli, the mean of 101 observations. A series of doses of a formolized extract was given to two horses, which had already received doses of heat-killed and living bacilli. The reactions were of the usual kind but were exceptionally severe and caused attacks of cardiac weakness verging on collapse; this symptom was perhaps associated with the sudden introduction of toxic substances in solution into the circulation.

The general tremor and the dyspnoea which often appear during the administration of the dose may be allergic in nature.

If the doses are given without due regard to the sensitiveness of the animal loss of appetite and of condition necessitating a prolonged rest may follow; a result which it is better to avoid, because, as already stated, the response is not likely to improve if the immunization should be resumed.

THE CONCENTRATION OF THE SERUM

The natural serum from immunized horses is obtained by allowing the blood to clot in glass jars and expressing the serum by means of a metal weight. A pool of the sera is formed in such proportions that the mean titres are adequate, namely, not less than 1 : 1200 for the Vi antibody and 1 : 50,000 for the O antibody. The method of concentration is designed to retain in the final product the whole of the euglobulin fraction of the serum and about half of the pseudoglobulin; the details of the process are as follows:

The serum, water and ammonium sulphate are taken in the proportions of 1000, 2000 c.c. and 660 g. and are well mixed so as to dissolve the salt; the specific gravity of the mixture is 1.125–1.126. The material is filtered through chain-cloth and the precipitate pressed in order to remove as much of the liquid as possible. The pressed precipitate is dialysed in Cellophane bags against cold running water for 3 or 4 days. The contents of the bags are pooled and distilled water is added in such a proportion that the solution of dialysed globulins corresponding to 1000 c.c. of the original serum is contained in a volume of 250 c.c. 1% NaCl and 0.35% tricresol are added. The product is filtered and tested for sterility.

This process represents a concentration of at least three times and yields a serum with a protein content of 12–14%. The final product, when tested after filtration, should give the following minimum titres:

In agglutination tests:

Vi titre 1 : 3000 to 1 : 3600 against the strain Watson;

O titre 1 : 120,000 to 1 : 150,000 against the strain O 901.

In mouse-protection tests against 3 M.L.D. of the living virulent strain Ty 2:

With a dose of 0.08 c.c. nearly 100% survivors;

With a dose of 0.04 c.c. 50% to 75% survivors.

In mouse-tests for the neutralization of 1 M.L.D. of killed typhoid bacilli (strain O 901):

With a dose of 0.3 c.c. nearly 100% survivors;

With a dose of 0.15 c.c. 50% to 75% survivors.

PRECAUTIONARY MEASURES AGAINST THE RISK OF INFECTION TO THE PERSONNEL

Despite the fact that the culture used in the living state for producing the Vi antibody is a rough variant, we consider it desirable to take every precaution to prevent the spread of the infection because we cannot disregard the possibility that a transformation of the living rough bacilli into the smooth highly virulent form may be effected by their utilizing the smooth antigen which may be present in the blood and tissues of the horse: an analogous change to that which has been experimentally produced *in vivo* and *in vitro* with rough cultures of *Pneumococcus*. Accordingly, horses, when undergoing treatment with the living bacilli, are kept in special loose-boxes in a separate block, the doors and windows of which are screened to prevent the entrance of flies. The bedding consists of compressed peat which absorbs the urine; when soiled it is incinerated. The attendants receive a dose of typhoid vaccine at appropriate intervals and are provided with rubber thigh boots which are soaked in foot-baths of antiseptic solution. In a room immediately adjoining the stable the syringes and needles intended for the doses or for blood samples are sterilized. When a horse is bled out the carcass is dismembered and incinerated *in situ*.

ACKNOWLEDGEMENTS. We are glad to acknowledge the kind help of Miss R. M. Pitt who assisted us in maintaining the cultures and performing agglutination tests. Our thanks are also due to our colleague Dr W. T. J. Morgan who arranged for the injections of the antigen kindly supplied by Prof. Topley.

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(*MS. received for publication 8. IV. 1938.—Ed.*)