The Preparation of Diphtheria Antitoxin and Prophylactics

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<Alvar Liddell narrates>

In this film we'll be showing you the stages in the preparation and testing of diphtheria antitoxin and prophylactics. The serological laboratories and stables were photographed under wartime conditions.

To combat diphtheria there are two needs: firstly, to inject patients suffering from the disease and susceptible contacts with antitoxin which has been produced artificially in animals; and secondly, to immunise every individual, preferably during infancy, with a harmless prophylactic so that when exposed to risk he does not contract the infection. Naturally today stress is being laid on the second of these measures of control, namely prevention.

Diphtheria is a disease caused by a bacillus, corynebacterium diphtheriae, or the Klebs-Löffler bacillus. The bacilli multiply locally on the surface of the body, the most common site being the fauces where a membrane forms consisting of fibrin, necrotic cells and bacteria. The bacillus rarely invades the internal organs or enters the blood

stream but it makes a very powerful poison or toxin which diffuses from the bacillus and is absorbed by the cells of various organs and tissues; many of these cells are destroyed. The toxin passes into the blood stream and is dispersed all over the body. Amounts large enough to cause death may reach the susceptible cells of the heart.

In the diagrams that follow, toxin will be represented by this symbol, and antitoxin by this.

In response to diphtheria bacilli in numbers too small to cause obvious disease, many human beings and animals produce antitoxin. If attacking bacilli produce more toxin than the antitoxin can deal with, the result will be disease. If there is more antitoxin than toxin, no harm is done by the toxin. The germs may survive and the immune individual who becomes thereby a carrier, may pass them on to less resistant, non-immune persons who will develop diphtheria whilst the carrier remains healthy.

The first consideration in the study of diphtheria must be an examination of the toxin which the bacilli produce. If 1/5000 of a millilitre of a potent diphtheria toxin is injected into a guinea pig, the animal will die; but the simultaneous injection of 1/100 000 of a millilitre of unconcentrated antitoxic serum may save its life. Thus an amount of antitoxin may protect against 20 times its volume of toxin.

We shall now see the toxin being prepared in the laboratory. A broth is made by digesting beef with a preparation of ox pancreas. Maltose, sodium acetate and sodium lactate are added as energy sources. The broth is filtered through filter paper. In order to sterilise it the broth is then passed through Berkefeld type filter candles. Here we see the candling apparatus being assembled. After assembly, the candling apparatus is itself sterilised by autoclaving. Then the broth is pumped through this apparatus into 4 litre bottles, 700 millilitres of broth to each bottle. The bottles are then heated for 10 minutes at 100˚C in a steam steriliser, and when cool are transferred to the incubator. Here the broth is inoculated with the bacillus – the Park-Williams no. 8 strain is particularly suitable. The bacillus is grown in this broth for 10 days at 34˚C. This is its appearance after 24 hours and at the end of 10 days.

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After this time the culture is transferred to the filter room where 5 millilitres of toluene are added to each bottle as an antiseptic. The bottles are shaken and left for 24 hours. After this period the cultures are filtered through filter papers. Then the toxin is bulked. To complete the preparation of the toxin, 0.5% of phenol is added as a preservative and the liquid is pumped through sterilising candles. The amount of specific toxin present in any preparation may be measured by its action on guinea pigs; the minimum lethal dose of toxin is the least quantity which, given subcutaneously, will kill within 5 days the majority of guinea pigs injected. This measurement of toxin is rarely made now except for experimental purposes. Injected intradermally, 1/5 000 000th of a millilitre of good toxin will produce necrotic lesions surrounded by areas of congestion. Here are lesions of this type 48 hours after injection.

The utility of a toxin is best measured by the flocculation reaction. Equal volumes of toxin are measured into a series of tubes and varying amounts of antitoxin are added. After mixing, the tubes are placed in a water bath at a temperature of 50˚C. A flocculent precipitate appears first in the mixture most nearly neutral; from the composition of this mixture we can calculate the amount of toxin with which 1 unit of antitoxin combines – that is the Lf dose.

We shall now consider the conversion of toxin into the harmless preparation called toxoid. To toxin which has not been treated with phenol, formalin is added and the mixture is kept for 2-3 weeks in an incubator at 37˚C. During this period the toxin has been rendered harmless so that it will neither kill guinea pigs nor produce intradermal lesions on injection. This preparation, toxoid, is a valuable immunising agent. In the laboratories toxoid is shown to be free from all active toxin by injecting 0.2 of a millilitre intradermally into rabbits. No reaction follows this injection although high dilutions of toxin produce well-marked reactions. Both toxin and toxoid can be measured by flocculation. But toxoid can be measured in animals only by blending with a known toxin.

Antitoxin is very closely associated with certain proteins of the serum of horses which have been injected with toxoid. At the laboratory stables, horses are kept for the preparation of antitoxic and other sera. The best animals for the production of antitoxin are those 6 years old and over. They should be sturdy and good-tempered. The amount of natural antitoxin possessed by these animals varies greatly. Horses which have a great number of human contacts, for example army horses, possess more antitoxin than, say, farm horses.

On arrival at the stables, horses are examined by a veterinary surgeon for their fitness and general suitability. Those found to be suffering from any form of infectious or constitutional disease are rejected as unsuitable. Subject to further examination, this horse is temporarily accepted and a full description of the horse is made. As soon as possible, the shoes are removed. The stable number is then burnt on the hoof; this is done in order to ensure accuracy in identifying the horse when it is to be injected or bled. This number accompanies the horse throughout his course. New horses must be kept in the isolation stables for 7 days and during this time they're under constant observation to ensure that they are free from any disease which may have been latent at the time of acceptance. The horse's record card and temperature sheet are filled in. This card contains details of the horse's history from the moment he enters the stables.

Here we see a test bleeding being made. About 500 millilitres of blood is taken from the jugular vein and sent to the laboratory where the serum is examined for any natural immunity. If the horse possesses considerable natural diphtheria antitoxin it means that its cells must have had previous training in antibody production and it will be particularly useful for diphtheria immunisation.

Within 48 hours of the horse's arrival it's injected with tetanus toxoid to protect it from accidental tetanus. A second injection of tetanus toxoid is given 4 weeks later. When the isolation period is over, the horse is taken to one of the immunisation stables. Now begins the animal's course of immunisation, prescribed in the laboratory from its preliminary blood test.

The primary course consists of a series of increasing doses of diphtheria toxoid. The site of injection is first clipped and cleansed thoroughly with warm disinfectant fluid. The injection is given intramuscularly into the neck. Injections are given 2 or 3 times a week, the amount being gradually increased from 5 millilitres to an average maximum of 600 millilitres.

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The condition and temperature of the horse are observed carefully throughout the course. If necessary a dose may be repeated or halved or even omitted altogether. Horses must be kept in perfect condition throughout immunisation if antitoxin of high value is to be obtained. During this course of injection, experimental bleedings are made each week and sent to the laboratory. Here they are tested to ascertain the amount of antitoxin the horse is producing.

Here is a graph showing the increase in the amount of antitoxin produced by the horse during its course of toxoid injections. This animal had some natural immunity although it's too small to show on the graph, and after 4 or 5 weeks its antitoxin value rose to 1000 units per millilitre. Some animals, as in this case, do not respond so well.

When the antitoxin content of the horse's blood is sufficiently high, the laboratories inform the stable staff that the horse is ready for bleeding. First the jugular vein is made prominent with a tourniquet, the pad being placed over the jugular groove. The skin is then cleansed with swabs soaked in warm disinfectant fluid. A groom holds the horses head being careful to keep the neck straight. A trocar and cannula are sterilised. They are passed from above downwards through the skin into the vein. The trocar is withdrawn and a [*unknown word*] joint inserted into the cannula; attached to the joint is a length of rubber tubing connected with a 4 litre bottle. The bottle, which has been sterilised, contains a small amount of potassium oxalate solution to prevent the blood from clotting. 3 bleedings are usually made over a

period of 8 days, 8 litres of blood being taken at a time. From small horses, however, only 6 litres are taken.

After the 3rd bleeding the horse is rested and carefully watched to see that it's still in perfect condition and has in no way suffered from its bleedings. When the veterinary surgeon has declared the animal fit, it begins its second and shorter course of immunisation. This consists of 5 injections given over a period of 9 days of increasing amounts of toxin and alum. The first injection is 50 millilitres of diphtheria toxin plus 2 millilitres of 10% alum solution. The last injection is 150 millilitres of diphtheria toxin plus 6 millilitres of 10% alum. 3 or 4 days after this last injection, the first bleeding is made. As in the first course, 3 8 litre bleedings are taken over a period of 8 days. The number of courses a horse will stand and still yield satisfactory antitoxin varies considerably.

This bottle of blood contains the antitoxin. The next state in the process is to extract the antitoxin from the blood in as pure a form as possible. If the serum were injected in the form in which it's obtained from horse blood, the introduction of foreign proteins into the human would cause a high incidence of serum sickness which has very unpleasant manifestations.

When blood is first received the red cells are allowed to settle. The supernatant plasma, which contains fibrin, euglobulin, pseudoglobulin and albumin is then siphoned off. The antitoxin is associated with the pseudoglobulin.

As it may be necessary to store the plasma for several weeks before processing, a small quantity of antiseptic is added to prevent the growth of bacteria. When a batch of antitoxic plasma is required for refining, the plasma from a number of bleedings is sorted out and a batch is made up from plasma having similar antitoxic values to a total of 140 litres. The plasma is bulked into a stainless steel butt and thoroughly mixed. Then its volume is measured by weighing. A process sheet is used to keep a record of all manipulations and chemical treatments from this point until the refined serum leaves the laboratories. The plasma is diluted with 2 volumes of warm water to give a liquid which can be handled more readily than the original somewhat viscous

and sticky solution and also to provide a more suitable temperature for the rapid action of pepsin. It's then made slightly acid with hydrochloric acid to give the optimum pH for this particular peptic action. Pepsin is added at this stage. The acidity is carefully adjusted to pH 3.2, the albumin is digested and the antitoxic pseudoglobulin is split into 2 halves, 1 half of which contains no antitoxin and can readily be made insoluble. After half an hour the pepsinised plasma is ready for the addition of ammonium sulphate which removes a large proportion of unwanted proteins. A small quantity of sodium pyrophosphate is now added to bring the pH up to 4.3.

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The fibrin and euglobulin are precipitated and we obtain a medium most suitable for heat denaturation. The next task is to render insoluble the non-antitoxic heart of the pseudoglobulin. The contents of the butt are pumped into a heating vessel and brought to a temperature of 55˚C which is maintained for 1 hour. During this time a heavy precipitate of non-antitoxic protein appears in the solution. This precipitate can be readily filtered off in a filter press. The use of this type of press ensures rapid filtration whilst avoiding the loss of antitoxin. A clear liquid is obtained. A carefully controlled amount of ammonium sulphate is now added to the liquid. The antitoxin is precipitated while the products of the digestion of the albumin are left in solution. This precipitated antitoxin is filtered off in a filter press. The waste liquid runs away and a cake of antitoxin is left behind. This cake is of a spongy nature however and still contains a large quantity of ammonium sulphate solution which would cause unpleasant reactions if it were injected with antitoxin.

The ammonium sulphate is removed by dialysis. The cake is placed in cellophane bags and the bags are suspended in running water. Gradually the ammonium sulphate diffuses from the bags into the running water and a certain amount of water diffuses into the bags. After 48 hours the bags contain a solution of antitoxic pseudoglobulin free from ammonium sulphate.

The contents of the bags are bulked and tricresol is added to prevent the growth of bacteria. The solution is not sufficiently concentrated to allow curative doses to be issued in very small volume. To concentrate it a process of ultra-filtration is used. This filter, which consists of a collodion membrane of carefully standardised porosity, supported on a [*unknown word*] filter is immersed in the solution and water is sucked out under a vacuum. The filter is so fine that water and salts can pass through but not the large antitoxin molecules.

Here is the concentrated antitoxic pseudoglobulin solution. To ensure that it's perfectly clarified it's then passed through paper pulp. Before it's passed for human injection we must make sure that it does not contain bacteria. A filter candle is immersed in the solution which is drawn through by suction. The porosity of this candle is such that though protein solution will pass through it, bacteria will not.

Here, in its final form is the concentrated, bacteria-free antitoxin. This refined plasma shows a 4-fold increase in purity and an 8-fold increase in volume. It will remain clear and will retain its value for several years.

Before it's sent out for human use, it's tested for sterility, non-toxicity and potency. For sterility testing a sample is withdrawn with sterile precautions and measured parts of it are run into bottles containing 2 types of nutrient medium. The bottles are taken to the incubator and left there at a temperature of 37˚C for 5 days. And inspection is made every day and should the contents of any of the bottles appear cloudy during this time it means that the batch is contaminated and must be re-filtered.

Each batch of antitoxin is tested in the laboratories for potency. Preliminary tests are made by means of the flocculation reaction. Final titrations are made by injecting mixtures of toxin and antitoxin intradermally into guinea pigs in order to assess the unitage. Here we see such mixtures being prepared in readiness for injection. The neutralising power of each new antitoxin under test is compared quantitatively with that of a control antitoxin of known strength which in its turn is compared with that of

a standard serum, issued by the National Institute for Medical Research at Hampstead, London.

Varying amounts of the unknown serum and of the control antitoxin are added to different series of tubes containing a fixed amount known as the test dose of toxin. 0.2 millilitre amounts of each mixture are injected intradermally into a guinea pig; the injections are made on a constant pattern so that they are readily identified. The reactions are examined after 48 hours. No reaction appears at the site of injection of those mixtures in which the toxin has been completely neutralised by the amount of antitoxin present. Reactions of varying size and intensity are produced by the other mixtures depending upon the degree of neutralisation which has occurred. It is thus possible to determine the relative amounts of both the unknown and the known antitoxin that are just sufficient to neutralise 1 test dose of toxin. The test dose of toxin is so chosen that this end point corresponds to 1 unit of standard antitoxin.

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2 or 3 tests are usually sufficient to determine the potency of an antitoxin to within 5%. In order to obtain a serum of the antitoxic value required for issue purposes, a number of batches of varying potency are blended aseptically. The potency of the bulk serum is now tested and an appropriate dilution made. The serum is then run into bottles of a size suitable for filling ampoules, and the sterility is tested. In the laboratory, potency is again checked and the final non-toxicity test is made in accordance with the regulations of the Therapeutic Substances Act.

Injections are made subcutaneously into healthy guinea pigs to demonstrate that the batch is free from all toxic substances which might cause illness or death of the test animal. The serum is filled into ampoules which are sealed. Later, the ampoules are examined for flaws in the glass and any traces of foreign matter. The serum is then tested for sterility and the ampoules are packed in readiness for issue.

We’ve seen that the serum that contains the antitoxin is subjected to stringent laboratory tests in accordance with the regulations of the Therapeutic Substances

Act. As issued to the medical profession it must be of satisfactory potency, non-toxic and sterile. It contains a small amount of antiseptic usually thimerosal. The experimental determination of the activity of diphtheria antitoxic serum can be made with such precision that the rate of deterioration can be determined with accuracy. Sera, especially refined sera, kept at from 2˚C to 4˚C may be expected to retain their full labelled value almost indefinitely. Kept at 15˚C the rate of deterioration is less than 5% per annum. The strength of the refined antitoxin at the time of filling is such that the products retain their advertised unitage for 3 years from the date of manufacture unless exposed to temperatures above 15˚C.

Immunity can be either passive or active. Passive immunity is conferred when antitoxin made in the horse is used either for treating diphtheria or as a prophylactic measure for contact.

Not every person can contract diphtheria, some are immune. It's possible to test a person's susceptibility to, or immunity from diphtheria by means of the Schick test. A minute amount of diphtheria toxin, known as the Schick dose, is injected intradermally into the left arm and a corresponding amount of heated toxin, the control fluid, into the right arm. Readings are made a week later. If the test dose gives no reaction it means that this child possesses enough natural antitoxin to withstand an ordinary attack of the disease.

This child is Schick positive and is therefore susceptible to the disease. She has been in close contact with a diphtheria case. 1000 units of refined antitoxin are being injected intramuscularly. She will be passively immune within a few hours and should remain immune for 2-3 weeks.

This chart of a patient's circulating antitoxin shows that passive immunity is conferred immediately after an intravenous injection of antitoxin. Unfortunately the curve of circulating antitoxin shows that this passive immunity does not last more than a few weeks owing to the gradual elimination of the foreign protein by the body.

Our aim must be to obtain a much more lasting protection through active immunisation and we shall now see the preparation of diphtheria prophylactics. These are made from formal toxoid, the production and testing of which were described earlier in this film.

Active immunity, where the body cells have been stimulated to produce antibodies themselves, is much more lasting than passive immunity where preformed antitoxin from some other animal is injected. Active immunity arises in man, not only as a result of natural recovery from a clinical attack of diphtheria, or of a latent infection with transient symptoms, or none at all; but also as the result of injections of prophylactic derived from formal toxoid.

Two main prophylactics are used. Namely, toxoid antitoxin floccules (TAF) and alum precipitated toxoid (APT). In making TAF, toxoid is mixed with a suitable volume of antitoxin to produce flocculation. After standing for about 3 weeks, the precipitated floccules are thoroughly washed free from impurities with phenyl saline until the supernatant fluid becomes colourless. After reduction in volume, the suspension of floccules becomes the prophylactic TAF. This causes less reaction than ATP but 3 doses are required instead of 2.

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The second prophylactic, APT, was first prepared for human use at the Wellcome Research Laboratories. As with TAF, we start with formal toxoid. The toxoid is mixed with charcoal to purify it. The mixture is passed through 2 sets of filter candles to remove the charcoal and sterilise the liquid. A predetermined amount of alum is added to form an insoluble precipitate. It is this precipitate, washed and further purified and concentrated which is used for immunisation. Here we see the final product. APT is absorbed slowly and the stimulus to the production of antitoxin is thus prolonged.

In the development of active immunity, the first injection of an antigen into a non-immune subject acts as a primary stimulus. It's followed by a latent period of 2 or more weeks during which the antitoxin producing mechanism becomes responsive.

When potent antigens such as APT are used, a further injection given 4 or more weeks later acts in many children as a secondary stimulus, the cells have become trained and antitoxin is formed much more rapidly. The peak of the curve is reached in about 10 days and represents a production of far more antitoxin than that produced by a single injection. In some children, the responsive mechanism is not fully developed 4 weeks after the first injection and the second injection acts as an intermediate stimulus resulting in a slower production of antitoxin. With a weaker prophylactic, TAF, 3 injections are needed in order that at least 95% of children reach the Schick negative stage.

This child is being actively immunised against diphtheria at a clinic. 2 or 3 injections, depending on the prophylactic, are given at intervals of 4 weeks. This baby is being inoculated with diphtheria prophylactic APT. She has not been previously Schick tested as the Schick test before immunisation is generally dispensed with in infants, and indeed in all children under the age of 8 years, who are almost all Schick positive unless they've already been protected.

A few months after the course of active immunisation, the Schick test may be made to confirm that a useful level of immunity has been obtained. If the test is negative, the child, who if left untreated would have fallen an easy victim to the disease, has been actively immunised. His chance of ever contracting the disease is greatly reduced.

This film has dealt with diphtheria antitoxin and prophylactics prepared in these laboratories. We now possess the means of combating one of mankind's most dangerous enemies – diphtheria. Much has been achieved. Before antitoxin began to be used generally in the treatment of diphtheria in 1894, 30-50% of diphtheria patients died. Today, the death rate in the London Fever Hospital rarely exceeds 5%. During recent years we've seen the development of active immunisation of children, nurses and others, by inoculations of diphtheria prophylactic. Our knowledge must be applied on an ever-larger scale, for during the war years there were more child deaths from diphtheria than from bombs – the figures being 9000 and 8000

respectively. Mass immunisation has already affected the prevalence of diphtheria and in particular the mortality. For example, the incidence of diphtheria during 1943 in England and Wales among children under 15, was 3 ½ times greater in the non-immunised group than in the immunised group, and the chance of dying from diphtheria was 25 times as great.

Much has still to be discovered. Research is going on all the time. But the battle is being fought successfully and if every child were immunised, the outcome would be the virtual stamping out of diphtheria.

<**End credits** >