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Titration of Diphtheria and Tetanus Antitoxins in Sera of Low Titre

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Available methods for titrating diphtheria and tetanus antitoxin at low concentrations in human or animal blood are surveyed, with special attention to the amount of serum required for the test.

In vivo methods, especially the rabbit or guinea-pig intradermal test for diphtheria and the mouse test for tetanus, are precise and reliable. If, however, serum levels as low as about 0.001 IU/ml have to be determined, rather large amounts of serum are required: moreover, the tests are rather time-consuming and expensive.

Tissue culture methods are available only for diphtheria antitoxin titration. The titres found coincide very well with those from animal tests. The titrations are less time-consuming and more economical. These methods seem to be very promising for the replacement of animal tests. Of the real in vitro methods, the haemagglutination procedure has been investigated most thoroughly and used most frequently. Low titres can be measured using small amounts of serum, but the titres thus obtained may differ considerably from those obtained in animal tests, at least for individual sera. For mass screening, the method is very suitable.

A number of other methods are briefly discussed, but they are either less suitable or have not yet been sufficiently investigated.

In many investigations in the field of diphtheria and tetanus, especially in those concerned with estimating the level of immunity or the response to a vaccination procedure, the titration of antitoxin in the blood of man or animals plays an important role. The chief points of interest in these methods are the precision, the minimum antitoxin level that can be measured, and the amount of serum required for the test. The titration of high-titre hyperimmune sera for prophylactic or therapeutic purposes forms a separate subject. This is omitted from the present discussion since the precision required is usually much greater whereas the minimum measurable level and the amount of serum required are relatively less important.

International standards for diphtheria antitoxin and tetanus antitoxin have been in use for many years and these standards—or national reference preparations calibrated against them in international units—are used for the titration of sera. The methods used to establish and assay these international standards are not very clear, as records are scarce.

According to the Reports of the WHO Expert Committee on Biological Standardization the International Standard for Diphtheria Antitoxin dates from 1922 and the International Standard for Tetanus Antitoxin from 1928.

It appears from information collected by Dr J. Spaun (Statens Seruminstitut, Copenhagen) that the first clear document on the International Standard for Diphtheria Antitoxin is to be found in the *Bull. Hlth Org. L.o.N.* (1935). Jensen (1936) provided the data about the International Standard that are used nowadays (1 IU = 0.0628 mg of the International Standard).

The International Unit for Tetanus Antitoxin was defined in 1928 in such a way that 2 IU would be equal to 1 American Unit (League of Nations. Health Organisation, 1928). In fact the first samples of a real standard preparation were distributed by the Statens Seruminstitut in 1935, 1 IU being 0.1547 mg. In 1949 the international unit was made equal to the US unit and since then 1 IU has been 0.3094 mg of the International Standard. In 1969, this first standard was replaced by the second standard, which is in use today (1 IU = 0.03384 mg; WHO Expert Committee on Biological Standardization, 1970).

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Although no good evidence is available, it is almost certain that the assays of the original preparations were done by means of *in vivo* tests, i.e., intradermal tests for diphtheria antitoxin and mouse tests for tetanus antitoxin, as is described be low.

The methods available can be divided into three main groups: (1) tests on living animals; (2) titrations on cultivated cells; and (3) *in vitro* titrations. In all methods the interaction of the antitoxin with the relevant toxin is a fundamental characteristic. Some of the methods are applicable to both diphtheria antitoxin and tetanus antitoxin, whereas others are strictly limited to one of these materials, depending on their specific properties.

TITRATION OF DIPHTHERIA ANTITOXIN

Some of the methods in use are based on the actions of diphtheria toxin on living animals or living cells and others are based on immunochemical principles: the latter are also, in general, applicable to tetanus antitoxin.

Tests on living animals

To this group belong the oldest known titration methods involving skin tests (intradermal test) in guinea-pigs and rabbits and, more recently, tests on chick embryos and chicks.

Lethal toxicity tests in mammals. As diphtheria toxin has a lethal effect on several mammals, the protection of antitoxin in toxin–antitoxin mixtures can be used for titrating the antitoxin. For this purpose guinea-pigs and hamsters are used (Fan & Lim, 1930; Olitzki et al., 1948). Although such tests have been described, they have never been widely used as they are expensive and require rather large amounts of serum. Mice would be more suitable, but they are not sufficiently sensitive to diphtheria toxin.

Guinea-pig skin test. The oldest titration method, first described by Römer (1909), is still in general use. It is based on the fact that small amounts of diphtheria toxin, when injected intradermally into the guinea-pig, cause various skin reactions at the site of injection after 1–3 days. If such a toxin dose is mixed with different amounts of antitoxin these reactions will be absent above a certain (neutralizing) antitoxin level. If this is done with a serum of unknown antitoxin content and at the same time with a few dilutions of a standard serum, the titre of the former serum can be determined by comparing the skin reactions. The great advantage of this method lies in the fact that all these injections can usually be administered into the skin of the same animal, thus eliminating individual differences in toxin sensibility. The titration can be performed with high precision and a level of 0.001 IU/ml can be titrated easily (Starke & Zillmann, 1960; Glenny & Llewellyn-Jones, 1931). A disadvantage is the fact that rather large amounts of serum are required if it is necessary to determine levels as low as 0.001 IU/ml. If a minimum level of 0.01 IU/ml is acceptable, the test can be performed with smaller amounts of blood, which can be collected by finger puncture on filter paper discs (Mirchamsy et al., 1968).

Rabbit skin test. A similar test in rabbits was described by Fraser & Wigham (1924) and modified by Jensen (1933). The test is essentially the same as the guinea-pig intradermal test, but is probably slightly more sensitive still (0.0003 IU/ml according to Starke & Zillmann, 1960). Up to 250–300 injections can be made in one rabbit (Jensen, 1933; Greenberg & Gibbard, 1949). An adaptation of this method for estimating the antitoxin content of small blood samples (0.2 ml) with a minimum titration level of 0.02 IU/ml was described by Taylor & Moloney (1960).

Chick test. The toxic effect of diphtheria toxin on birds was first shown by Loeffler (1884) and has subsequently been demonstrated by Frobisher (1940). Branham & Wormald (1954) made use of this susceptibility for the titration of antitoxin. They injected toxin–antitoxin mixtures into 8-day-old chicks; by comparing survival ratios after the injection of toxin mixed with dilutions of the unknown serum and of a standard serum it was possible to determine antitoxin values that corresponded very well with the values obtained by guinea-pig skin tests. The results suggest, however, that the method is less suitable for very low titres. It is doubtful whether this method has any advantage over the intradermal tests.

Chick embryo test. Dishon (1957) used groups of 9-day-old embryonated eggs. The embryos are very sensitive to diphtheria toxin injected into the allantoic cavity. When toxin–antitoxin mixtures are injected the survival ratios of the embryos in each group depend on the antitoxin level. The method is interesting but is not very attractive for practical use.

Titrations on cultured cells

Levaditi & Muttermilch (1913) demonstrated the toxic action of diphtheria toxin on cultured cells.

This action can be prevented by the addition of suitable amounts of antitoxin, and tissue culture titration techniques have been developed. Okabe & Teruuchi (1930) described a method of standardizing diphtheria antitoxin by a tissue culture method. They compared a number of therapeutic sera by this method and by *in vivo* tests and found a good correlation. It is remarkable that the tissue culture titration was not subsequently used for more than 25 years. This was probably because tissue culture was still a rarely used and difficult technique at that time. The development of virological techniques led to a renewed interest in this method.

Disk-plate assay. Farrel & Reid (1959) described a disk-plate assay method for the titration of poliomyelitis antibodies. Trypsin-dispersed monkey cells were grown in Petri dishes, living virus was added in an agar layer on a plain agar overlay, and small disks containing antiserum dilutions were placed on the surface.

The zone of inhibition of virus activity was used to measure the antitoxin content of the serum. This system was adapted by Farrel et al. (1963) to the titration of diphtheria antitoxin. Several types of cell appeared to be suitable and diphtheria toxin in agar was used instead of the virus. The method appeared to be rather sensitive and the results corresponded well with those from rabbit skin tests. The lowest titratable level was 0.02 IU/ml.

Tissue culture tests (cytopathic effect and colour tests). These tests are based on the same principle as the disk-plate assay, but are discussed separately as they are completely different from a technical point of view. These methods too are modifications of techniques in use for the titration of poliovirus antibodies (Salk et al., 1954).

The principle is as follows: cups in plastic trays, or small tubes, are filled with a suitable tissue culture medium, serum dilutions, a constant amount of toxin, and a standardized cell suspension. The whole is covered with medicinal paraffin and incubated. After an appropriate lapse of time—usually about 5 days—the results are read either by microscopic estimation of the cytopathic effect or by visual estimation of the colour of the pH indicator in the medium. Living cells produce acid and this leads to a colour change (from red to yellow); if the toxin is not neutralized the cells die and the colour change is inhibited. The results are evaluated quantitatively by comparing the cytopathic effect or change in tubes or cups containing an unknown serum with that for a standard serum.

Placido Sousa & Evans (1957) described the aconti of diphtheria toxin on tissue cultures and its neutralization by antitoxin, and stated that the metabolic inhibition test (pH colour method, Salk et al., 1954) could be used for the titration of antitoxins (therapeutic sera). Stănică et al. (1964) used this test for the titration of low-titre guinea-pig sera and were able to measure titres of 0.02 IU/ml and even lower.

Topciu et al. (1966) performed similar tests with human sera and found that the method was very sensitive and gave reproducible results and that antitoxin concentrations of less than 0.005 IU/ml could be measured. Both these groups of investigators used the cytopathic effect of the toxin as an indicator. Considerable experience with these tests was gained in Czechoslovakia (Izbický, 1968, 1969a, 1969b; Kříž et al., 1967; Jandasek & Svobodova, 1967). The minimum level of titration in their tests was about 0.005 IU/ml. It was shown that the results corresponded very well with those obtained by *in vivo* titration. This has been confirmed in the author's laboratory (van Ramshorst, 1970). Probably it is possible to measure even smaller quantities of antitoxin. Research on this point is in progress. The tests can be performed with very small amounts of serum and many sera can be titrated in a fairly short time.

In vitro *titrations*

Whereas tissue culture methods can be considered on the borderline between *in vivo* and *in vitro* methods, the methods discussed in this section can be performed without living animals or living cells. For a long time the only method of this type was the flocculation reaction discovered by Ramon (1922, 1923). The method is important for titrating hyperimmune horse sera, but is unsuitable for low-titre sera or sera from humans or guinea-pigs. For this reason it will not be further discussed here. Other *in vitro* methods are gel diffusion, microtitration on sensitized collodion particles, and haemagglutination.

Gel diffusion. If toxin and antitoxin are pipetted into suitable wells in an agar surface they will react somewhere in the agar, forming a precipitate if the concentrations of the reactants are within a certain ratio. In principle, the position of the precipitation line can be used to measure the concentration of antitoxin if the toxin concentration is known. The author's experience has shown that this method is of limited value (unpublished data). Usually there are no problems with high-titre sera, as they can

be diluted. However, the minimum concentration of the antitoxin necessary is in the order of 1 IU/ml. Moreover, the precipitation patterns produced by undiluted low-titre sera—even if they contain 1 IU/ml or more—are often obscured by lipids, etc., in the serum. For these reasons the method was found unsuitable for routine titration of low-titre human or guinea-pig sera.

A gel-diffusion test for the titration of diphtheria antitoxin was also described by Beys l'Hoest (1968). The method was found suitable for sera with a minimum antitoxin level of 1–2 IU/ml, and this is in agreement with the author's experience.

Microtitration on sensitized collodion particles. This method was described by Sasagawa et al. (1966). It can be considered as a variant of the haemagglutination method to be described below and the authors found the results to coincide well with those obtained by haemagglutination. They state that the test has 10–100 times the sensitivity of the intracutaneous test in rabbits. Probably similar techniques could be, or may have been, devised using other particles (e.g., latex or bentonite).

Haemagglutination. This method is based on the principle that sheep erythrocytes sensitized (coated) with toxin or toxoid can be agglutinated by antitoxin.

The test can be performed in two ways (see Tasman et al., 1960). In the direct test the sensitized erythrocytes are mixed with different serum dilutions. If the antitoxin concentration is above a certain level, the contents of the cells will agglutinate. In the haemagglutination inhibition test the serum dilutions are mixed with a certain quantity of " test toxoid " and these mixtures react with sensitized erythrocytes. If the serum dilution is too low to neutralize the test toxoid, the erythrocytes will not agglutinate. The haemagglutination test has been studied by many investigators (Boyden, 1951; Fisher, 1952; Stavitsky, 1954; Landy et al., 1955; Scheibel, 1956; Fulthorpe, 1957, 1958, 1959; Butler, 1963; Surján & Nyerges, 1962; Cagall & Levy, 1968; Mai & Rosin, 1969, etc.). The great number of papers on this subject reflects in a way the controversial nature of the method.

There are different methods of preparing and preserving the coated erythrocytes. The antigen is usually fixed to the blood cells by means of tannic acid, although other methods can be used (Lavergne et al., 1965). The sensitized cells can be used fresh or after preservation (usually with formalin) for up to 2 months (Mai & Rosin, 1969). They can also be preserved by freezing at –70°C (Hubert et al., 1963). A number of things can be said in favour of this method. The titres are reproducible and the test is fairly simple and economical and may be used to measure very low antitoxin concentrations. Diphtheria antitoxin can still be measured at a level of about 0.00015 IU/ml (Butler, 1963), although some authors state that animal tests are more sensitive at very low concentrations. The tests can be performed with small quantities of serum. In spite of all these advantages the haemagglutination test has certain drawbacks that limit its general applicability. Scheibel et al. (1962) found the results different from those obtained with *in vivo* methods in certain sera. Surján & Nyerges (1962) found 5–6-fold differences in some sera. Chatterjee (1964) stated that the test, although useful for screening purposes, could not replace the *in vivo* test. Titres determined by this test and by *in vivo* methods do not usually differ by a factor greater than 2–3, but may sometimes show 5-fold differences. Other investigators (Landy et al., 1955; Fulthorpe, 1957, 1958, 1959) found larger individual differences in human and guinea-pig sera. Cagall & Levy (1968) also state that the haemagglutination test cannot replace the neutralization test.

As a general conclusion, it might be said that the haemagglutination test is useful and is especially suitable for investigations with large numbers of sera, where the individual serum titres are not very important.

Other possibilities. It may be assumed that radioimmune assay techniques will lead to new methods of antitoxin titration. At present no published data are available.

TITRATION OF TETANUS ANTITOXIN

The number of methods for titrating tetanus antitoxin is considerably smaller than is the case for diphtheria. Since tetanus toxin does not produce any skin reaction upon intradermal injection, intradermal tests do not exist. Furthermore, there are not at present any easily cultivated cells that are sensitive to tetanus toxin. Even high concentrations of the toxin do not kill or appreciably inhibit the growth of any cell type in use for tissue culture. Nervous cells might be sensitive, but simple methods of growing these cells on a large scale are not available. Consequently, there are no tissue culture methods for titrating tetanus antitoxin. There remain the tests on living animals (lethal toxicity and/or general tetanus symptoms) and *in vitro* methods.

Tests on living animals

The only *in vivo* method available is the following: a dose of tetanus toxin is chosen that produces local symptoms of tetanus (Chen et al., 1956; Taylor & Moloney, 1960) or that kills the animals (Ipsen, 1959). In principle such a test could be performed in several animal species, but in practice mice are always used. If dilutions of antitoxic serum are mixed with the toxin test dose and these mixtures are injected, each in one or more mice, the mice will survive or remain free from tetanus symptoms if the toxin is completely neutralized. Mice infected with the same toxin dose mixed with dilutions of a standard serum serve as controls.

Titres as low as 0.001 IU/ml can be measured in this way if a sufficient quantity of serum is available. If only small amounts of serum are available (e.g., blood taken by skin puncture) it must be diluted and the minimum level becomes higher. Mirchamsy et al. (1968) were able to determine antitoxin titres of 0.0025 IU/ml in finger-puncture blood absorbed on filter paper.

The chief disadvantage of the method lies in the fact that several animals must be used for each titration.

Titrations on cultivated cells

This technique is not possible at present.

In vitro *tests*

Here the possibilities are in principle the same as for diphtheria antitoxin titration.

Flocculation and gel diffusion are possible, but with the same limitations as described above for diphtheria, and these methods are not practicable for low-titre sera. A double diffusion technique in agar gel was used by Alexander & Moncrief (1966) for the titration of antitoxin in human sera. The minimum level that could be detected was 0.5 IU/ml. Recently the use of an immunofluorescence test for the titration of tetanus toxoid was described by Wojtyła et al. (1969). Such a method could probably also be used for antitoxin titration, but according to the authors its sensitivity is equal to that of the precipitation test and it is therefore unsuitable for measuring low levels.

Microtitration on sensitized particles has not been described, but there is no reason why it could not be carried out as for diphtheria antitoxin.

Haemagglutination can be performed exactly as for diphtheria antitoxin. In most of the papers mentioned above this technique is described for diphtheria and for tetanus antitoxins. Some authors state that the correlation with *in vivo* tests is slightly better for tetanus antitoxin than for diphtheria antitoxin. The minimum antitoxin level that can be detected is in the order of 0.002–0.01 IU/ml.

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RÉSUMÉ

TITRAGE DES ANTITOXINES DIPHTÉRIQUE ET TÉTANIQUE DANS LES SÉRUMS DE FAIBLE TITRE

Le présent article est consacré à une revue et à une brève analyse des méthodes actuellement disponibles de titrage des antitoxines diphtérique et tétanique. Parmi les principaux points examinés figurent la précision des diverses techniques et la quantité de sérum nécessaire à leur exécution.

On peut classer les méthodes en trois grands groupes: épreuves sur animaux vivants, titrages sur cultures tissulaires et tests *in vitro*.

Titrage de l'antitoxine diphtérique. On dispose d'une série de tests sur animaux: épreuve de toxicité létale pour les mammifères (rarement utilisée), tests cutanés sur le cobaye et le lapin (très précis), tests sur le poulet et sur l'embryon de poulet. Parmi ces méthodes, seuls les tests cutanés sont couramment employés. Ils ont le désavantage d'exiger d'assez grandes quantités de sérum pour la détermination des titres faibles.

Les cultures tissulaires se prêtent au titrage de l'antitoxine diphtérique, soit par la technique des disques, soit par la mesure de l'effet cytopathogène ou de l'inhibition du métabolisme cellulaire. Ces méthodes sont précises et n'utilisent qu'une petite quantité de sérum.

Les titrages *in vitro* comprennent: la réaction de floculation de Ramon (applicable uniquement aux sérums équins hyperimmuns), la diffusion en gel (seulement pour les sérums dont la teneur en antitoxine est assez élevée), le microtitrage sur particules de collodion sensibilisées et l'épreuve d'hémagglutination des érythrocytes de mouton sensibilisés. Cette dernière épreuve est particulièrement adaptée au titrage de nombreux sérums,

mais dans certains cas les résultats qu'elle fournit diffèrent sensiblement de ceux obtenus par les épreuves *in vivo*.

Titrage de l'antitoxine tétanique. On ne peut recourir aux tests cutanés et il n'existe aucun système de culture tissulaire sensible à la toxine tétanique. Il faut s'adresser à l'épreuve de toxicité pour la souris, précise mais coûteuse, ou aux épreuves *in vitro* : floculation, diffusion en gel, immunofluorescence (inapplicable aux sérums de faible titre), hémagglutination. Ces épreuves ont les mêmes avantages et les mêmes limites que leurs homologues servant au titrage de l'antitoxine diphtérique.

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