A Method for the Rapid Determination of Diphtheria Antitoxin in Clinical Practice

By

G. Nyerges, Georgette Nyerges, Margaret Surján, J. Budai and J. Csapó

First Section of Paediatrics (Head: Prof. J. Csapó), Municipal László Hospital, and Vaccine Control Department (Head: Dr. L. Erdős), State Institute of Hygiene, Budapest

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While in Hungary the incidence of diphtheria has become negligible since the discovery and widespread adoption of anatoxin [19], diseases of the tonsil that bear a striking resemblance to diphtheria are still frequent [3, 30], a fact involving problems of differential diagnosis. Although it is desirable that adequate therapy should start at the earliest possible moment, the number of unnecessary serum treatment would be inordinately high if all such cases were subjected to serotherapy, the more so as heterologous serum is not quite harmless and involves sensitization. The indications of serotherapy have recently been studied by Vigh et al. [30]. In view of their findings, before resorting to serotherapy, for a time we took into account not merely the clinical picture but whenever it was possible to await also the bacteriological result, further the time since the onset of the disease. Although the number of serum applications thus became considerably less, it still often happened that we started treatment with serum in cases where neither the subsequent course of the illness nor the subsequent bacteriological result confirmed the diagnosis of diphtheria.

Knowledge of the patient's actual antitoxic immunity would be a great help in the decision regarding the necessity of serotherapy. Therefore, a method allowing rapid and so-to-say bedside determination of whether the patient's blood contains an adequate amount of diphtheria antitoxin must mean a great facility. Namely the administration of serum cannot be delayed until the result of the current procedures for the determination of antitoxic immunity (Schick's skin test, biological assay in animals) becomes available.

The haemagglutination test (HA) seemed to be promising in this respect. Boyden [5] found that non-haemolyzing proteins were readily adsorbed on tannic-acid-treated red blood corpuscles and that the latter were agglutinated by the antiprotein. Fisher [9] was the first to utilize this method for the titration of diphtheria antitoxin. The reliability of the test has been confirmed by several authors [15, 21, 28] who found the HA-values to agree well with the results of biological assays. While the HA results are not available before 18 to 24 hours if the test is made according to the original prescription, in tubes, not

more than 30 to 60 minutes are needed if TAKÁTSY's plate technique [26] is used. This technique had been used by BACKHAUSZ [2] in studying the adsorption of antigens. SURJÁN and NYERGES [24], applying this technique, examined a number of immunized children and found a close and direct correlation between the results of the HA micromethod and those of JENSEN's biological assay. The said authors introduced moreover the preservation of erythrocytes with formalin, allowing protracted storage of the suspension and enhancing the reliability of the HA test. The method seemed, therefore, suitable for the rapid determination of antitoxin in patients suspect of diphtheria and thus to facilitate decision of whether or not to apply serotherapy.

METHOD

The diphtheria suspects referred to our hospital, were referred to the observation ward and the blood antitoxin level was determined immediately after admission.

The technique of the HA micromethod has been described previously [24]. The principle is that purified diphtheria anatoxin is adsorbed onto the surface of tannic acid-treated sheep erythrocytes and the erythrocyte suspension is preserved with formalin. Serial 1:2 dilutions are then made on Takátsy's plate [26] of both the test serum and the Copenhagen standard diphtheria antitoxic serum, and the sensitized erythrocyte suspension is added to the dilutions. The result can be read after 30 to 45 minutes of incubation. A comparison of the titre of the test serum with the standard preparation gives the sought antitoxin level.

In addition, routine tests were made (throat swab bacteriology, blood counts, ESR, Paul-Bunnel's test, urine analysis, ECG* in every case. At discharge the patients were instructed to report for a detailed follow-up after four weeks, and if necessary also at later dates.

At the outset, we were faced with the question as to what antitoxin titre should be regarded as affording protection against diphtheria.

The limit of immunity generally accepted in the literature was from 0.01 to 0.03 I. U./ml, but at first we had to work with an adequate safety margin and thus established an about ten times higher limit, i.e. 0.2 to 0.4 I. U./ml. The antitoxin titre alone was of course not our only basis for deciding for serotherapy. Decision in this respect was based on the joint evaluation of the clinical picture, antitoxin titre, and (if available) the bacteriological finding.

Repetition of the antitoxin assay was expected to allow a retrospective establishment or exclusion of the diagnosis of diphtheria. It was supposed that if diphtheria toxin gained access to the organism of children who had earlier been vaccinated, their antitoxin titre was bound to rise. If antitoxic serum had been applied, its inhibitory effect on active antitoxin production was duly taken into account.

MATERIAL

Two hundred children suspect of having diphtheria were referred to our Department from September 1, 1961, to April 30, 1963.

* The ECG tracings were interpreted by Dr. E. FARKAS who had no previous knowledge of the cases. We wish to express our thanks for her valuable assistance

TABLE 1

Classification of children, admitted as diphtheria suspects, according to clinical picture, bacteriological findings, and serum antitoxin titre

Clinical picture	Bacteriology	Antitoxin titre (I.U./ml)	
		<0.2	>0.4
Membrane pointing to diphtheria	+	7	—
	—	4	19
Membrane, not characteristic of diphtheria	+	2	2
	—	26	26
Lacunar, follicular, catarrhal tonsillitis	+	13	18
	—	29	54

Clinically, they belonged to the following categories.

(i) Patients who at admission displayed faucial symptoms characteristic of, or pointing to diphtheria, such as a greyish white or darkish grey adhering membrane covering or even extending beyond the tonsils which, if removed, left behind haemorrhages; peritonsillar oedema; oedematous cervical lymph nodes. This category thus comprised patients to whom, before, we should have administered serum without awaiting the bacteriological result.

(ii) Cases of non-diphtheritic tonsillitis with continuous or islet-like purulent, crumbly and easily removable membrane. This often consisted of necrosed tonsillar tissue, after the detachment of which, there remained practically no tonsillar substance. This category comprised patients to whom, before, we should have administered serum only after a positive bacteriological finding.

(iii) Cases of follicular or lacunar tonsillitis and patients whose throat showed neither exudate nor follicles so that only faucial hyperaemia pointed to previous disease. These patients were referred to the hospital partly on account of positive bacteriological findings and partly because they had not responded to treatment at home. This category contained patients who were not given serum even in cases with positive bacteriological result.

RESULTS

Table 1 shows the classification of the material according to the above clinical symptoms, the bacteriological result and the initial antitoxin titre.

ad (i). In this category there were 30 cases of which 7 were bacteriologically positive. One of the patients had malignant diphtheria; at admission, he showed a serum antitoxin titre of 0.01 I. U. per ml. The other 6 positive patients had diphtheric tonsillitis without early toxic symptoms and an initial titre of from 0.006 to 0.2 I. U./ml. The clinical picture and the low antitoxin titre in these 7 cases justified the administration of serum without awaiting the bacteriological finding. The child suffering from malignant diphtheria had never been vaccinated and died in spite of having promptly been given high doses of serum. The ECG showed in this case a grave disturbance of impulse formation and conduction. The other 6 positive patients recovered. One of them exhibited symptoms of myocarditis, further palatoplegia

TABLE 2

Antitoxin titre in clinically characteristic and bacteriologically confirmed cases of diphtheria, before and after serum treatment

Initials of patients Dose of serum (I.U.)	Before serotherapy (I. U./ml)	2	4	7	14	30	60
		days after serum administration (I.U./ml)					
I. K. 140,000	0.006	12	50	50	100	100	50
E. K. 40,000	0.02	25	25	50	100	100	100
J. K. 80,000	0.1	25	12	—	3	25	—
B. F. 100,000	0.1	25	25	50	6.4	1.6	1.6
K. V. 100,000	0.2	25	25	—	6	0.8	0.2

and peroneal paresis, while no complications supervened in the other 5 cases, except on the ECG a transitory disorder of impulse formation in one, and a disturbance of repolarization in another case.

The antitoxin titre of 5 patients was determined repeatedly (Table 2). The level at which the titre had stabilized after the elimination of the introduced serum was considerably above the initial level in four cases, and returned to the initial value in one case. The latter phenomenon may have been due to an inhibition of active antibody production by the introduced antitoxic serum.

Four of the 23 bacteriologically negative children exhibited a low initial antitoxin titre, and so they received antitoxic treatment. One of these cases subsequently proved to be one of infectious mononucleosis, and another of staphylococcal tonsillitis. Two patients had pseudomembranaceous pharyngitis. Their antitoxin level returned to the initial value after the elimination of the serum; since these cases received serum treatment, the possibility of diphtheria could not be excluded retrospectively. The ECG showed a transitory slight disturbance of repolarization on the 3rd day in one of these cases. The initial antitoxin titre was high in the remaining 19 children, so that no serotherapy was instituted. Seven of them turned out to have mononucleosis, two staphylococcal tonsillitis, one streptococcal tonsillitis, two Plaut—Vincent's angina, while no pathogenic agent could be isolated in seven cases. All these patients recovered without clinical complications. The ECG in one case showed a temporary irregularity of impulse formation (coronary sinus rhythm). At follow-up in 15 cases no change in the antitoxin titre was revealed.

As expected, we encountered no clinically characteristic and bacteriologically positive case with a high degree of antitoxic protection.

ad (ii). Four of the 56 children in this category were bacteriologically positive. Two of them had a low antitoxin titre (0.01 and $<$ 0.01 I. U. per

ml, respectively) and received, therefore, antitoxic serum after the bacteriological result had been received. The consequent rise of titre pointed to diphtheria in both cases. The ECG in one of them showed an irregular T_1; there were no pertaining clinical signs. The other two bacteriologically positive patients had a satisfactory degree of protection and received, therefore, no serum treatment. In one of these children was it only possible to determine the subsequent antitoxin titre; its rise pointed to diphtheria. The ECG was normal in both patients. All bacteriologically positive patients recovered without complications.

As regards the 52 bacteriologically negative members of this group, we isolated from their throat swab *Streptococcus haemolyticus* in 10 cases, *Staphylococcus aureus* in one case, while infectious mononucleosis was diagnosed in four cases. No pathogen was revealed in the remaining 37 cases. In view of the atypical clinical picture and the negative bacteriological finding no serotherapy was instituted in any of these cases, irrespective of the antitoxin titre. Subsequent determinations of the titre were made in 25 cases, 13 of which had had a low, 12 a high initial titre. A rise of titre was observed in 4 patients, 3 of whom had been vaccinated in the meantime. The rise of titre in one of the children whose original blood antitoxin level was low justified the diagnosis of diphtheria, so that this patient was readmitted for observation. No complications supervened. In the bacteriologically negative group the ECG revealed a slight disturbance of repolarization in one case, and transitory sinus tachycardia in two cases.

A typical membrane or an atypical membrane associated with positive bacteriology would have induced us to administer antitoxic serum to 34 children in this two groups, had we adhered to the old routine. The rapid determination of the antitoxin titre has made it possible to restrict serotherapy to 13 cases, so that 21 patients were spared the risks of superfluous serum treatment. The omission of serotherapy caused no harm in any of the patients, most of them later turned out not to have had diphtheria, while diphtheria patients with a satisfactorily high level of self-produced antitoxin were protected without the introduction of serum.

ad (iii). This was the largest group, 31 members of which were bacteriologically positive. The clinical picture was so mild as to render serotherapy superfluous. These patients received penicillin and in consideration of the favourable reports [1, 14, 25, 30] in this respect — some were given a 3-day erythromycin treatment in addition. All patients turned negative and recovered without complications. Subsequent determinations of the antitoxin titre in 26 out of these 31 cases revealed that nine of the 12 children whose initial titre had been below 0.2 showed a rise in the antitoxin titre, thus they had had diphtheria. There was only one among the 14 patients with initially high titres whose antitoxin level increased; that of 13 chil-

dren remained unchanged. We would formerly have diagnosed cases of this nature for tonsillitis of diphtheria carriers, whereas the sudden rise of antitoxin titre in patients whose original protection was low showed that they had actually had diphtheria. Except for a temporary slight disturbance of repolarization in 2 cases, as revealed by the ECG, all these patients recovered without complications.

The bacteriologically negative group of patients with slight tonsillitis comprised 83 members. We isolated other pathogenic organisms in 19 cases, diagnosed infectious mononucleosis in 6 cases, and failed to clear up the aetiology in the rest. The initial value of the antitoxin titre varied between 0.006 and 12.8 I. U./ml. All these patients were treated with penicillin. Cervical lymphadenitis supervened in 2 cases, the others recovered without complications. The ECG revealed in 9 cases abnormalities that may have been due to the primary disease (3 cases of sinus tachycardia; 6 cases of transitory repolarization disturbance with irregular impulse formation in one of the latter); these changes were not clinically manifest and subsequently all disappeared. At the follow-up in 49 cases the antitoxin level was found to have significantly increased in one single case where the child had been vaccinated between the two determinations. Thus, none of the bacteriologically negative cases of slight tonsillitis could be proved to have been of diphtheritic origin.

Reliability of the HA test

With a view to ascertaining whether the titres obtained were reproducible, after the lapse of a month we again estimated the titre in those bacteriologically negative patients whose clinical illness had not pointed to diphtheria. Children who had been vaccinated between the two determinations and those who had received antitoxic serum prior to admission were not reexamined. The results are shown in Table 3. It can be seen that the difference did in no case exceed two steps of dilution, and that the deviations were uniformly distributed towards increase and decrease, so that they were not due to specific factors. It should be taken into account that, although both the first and the second tests were made under apparently identical conditions, there was a temporal interval between them and the

TABLE 3

Changes in the antitoxin titer of 70 non-diphtheritic children at repeated titrations

Decreased by			Unchanged	Increased by		
3	2	1		1	2	3
steps of dilution				steps of dilution		
—	2	12	44	10	2	—

TABLE 4

Distribution of confirmed cases of diphtheria according to initial antitoxin titre

I. U./ml	0.01	0.02	0.05	0.1	0.2	0.4	0.8	1.6	3.2
No. of cases	9	3	2	4	1	—	2	—	—

erythrocyte suspension employed was not invariably the same. If we further consider that the evaluation of titrations is always somewhat subjective and that titres of children may undergo slight changes independently of specific stimuli, we feel justified in regarding the HA-test as well reproducible and reliable. It further follows from the data of Table 3 that changes in titre exceeding two steps of dilution were due to specific factors.

The fact that all clinically grave cases were associated with low antitoxin titres is a further argument for the reliability of the method. There was an unequivocal correlation between the gravity of the faucial process and the increase in titre. The initial titre rose, on the average, 12 steps of dilution in patients with a confluent membrane, against 7 steps in the case of diphtheria manifesting itself with banal tonsillitis.

Limit of safety

The next problem was the determination of that antitoxin value above which serotherapy could be dispensed with. A diagnosis of diphtheria was made in all clinically typical, bacteriologically positive cases, further, retrospectively, in those atypical ones in which a subsequent determination revealed a rise of the antitoxin titre. Table 4 shows the initial antitoxin values of confirmed cases of diphtheria. The greatest number was associated with low initial titres, there were only two cases with a somewhat higher initial titre (0.8 I. U. per ml). Both these children had been ill for some (7 to 10) days, their tonsillar finding was atypical, and they displayed no toxic symptoms. Their original antitoxin titre may have been low, but it was during its rise that we estimated it at admission. This supposition is borne out by the fact that their titre rose continuously since the very first day of observation, while that of the other diphtheritic patients did not start rising until the 6th to 9th day of the disease. We found the upper value to lie between 0.1 and 0.2 I.U./ml; taking into account the margin of error inherent in the method we are considering 0.2 to 0.4 I. U./ml to be the limit of safety.

DISCUSSION

Among 200 children referred to our Department for the suspicion of diphtheria only 7 had typical diphtheria, 35 cases of apparently banal tonsillitis proved to be diphtheritic only by the bacteriological result, while 78.5 per

cent of all diphtheria suspects were found to suffer from different other forms of tonsillitis. It is, therefore, only natural that we welcomed the possibility of using the antitoxin titre as an indicator of immediate serum treatment.

The present studies were principally designed to ascertain the clinical reliability of the method. Earlier investigations have made it clear that although — like biological titrations — it gives a true picture of the antitoxic immunity of a whole population, individual deviations from the biological antitoxin assay may nevertheless be quite considerable. In a previous paper [24] we have pointed to certain shortcomings of the biological antitoxin assay, which made a comparison of the two methods difficult. What we wished to ascertain in the present studies was, therefore, not whether and how far the HA values agreed or disagreed with those obtained with the biological method, but the reliability of the HA test in giving a true picture of the organism's immunity. Apart from animal experiments, observations on clinical material seem to be most suitable for this purpose. SCHEIBEL [21] observed in immunized guinea pigs a good correlation between the serum antitoxin titre determined with the HA test and the resistance to toxin. Our view that the method is reliable and its values are in accordance with the clinical picture has been based on the following considerations.

(i) In non-diphtheritic patients repeated HA tests showed no essential changes. The maximum difference amounted to two degrees of dilution in some cases, and it was obviously due to methodological inaccuracies. Similar results were obtained previously [24] on repeated titrations of the same sera.

(ii) The HA test proved to be sensitive to the following specific changes in the serum antitoxin level. (a) Passively administered antitoxin raised the titre to approximately the precalculated level. (b) In non-diphtheritic patients who had received serum treatment the antitoxin titre decreased in proportion to the elimination of serum. (c) The blood antitoxin titre showed a considerable increase after active immunization. (d) Except a few serum-treated cases, children with diphtheria responded to the infection with antitoxin production.

(iii) The reliability of the method has clearly been proved by the fact that in our material there was not a single case of clinically characteristic and bacteriologically positive diphtheria with a high antitoxin titre and that, further, there was a clear correlation between the serum antitoxin titre and the gravity of illness. If in children with a high initial antitoxin titre the clinical symptoms were characteristic of diphtheria, the disease later proved to be due to some cause other than diphtheria.

Our results have made it possible to determine that antitoxin titre above which serotherapy becomes superfluous. The currently accepted limit of antitoxic protection is 0.01 to 0.03 I. U./ml. There is, however, a

number of reports on the occurrence of diphtheria in patients who had been vaccinated, or were Schick-negative, or showed an antitoxin titre exceeding Schick's limit value [6, 7, 8, 10, 11, 12,16, 17, 18, 20, 22, 23, 27, 29]. We have observed similar cases, and for this reason we established the safety limit at 0.2 to 0.4 I. U./ml.

In vaccinated children diphtheria mostly appears in an atypical form and imitates banal tonsillitis without toxic symptoms. It remains to be elucidated whether *Corynebacterium diphtheriae* is the actual pathogen in those bacteriologically positive cases which appear to be banal tonsillitis and where a failure of the antitoxin titre to rise argues retrospectively against the possibility of diphtheria.

The value of the bacteriological result has to be emphasized. Its value is best shown by the fact that of all our cases with diphtheria a single one only was negative bacteriologically.

The present results have shown that the HA test as applied by us, together with the clinical picture and (if available) the bacteriological result, is eminently suited for deciding for serum treatment, provided the following principles are observed.

(i) If a case displays the characteristic or suspicious symptoms of diphtheria, one should not await the bacteriological result, but decide for serum administration on the basis of the antitoxin titre. If the latter has a value of 0.4 I. U./ml or above, the process is either not diphtheria or, else, the existing amount of antitoxin affords sufficient protection. If the symptoms are pointing to a malignant process, one must not even await the result of the HA-test but administer antitoxic serum without the slightest delay [2, 13].

(ii) In the presence of a membrane or of necrosis not characteristic of diphtheria one should await the bacteriological result and only if this is positive and the antitoxin level is low must serum be administered. If the bacteriological finding is negative, the institution of serotherapy depends on the gravity or progress of the clinical picture considered in conjunction with the antitoxin level.

(iii) We do not administer antitoxic serum to bacteriologically positive patients suffering from follicular or lacunar tonsillitis. A considerable rise of the antitoxin titre in patients with low initial titre shows that they had suffered from diphtheria; toxic phenomena are, however, counteracted in vaccinated patients by the rapidly augmenting amount of antitoxin so that no complications arise. However, if the disease is grave and shows progress, and the child has not been vaccinated, it will be advisable to administer serum.

We wish to emphasize that there exist no sharp boundary lines either in respect of the clinical symptoms or in that of antitoxic protection, so that each case has to be treated individually.

Summary

A modified haemagglutination test for the rapid determination of the serum antitoxin level has been studied in 200 children admitted with the suspicion of diphtheria. The test has been found reliably to indicate changes in the antitoxin titre; its values agreed well with both the clinical and bacteriological results. It has been found that serotherapy is not indicated if the titre of antitoxin amounts to 0.2 to 0.4 I. U./ml. The method under review, in conjunction with the clinical picture and, if available, the bacteriological result, helps the physician in deciding whether or not to resort to serotherapy in doubtful cases. Of the 200 children, 34 would have been given antitoxic serum according to the traditional principles; the new method has made it possible to reduce this number to 13, so that 21 patients were spared the risks of heterologous serum treatment.

The different aspects and indications of serum treatment have been discussed.

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Dr. G. Nyerges
Gyáli út 5.
Budapest IX., Hungary