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THE JOURNAL OF TRAUMA
Vol 7, No. 2
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IN VITRO DETERMINATION OF TETANUS IMMUNITY

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Immunological methods for the prevention of tetanus are extremely effective when properly applied, and their widespread use has resulted in a marked reduction in the incidence of tetanus. A full course of active immunization gives virtually complete protection which persists for years in most individuals and probably for a lifetime in many. In addition, prompt production of protective levels of antibody is invariably elicited by a single booster injection of tetanus toxoid. Unfortunately, a course of active immunization will not provide protection against the development of tetanus when started at the time of a given traumatic injury since the development of protective levels of antibody requires several weeks. In injured patients without previous active tetanus immunization, protection must be provided by the passive administration of antitoxin. Passive immunization with human antitoxin is safe and effective, but it is relatively short lived, expensive, and sometimes not readily available. Reactions to human tetanus antitoxin have been seen, but they are rare and usually not severe. The administration of heterologous antitoxin, usually horse antiserum, is less effective than human antitoxin and may cause severe allergic reactions which are disabling and occasionally fatal. Its use should be condemned.

The type of tetanus prophylaxis indicated for a given injury depends entirely upon whether or not the patient has been actively immunized in the past. When accurate information can be obtained, the indications for active or passive immunization are usually obvious. Unfortunately, accurate information is often lacking, and the decision for the type of therapy to be used is necessarily based upon guesswork, intuition, or nothing at all. It would be helpful in such instances to have a suitable laboratory test for the determination of tetanus immunity to provide a rational basis for the selection of the optimal type of therapy for tetanus-prone individuals. Neutralization tests and bio-assays are unsatisfactory for this purpose because of their complexity, the time required for their performance, and their expense. Since *in vitro* methods avoid some of these disadvantages, it is the purpose of this manuscript to review four *in vitro* tests which are considered to have potential usefulness in the clinical laboratory, including a newly developed passive latex agglutination test. Specific details of the first three tests may be obtained by referring to the original articles.

Read before the Twenty-Sixth Annual Session of the American Association for the Surgery of Trauma, Santa Barbara, California, October 6-8, 1966.

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PASSIVE HEMAGGLUTINATION

The passive hemagglutination test was used by Fulthorpe[7] in 1957 for measuring tetanus antitoxin levels and has been used extensively by Levine and coworkers in this country since 1960.[12] Performance of the test requires the use of washed and tanned or formalinized sheep erythrocytes which are sensitized with tetanus toxoid. After the serum to be tested has been inactivated, serial dilutions are incubated at room temperature with the sensitized erythrocytes, and titers are determined by agglutination patterns. Standard antisera must be run with each group of tests, and the true tetanus antitoxin titers must be calculated according to a regression equation.[11] Approximately six hours are required for performance of the test.

The passive hemagglutination technique has several desirable features. Titers may be run on very small serum samples, it is extremely sensitive, large numbers may be run at once, and it is inexpensive when compared to *in vivo* assays. However, the test requires an exacting technique, and experienced personnel are necessary for its performance. The antigen is relatively unstable and must be prepared within 48 hours of use. In addition, a linear relationship between the hemagglutination titers and actual tetanus antitoxin titers is not obtained by the test, and there are many opportunities for the introduction of errors. Benenson et al.[2] found a poor correlation between the hemagglutination titers and titers obtained by toxin neutralization tests in the sera of animals given a booster injection of toxoid following radiation. Chatterjee,[3] on the other hand, has reported good correlation between hemagglutination and bioassay methods.

The passive hemagglutination test has found its best use as a research tool. It is not practical for use in clinical laboratories because of its complexity and the use of relatively unstable reagents.

REVERSED PASSIVE HEMAGGLUTINATION

Cook[4] developed the reversed passive hemagglutination test for determining tetanus antitoxin levels. This test utilizes formalinized sheep erythrocytes which are sensitized with tetanus antitoxin and which may be lyophilized for storage. After reconstitution with buffered glycerin, the cells apparently may be used for a five-day period before deterioration. The test is run by standardizing toxin or toxoid by neutralization with known quantities of antitoxin before incubation of the material with the sensitized cells. After the toxin or toxoid has been standardized, it is neutralized by the serum sample to be tested, the mixture is incubated with the sensitized erythrocytes, and the agglutination titer is obtained. The test thus measures the amount of toxin or toxoid not neutralized by the test serum. From this, the amount of antitoxin in the test serum can be calculated.

The reversed passive hemagglutination test has no advantage over the passive hemagglutination test, but apparently it has all of its disadvantages, is more difficult to perform, and is not as accurate. For these reasons, it also would be an unsuitable test for use in clinical laboratories.

QUANTITATIVE GEL DIFFUSION

A microdiffusion technique in agar gel has been described by Alexander and Moncrief[1] for the semiquantitative measurement of tetanus antitoxin levels. It is performed on a microscope slide, with the use of a template, by diffusion of a standard concentration of tetanus toxoid against serial dilutions of serum. After incubation at 4° C. for 72 to 96 hours, the template is removed, and the sheet of agar on the microscope slide is washed, fixed, and stained. The preparation is viewed through a low-power dissecting microscope, using indirect lighting, and the end-point is read as the dilution having the last visible precipitin band. A standard is run with each group of tests to assure that optimum conditions for immunodiffusion have occurred.

The tetanus toxoid used as the antigen is stable, inexpensive, and commercially available. The test is accurate and easy to perform, although strict adherence to technical details must be observed. Very small samples are required, and a linear relationship is obtained between the titers by gel diffusion and mouse protection assay. The test has two major disadvantages. It is not suitable for the measurement of titers in sera containing less than 0.5 units per milliliter, and the test requires four to five days before results can be obtained.

The gel-diffusion technique has been found to be useful for investigative problems measuring anamnestic responses to tetanus toxoid, but because of its limited sensitivity, it is usually not suitable for the determination of prior tetanus immunity in individuals before a booster of toxoid is administered.

PASSIVE LATEX AGGLUTINATION

The passive latex agglutination test is a new test which has been developed in our laboratory for the determination of tetanus antitoxin titers. It is similar to passive hemagglutination in that it is dependent upon the aggregation of toxoid-sensitized particles by tetanus antitoxin. Since it is a new procedure, details of the technique will be described in a step-wise manner.

A suspension of latex particles (Difco Bacto-Latex 0.81) is incubated with an equal volume of tetanus toxoid (1,000 Lf per ml.) at 37° C. for 60 minutes. The suspension is centrifuged at 2,000 × G for 20 minutes, the supernatant discarded, and the precipitate washed three times with normal saline to remove excess tetanus toxoid. After the final centrifugation, the latex particles are suspended in five times their original volume in normal saline for a stock suspension. The stock suspension has been found to remain stable at 4° C. for at least three months. A working suspension is made from the stock suspension by diluting it 1:10 with normal saline.

Inactivation of complement and adsorption with latex to remove nonspecific agglutinating factors are performed in a single stage by incubation of 0.5 ml. of the serum to be tested with 0.6 ml. of untreated latex suspension at 56° C. for 30 minutes. After incubation, the mixture is centrifuged at high speed to remove any latex aggregates. In removing the supernatant, care must be exercised to avoid the transfer of latex aggregates. If these should be present upon examination with a low-power microscope, recentrifugation is necessary. A high gravita-

tional force is desirable. The supernatant approximates a 1:2 dilution of serum. Serial dilutions are made, and 0.3 ml. of the working antigen (diluted suspension of latex-toxoid complex) is added to each. These mixtures are incubated in Wasserman tubes in a water bath at 37° C. for 120 minutes. The tubes are then centrifuged in an angle-head Adam's serofuge at 3,400 r.p.m. (1,000 × G) for 60 seconds to enhance the formation of aggregates. A constant period of centrifugation is necessary to avoid discrepancies in interpretation of the tests. The tubes are examined with a 30-power binocular microscope for evidence of aggregates of latex particles after slight agitation. The test is regarded as being positive when definite aggregation is observed.

The passive latex agglutination method offers several advantages over the previously described tests. The antigen is relatively stable at 4° C., making it unnecessary to prepare the material repeatedly. The test is inexpensive and considerably easier to perform than the other tests described in this paper. Only three to four hours are required for completion of the test, the majority of this time being required for incubation. It is sensitive for levels of tetanus antitoxin as low as 0.02 units per milliliter, and a linear relationship is obtained when passive latex agglutination titers are compared with titers obtained by mouse protection assays. The test may be modified for very small samples of serum if desired. Obviously, it cannot be performed at the bedside, but any well-equipped laboratory has the necessary equipment available.

The latex-toxoid test has been performed on serum samples from 59 individuals. Thirty-one of these samples were from patients with a positive history of immunization, 15 were from patients who denied any history of tetanus immunization, and 13 were titered sera obtained from Parke, Davis and Company. These

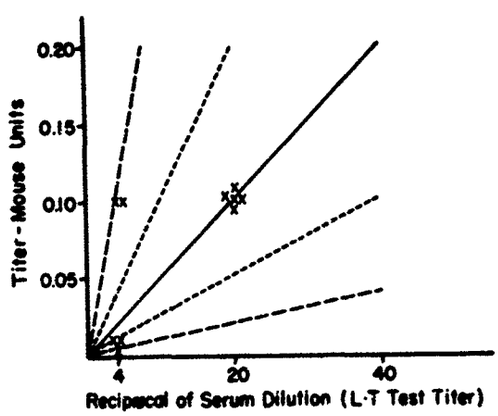

FIG. 1. Comparison of sera with low tetanus antitoxin titers by mouse protection assay and passive latex agglutination technique (L-T test). The mouse test was done at ten-fold increments (0.001 u., 0.01 u., 0.1 u., 1.0 u.), and the passive latex agglutination with five-fold dilutions. Within these dilutions, a five-fold difference between the titers obtained by the tests is acceptable (points should be within the outer dashed lines).

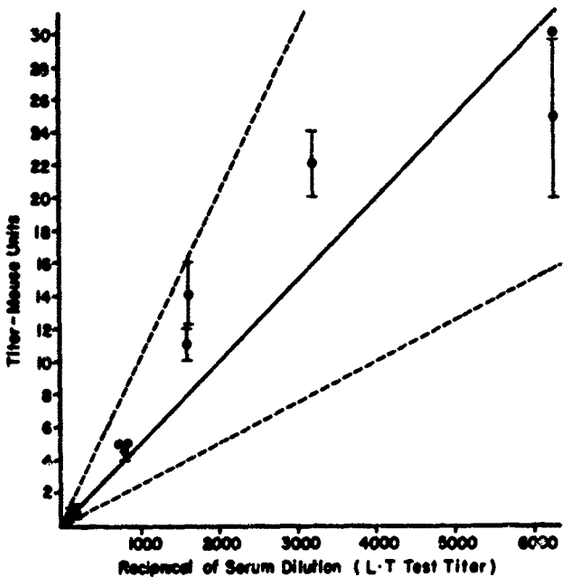

FIG. 2. Comparison of sera with high titers of tetanus antitoxin by mouse protection assay and latex agglutination technique (L-T test). Vertical line, where present, represents range of possible value for mouse units. Dotted lines show the range of error for doubling dilutions. Good correlation is obtained by the two tests.

last 13 sera and an additional 11 sera from patients were examined by both the passive latex agglutination test and mouse protection assay (antitoxin titration by L+ dose). Good correlation between the two tests was found both at high and low antitoxin levels. Each of the determinations fell within the limit of error for the dilutions used (Fig. 1, 2). Of the 31 serum samples from patients who gave a positive history of tetanus immunization, only one was found to have a negative reaction with the passive latex agglutination test. This individual was a physician's 26 year old wife, who obtained the information from her mother that she had received a series of shots before the age of one, but no immunization had been given since that time. Mouse protection assay of her serum sample showed that there was less than 0.001 unit per milliliter, indicating that she was not adequately immunized. Four other patients who had received their last booster shots of tetanus toxoid 21 or more years previously had titers of greater than 0.02 units per milliliter, and one of these had a titer of greater than 1.0 unit per milliliter by both mouse protection test and the passive latex agglutination test (Table I). Four of the 15 patients who denied a history of prior tetanus immunization were found to have a positive passive latex agglutination test (Table II). When mouse protection assays were run on these four sera, it was found that three of the four had antitoxin titers of 0.1 unit per milliliter or greater. Serum samples from the fourth individual showed no protective activity with a titer of less than 0.001

TABLE I

Patients with a Positive History of Tetanus Immunization

Years Since Last Immunization	Number of Patients	Number with Positive Titers		
		1:4	1:20	1:100
0-1	5	5/5	5/5	5/5
1-5	9	9/9	9/9	6/7
6-10	8	8/8	7/8	1/5
10-20	4	4/4	3/4	1/3
21 or >	5	4/5	2/5	1/3
Total	31			

TABLE II

Patients Denying History of Immunization

Negative at titer of 1:4	11
Positive titer at 1:1*	1
Positive titer at 1:20*	3
Total	15

* See discussion in text

unit per milliliter. The reason for the discrepancy in this one serum is as yet unexplained, but it appears that this individual has serum antibodies which cross react with tetanus toxoid but which do not neutralize tetanus toxin. Through the patient's cooperation, a single injection of tetanus toxoid was administered, and serum titers were drawn eight and 15 days later to see if an anamnestic-like response would be obtained for tetanus antitoxin. No such response could be demonstrated.

Since the passive latex agglutination test has provided accurate results on the sera of 58 of 59 individuals and is a simple, rapid, and inexpensive method, it would appear suitable for use in clinical laboratories.

DISCUSSION

A review of four *in vitro* methods for the determination of tetanus immunity has been made. Of these, only the passive latex agglutination technique seems to offer any real promise as a test which could be used routinely in clinical laboratories. It is realized that only preliminary studies have been made with this test, and more information must be obtained before it can be recommended as the sole basis for selection of the type of tetanus prophylaxis to be administered to injured patients.

At the present time, a preliminary protocol for tetanus prophylaxis of the injured patient can be visualized based upon results obtained by the passive latex agglutination test. Injured patients who are absolutely certain that they have never received any tetanus prophylaxis should be given passive protection with

human tetanus antitoxin and active immunization begun with alum precipitated toxoid.[3] Booster injections of the toxoid should be given at the end of one month and again after six months to one year. The passive latex agglutination test would provide useful information in the remainder of patients. The sera from those individuals should be run in three dilutions, 1:4, 1:20, and 1:100. Using standardized sensitized particles, positive titers at 1:4 represent a serum level of 0.02 units of antitoxin or greater, positive titers at 1:20 represent 0.1 unit or greater, and positive titers of 1:100 represent 0.5 units per milliliter serum or greater. If no positive agglutination is obtained, the individual must be considered to have had no prior active immunization. These individuals should be started on a course of active immunization with alum precipitated toxoid as well as passive immunization with human tetanus antitoxin. Individuals with positive titers of 1:100 would have circulating antitoxin levels of 0.5 units per milliliter or greater and probably need no further immunization. A booster injection of tetanus toxoid may not only be unnecessary but may be contraindicated in these individuals since the rare reactions that have occurred with toxoid have generally been in those patients with high antitoxin levels.[6] If a positive reaction occurs only at a serum dilution of 1:4 or 1:20, the patient is probably adequately protected against the development of tetanus, but, because of the comparatively low basal titer, a booster injection of tetanus would be recommended.

Routine utilization of the passive latex agglutination test for the determination of tetanus immunity would allow a rational basis for the selection of antitetanus prophylaxis. Many individuals with traumatic injuries receive booster injections of tetanus toxoid where these are not needed, and, in fact, may be contraindicated because of the occasional reaction to tetanus toxoid. Although these reactions are not frequent, they have been emphasized by Edsel[5, 6] and by Kittler, et al.,[10] who described 13 patients with rather marked reactions to tetanus toxoid, three of whom had systemic reactions. Further boosting of the tetanus antitoxin titer is unnecessary in individuals with greater than one-half unit of tetanus antitoxin per milliliter in their serum. Omission of the booster injection in these individuals would save them both the expense of the medicine and the discomfort of the injection. In the recent study of Gottleib, et al.,[9] 28 of 79 individuals who had received their last booster injections of tetanus toxoid 14 to 21 years prior to the time of study had prebooster titers of 0.4 units or greater, and 18 of the 79 individuals had titers of 0.8 units or greater.

Patients whose status of tetanus immunization is uncertain are received daily in the emergency departments of our major hospitals.[8] Many of these individuals have been actively immunized with tetanus toxoid in the past; for them the administration of human tetanus antitoxin and the performance of a new series of active immunization is unwarranted since a simple booster injection of tetanus toxoid will invariably suffice to give adequate protection against the development of clinical tetanus. The determination of totally nonimmunized individuals is also important so that additional emphasis may be placed on encouraging them to return at the necessary intervals for a full course of active immunization.

SUMMARY AND CONCLUSIONS

Four *in vitro* methods for the determination of tetanus immunity are discussed, including a new test which utilizes a passive latex agglutination technique. Of these tests, the passive latex agglutination technique appears to have the greatest potential for practical clinical value since it utilizes a relatively stable antigen and can be performed inexpensively in any well-equipped clinical laboratory within a matter of hours. The results obtained by the test may be valuable as a guide for th tional selection of the optimal type of therapy for tetanus prone individuals, both when a definite history of past immunization is obtained and when no history is available.

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DISCUSSION

Dr. Wesley Furste (Columbus, Ohio): Several years ago at the January meeting of the American College of Surgeons Committee on Trauma and last year at our Association meeting, it was pointed out that tetanus and the side effects produced by some agents used for tetanus prophylaxis can be eliminated by a four-point program.

Proper surgical wound care (point 2) and emergency medical identification devices (point 4) which have information about the immunization status of the wounded individual are part of the base upon which the pyramid of tetanus prophylaxis is built.

As Dr. Alexander pointed out, the passive latex agglutination test might allow us to know whether or not tetanus immune globulin (human) (point 3) should be administered and whether or not tetanus toxoid should be given (point 1).

Dr. Oscar P. Hampton, Jr., chairman of the A. C. S. Committee on Trauma, has repeatedly pointed out that passive immunization with heterologous antitoxins should not be done without proper consideration. As Dr. Hampton's data show, and as Dr. E. Eriksson of Sweden emphasized last July in Switzerland at the Second International Conference on Tetanus, the risk of tetanus with civilian wounds is less than the risk from the administration of heterologous antitoxins.

Also, Dr. Hampton has repeatedly urged that human tetanus antitoxin should not be given unless there are clear-cut reasons for doing so, for TIG(H) is a valuable product obtainable from only human donors.

In his manuscript, Dr. Alexander noted that many wounded individuals receive booster injections of tetanus toxoid when they are not needed and, in fact, may be contraindicated because of the occasional reaction to toxoid. Such was the attitude, also, at the International Conference on Tetanus. Dr. Inga Scheibel of Denmark reported at the conference that, in Denmark, if a patient has an adequate basic immunization, revaccination is not done except for injuries demanding medical attention. Dr. D. G. Evans of England remarked that the period for a wound booster may be as long as four years.

Nevertheless, at present, in view of legal judgments for tetanus cases, as the $174,748.50 award recently granted in a United States court, a one-year wound booster interval may be advantageously recommended, unless a higher serum antitoxin level is evident, such as Dr. Alexander's test might prove.

Just as Dr. Alexander is concerned, I am worried about the one individual in his series who had a positive latex agglutination test, but whose serum showed no protective activity with the mouse protection assay.

If the latex agglutination test can be performed as quickly and as inexpensively as the serum amylase determination, such a test might become as important for the trauma surgeon as the amylase test is for the cavity surgeon. A commercially-prepared antigen, i.e., the latex toxoid complex, which the technician would have immediately available without having to prepare it, could make this test a rapidly performed one.

I congratulate Dr. Alexander and Col. Moncrief on their stimulating approach to determining the patient's potential resistance to tetanus and certainly appreciate their letting me see their manuscript in advance.

Dr. Householder said he would permit me to take another thirty seconds to ask the audience some questions.

As some of you might know, Dr. Hampton, Dr. Paul A. Skudder and I are quite interested in the supply of human tetanus antitoxin. In going through the literature, I found 24 synonyms for this particular product, which is properly known as tetanus immune globulin (human) or TIG(H). Commercially, it is sold in the United States as Hyper-Tet®, Hu-Tet®, Homo-Tet®, and Gammatet®; in Austria it is called Tetabullin and in Switzerland it is known as Tetuman Berna.

What I want to ask is: How many in the audience are able to obtain tetanus immune globulin (human) when they want to give it to a patient? I see from the hands raised that practically all of you can now provide TIG(H) for your patients. And now may I ask: How many in the audience are unable to obtain TIG(H) when they want to administer it to a patient? I see three raised hands. Where do these three surgeons practice?

ANSWER: We had some trouble in obtaining TIG(H) in Milwaukee, Wisconsin, about three months ago. Now our supply situation is much better.

DR. FURSTE: With such a supply situation for TIG(H), we should no longer have anaphylactic problems or serum sickness on account of patients being given the heterologous equine or bovine tetanus antitoxins.

CAPTAIN J. WESLEY ALEXANDER (Cincinnati, Ohio): I appreciate the comments of Dr. Furste and I have only one comment to make concerning possible commercial availability. We are now working with one of the pharmaceutical companies to see if we can make a better complex that will last for an even longer period of time, and also to see if we can determine why an occasional patient has a false positive reaction. Thank you.