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Overview of currently approved serological methods with a focus on diphtheria and tetanus toxoid potency testing

James E. Keller *

FDA, Center for Biologics Evaluation and Research, Bethesda MD 20892

Abstract

Vaccines are biological products made from living organisms. The natural complexity of biological molecules along with the inherent uncertainties of product manufacturing introduces the likelihood that random alterations can impact the quality of the vaccine each time it is made. The factors that can affect the final product are often unknown. Testing for potency of vaccine bulk or product dispensed into final containers was designed with the hope of ensuring that a vaccine is effective when used during its approved dating period and that its protective activity was not inadvertently altered during any phase of production. Ideally, potency testing measures a biological or biochemical property of the vaccine that is related to its ability to elicit protective immunity in the target population and provide some assurance that consistent clinical benefit is derived from each lot of product. Potency methods vary depending on the nature and composition of the vaccine. *In vivo* potency testing might entail immunizing groups of laboratory animals and then challenging them directly to measure survival, or involve serological potency assays in which sera from immunized laboratory animals are tested for the ability to neutralize pathogens or toxins. In the U.S., diphtheria toxoid and tetanus toxoid potency tests have customarily involved a serological method. This approach uses fewer animals than would have been required using a direct challenge method, while providing satisfactory evidence that each toxoid lot could induce protective immunity. This paper will discuss the details of the original U.S. test method for diphtheria and tetanus toxoid potency and present issues that must be considered when developing and validating non-animal-based approaches to refine or replace these tests.

Keywords: tetanus; diphtheria; toxoid; potency

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* Corresponding author *e-mail address*: james.keller@fda.hhs.gov

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1. Vaccine potency

Once a vaccine is licensed by the U.S. Food and Drug Administration's Center for Biologics Evaluation and Research (CBER/FDA), the manufacturer assures through consistency of the manufacturing process and product testing that future lots of vaccine will have the same biological and biochemical properties as the lots that were tested and shown to be safe and effective in the clinic. A good potency measure is key to ensuring consistency of the vaccine. Vaccine potency testing consists of one or more laboratory test methods to ensure that the final packaged vaccine product will elicit a desired protective immune response in the target population. Ideally, potency test data would be predictive of efficacy; however, establishing a direct correlation between potency and clinical outcome can be difficult, if not virtually impossible [1]. While a direct link between potency and efficacy may not be possible to demonstrate, a properly designed potency test helps to ensure that the vaccine was manufactured in a consistent manner and has the same biological and biochemical properties as lots shown to be effective in the clinic [2]. The design of a relevant potency assay may require substantial laboratory investigation, followed by reagent production, protocol design, and assay validation.

Potency test methods can be divided into three broad categories: (1) *in vivo* immunization with lethal challenge, (2) *in vivo* immunization with serological analysis, and (3) physicochemical/*in vitro* or *ex vivo* analytical testing. Viral vaccine potency assays in the United States tend to fall within all three categories, but most frequently they utilize antigen quantification, measure viral viability (PFU, infectivity of cell cultures, etc.), or use some combination of similar analytical tests. In contrast, bacterial vaccine potency assays tend to rely upon animal immunization with either a challenge aspect or a serological measure of the immune response. They less often rely entirely on biochemical analysis. Bacterial vaccines derived from polysaccharide antigens are a rare example of a class of vaccine that does not use animals for potency testing: potency is determined exclusively from biochemical analyses [3]. In contrast to polysaccharides, protein-derived bacterial vaccines, especially vaccines containing antigens that have been chemically inactivated, are more difficult to biochemically characterize in a precise manner, even using state-of-the-art techniques. Consequently, potency assays for these types of vaccines generally involve the direct immunization of animals with a serological and/or survival endpoint. For example, potency of acellular pertussis vaccines is established from a series of serological *in vitro* endpoints based on antibody levels elicited by each pertussis protein in the vaccine. Potency testing of tetanus, diphtheria, and anthrax vaccines involves either the induction of a protective antibody response or animal protection.

Over the past 35 years, several efforts have been undertaken to develop *in vitro* tests for diphtheria toxoid and tetanus toxoid potency either to replace the *in vivo* survival portion of the potency test or to eliminate animals entirely. The following section will provide side-by-side outlines of diphtheria and tetanus toxoids to describe how each disease is caused, how each vaccine protects, and how potency testing has contributed to the successful implementation of each vaccine to help protect public health.

2. Diphtheria and tetanus toxoids

Corynebacterium diphtheriae are aerobic, highly contagious non-spore formers, and are not known to cause respiratory disease in any animals other than humans [4]. *Clostridium tetani* are spore formers that are ubiquitous within soil, are commonly found within the intestinal flora of grazing animals, require anaerobic conditions to germinate, and are not contagious or transmissible [5]. In spite of these substantial differences, both bacteria cause disease by similar general mechanisms: an initial localized infection followed by the release of protein toxins into the general circulation. Diphtheria toxin and tetanus neurotoxin are large proteins that diffuse from the infection site into local tissues where each toxin binds to and acts upon specific cells [6,7]. Much research has examined the biochemistry of these toxins, generating a relatively clear understanding of how each functions at the molecular level and how each toxin acts to cause illness [6-11]. The toxins are solely responsible for the lethal outcome of each disease. Therefore, although both diseases have few specific commonalities, they share the critical quality of being toxin-mediated illnesses.

In 1897, Ehrlich reported that tetanus toxin could be chemically inactivated to produce a nontoxic tetanus molecule that interacted with tetanus antibodies *in vitro* and also elicited protective immunity in mice [12]. Ehrlich had found a similar phenomenon while studying diphtheria toxin, leading him to coin the term “toxoid” to describe nontoxic toxin preparations that retain antigenic similarity to the parent toxins. Löwenstein (1909) was the first to

provide documented evidence that formalin would detoxify tetanus toxin to produce tetanus toxoid more efficiently than Ehrlich's method [13,14]. Löwenstein was studying the degradation of tetanus toxin in the presence of artificial light and solar light. By accident, one batch of toxin was treated with formalin instead of the typical preservative, phenol. Formalin rendered tetanus toxin harmless without compromising its immunological similarity to native tetanus toxin. Though Löwenstein's work did not directly lead to desirable breakthroughs for vaccine development, after World War I separate groups led by Ramon and Glenny reported the use of formalin to detoxify diphtheria toxin for the safe immunization of animals, and eventually people, for antitoxin production [15,16]. Ramon's group was the first to develop adequate testing methods to assure that toxin quality, detoxification conditions, and toxoid antigenic content were well controlled, which facilitated subsequent clinical studies to be performed in children in the U.S. [17,18, 29].

A limitation that Ehrlich encountered in studying toxoid chemistry was that direct identification and quantification of toxoid molecules was not possible. Decades after Ehrlich, this problem remained a significant scientific obstacle that hindered establishing standard toxoid dosing for clinical testing. Ramon created a practical *in vitro* method to quantify antigen content by measuring the amount of antitoxin that would precipitate the toxoid [15]. He eventually optimized his flocculation method for both diphtheria and tetanus toxins and toxoids. Glenny contributed much to confirming the utility of and then modifying Ramon's original diphtheria flocculation methodology [16,19]. Today the Ramon and Glenny procedures for measuring the Limit of Flocculation are routinely performed during diphtheria and tetanus toxoid manufacture. The flocculation values derived from the test are used to establish human dosage for each final lot of vaccine [20].

The expediency and quickness of the Limit of Flocculation test represented a critical contribution from Ramon to the manufacture of diphtheria and tetanus toxoids for human immunization. With the development of the flocculation test, there was hope that animal testing would be unnecessary because the procedure measured the ability of protective antiserum to bind to the toxoid. Thus, the presumption was that, because Ramon's assay quantified the ability of a strongly neutralizing antiserum to react with each toxoid batch, the toxoid would have the critical antigenic qualities needed to elicit protective immunity *in vivo*. However, within several years it became clear that flocculation or antigenic content of a toxoid as provided by Ramon's assay did not always correlate with the *in vivo* immune response elicited by the toxoid [21,22]. By the mid 1930s, it was generally understood that the flocculation test provided useful data for establishing dosing, but independent animal-based potency testing was required to ensure that the toxoid batch was immunologically active *in vivo*.

Potency test methods used in the U.S. for diphtheria and tetanus toxoid can trace their origins to Ehrlich's original work in the 1890s, when he developed the standard method for quantifying protective horse antibodies to diphtheria toxin. The Ehrlich procedure calls for mixing serial dilutions of a standard toxin preparation with a fixed volume of equine immune serum. These mixtures are then injected into different groups of nonimmunized guinea pigs. By testing several dilutions of toxin, the survival of some animals and death of others quantifies the neutralizing ability of each batch of equine antiserum.

Forty years later, potency testing of diphtheria and tetanus toxoids in the U.S. came to rely upon mixing sera from immunized guinea pigs with a fixed quantity of standard toxin, which was then injected into naïve guinea pigs. Animal survival indicated that the serum from the immunized animals neutralized each toxin. Potency determined in this manner is a limit test and assures that the immune response elicits a desired threshold level of protective antibody. The potency method does not require precise value determination of how high the antibody level is.

This type of potency testing was first described for alum-precipitated tetanus toxoid in 1934 [23,24]. One of the methods described by Bergey and Etris, which is the basis for establishing tetanus toxoid potency today, entailed the immunization of five guinea pigs. Six weeks later, immune serum was collected from all animals and pooled. Potency was measured by Ehrlich's method of mixing dilutions of the pooled serum with a fixed dose of standard tetanus toxin. In a series of papers, Bergey and Etris (1934-1939) presented clinical studies showing that immunization with potent tetanus toxoid elicited protective antibody titers in adults and children [23,25,26].

Because diphtheria was a severe, contagious health threat to young children, diphtheria toxoid potency testing in the 1920s and 1930s went through many stages of development. The initial potency tests were performed on formalin diphtheria toxoid without adjuvant, which entailed active immunization of groups of guinea pigs followed by lethal or subcutaneous challenge with a given quantity of toxin [18,27-31]. Research by Glenny and others indicated that diphtheria toxoid bound to alum led to much higher antibody titers in animal studies. This led to using a potency test similar to that used for tetanus toxoid [32]. Sickles (1937) provides the first U.S. report of active

immunization of a group of guinea pigs with one dose of diphtheria toxoid, from which serum was collected at the end of four weeks and pooled. Then a single serum dilution was mixed with a standard quantity of diphtheria toxin and injected into a naïve group of guinea pigs to determine whether immune serum protected animals [33]. By 1947, this approach had gained prominence in the U.S. and was adopted as a standard test for precipitated diphtheria toxoid.

The details of both diphtheria and tetanus toxoid potency methods are described in the Minimum Requirements established and amended by the National Institutes of Health between 1947 and 1953 [34-36]. Separate methods are outlined for testing different forms of toxoid such as plain diphtheria or tetanus toxoids or for measuring potency when both toxoids were combined within one vaccine product. The overlapping similarities for measuring the potency of alum-containing diphtheria and tetanus toxoids allowed the potency testing of both vaccine components using a single group of immunized animals. For a combined vaccine using alum adjuvant, the Minimum Requirements allow for immunizing four or more guinea pigs with the final combined bulk diphtheria toxoid/tetanus toxoid product (**Figure 1**). Immune serum is collected four or six weeks later. A defined dilution of immune serum is combined with a standardized quantity of either tetanus toxin or diphtheria toxin and then injected into test animals. Protection of the test animals, which equates to toxoid potency, is determined using three mice or pairs of guinea pigs for tetanus toxoid or pairs of guinea pigs for diphtheria toxoid. If the final stage of testing for both toxoids is limited to guinea pigs, then in principle the potency of diphtheria and tetanus toxoids can be measured using as few as 12 guinea pigs (**Figure 1**). A valid test requires the use of two additional control guinea pigs to assure that the proper quantity of toxin was used in the test animals.

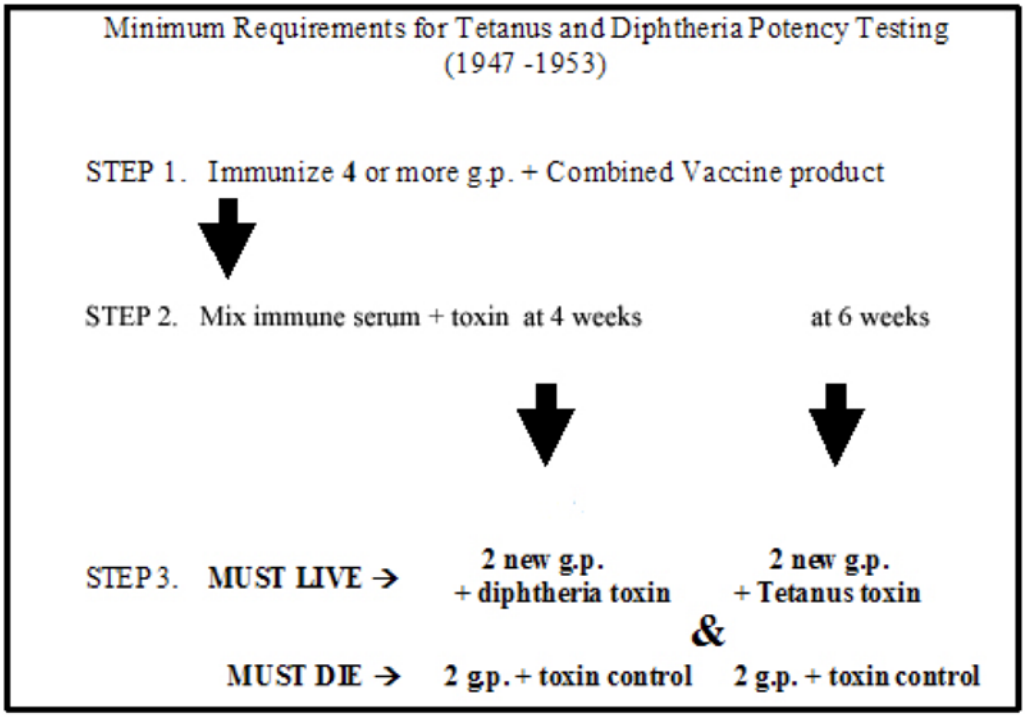

Figure 1. Outline of the U.S. minimum testing requirements for establishing potency of diphtheria and tetanus toxoids in the U.S. A minimum of 12 guinea pigs (g.p.) are required to certify the potency of both toxoids. For pediatric use, each toxoid must elicit at least 2 antitoxin units/mL within the defined timeframe of the test.

Toxoid strength is derived from the first two steps of the potency test: the biological immune response within the immunized guinea pigs and the biochemical ability of the serum to neutralize toxin *in vitro* (**Figure 2**). The third step of the assay, injection of the mixed immune serum and toxin into nonimmunized guinea pigs, does not directly measure the quality of the toxoid or antiserum but rather is used to detect whether active toxin remains within the

antitoxin–toxin mixture. Currently, there are no *in vitro* analytical methods available that can detect trace active toxin within toxin-antibody mixtures. Naïve animals used in Step 3 of testing serve primarily as biochemical detectors of free toxin that was not neutralized during the *in vitro* reaction between antibodies and toxin.

3. Alternative assays

Based on the above description of the U.S. potency tests, a potential approach for an alternative test would involve replacing the challenge portion of the test with an *in vitro* method to detect free toxin within a mixture of immune serum. Unlike tetanus toxin, diphtheria toxin kills a variety of cells that can be readily grown in culture, raising the possibility of using cell culture methodologies to quantify traces of toxin within complex biological mixtures. Validation studies must demonstrate that cell survival is steadily reduced by increasing levels of toxin, while showing that increasing concentrations of immune serum incrementally and proportionally stop toxin action on the cells and consequently allow cell survival. The most critical feature of the alternative method would be the ability to reliably discriminate between acceptable and unacceptable toxoid lots. The validation of a cell-based assay for antitoxin would also include verifying that the cell line can be maintained without changing sensitivity to toxin, reliably expresses toxin receptors, and replicates uniformly during the time-course of the test.

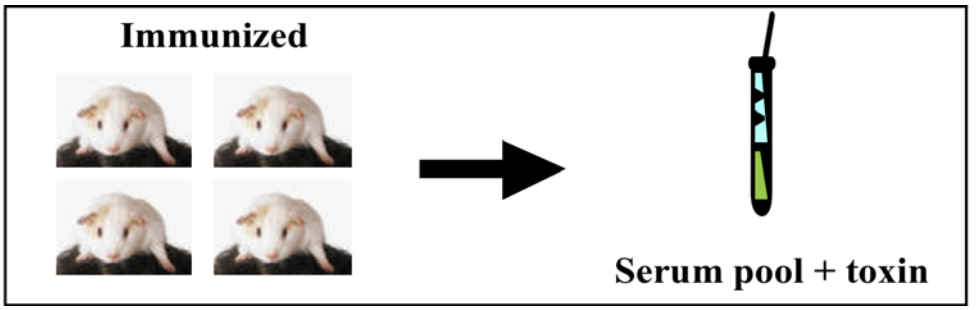

Figure 2. Measuring potency of diphtheria and tetanus toxoid lots is a combination of the biological response to immunization and the biochemical strength of the immune response measured *in vitro*. The final group of nonimmunized guinea pigs (not shown) is used not to gauge toxoid effectiveness but to determine the extent of the neutralization reaction between antibodies and toxin.

Diphtheria toxin action on cultured cells was first demonstrated in 1957 [37-38]. Later, Miyamura et al. modified the cell culture method using multiwell microculture plates and monitoring changes in medium pH to facilitate high throughput screening of antitoxin samples [39]. This type of cell culture-based test has been primarily evaluated in relation to *in vivo* testing of diphtheria toxin activity in the rabbit intradermal skin test as used outside of the United States [40-42]. Rabbit and cell culture methods produced similar data for quantifying antitoxin neutralization. Dular was the first to examine the neutralizing titers of immune serum collected following the immunization procedure used for the U.S. potency test [43]. Dular's results showed that guinea pig immune serum protected cells from toxin in a manner that did not correlate well with animal protection, suggesting that the different sensitivities of cultured cells and guinea pigs required greater optimization before cell-based serum analysis could be adopted. Gupta et al. altered the amount of toxin used to treat cells, which produced a narrow set of conditions where data correlated well with *in vivo* protection [44-45]. The modified assay has some limitations, however, and implementing the cell-culture based test for routine Good Laboratory Practice (GLP) testing may require the development of a low-avidity reference antitoxin to reflect the low-avidity antitoxin produced during each U.S. potency test. Studies by both Dular and Gupta support the notion that a separate cell-based assay could be implemented to detect non-neutralized diphtheria toxin within control and test samples.

An advantage of using a cell-based method in place of Step 3 of the *in vivo* test is that the cell culture test quantifies the critical functional aspect of antibody-mediated neutralization of the active toxin. Other *in vitro* antibody methods have been used to measure diphtheria and tetanus toxoid potency but, unlike cell-based survival, these *in vitro* assays do not measure functional antibody titers or biological activity of residual toxin. Diphtheria and

tetanus toxoids induce neutralizing and non-neutralizing antibodies in unpredictable abundance. Quantifying the functional neutralizing titer provides more relevant data regarding both the magnitude and protective ability of the resulting immune response than methods that do not distinguish between types of antibody. Two simpler potency methods for diphtheria and tetanus toxoids have been used to varying extents around the world. These enzyme-linked immunosorbent assays (ELISAs) do not distinguish between neutralizing and non-neutralizing antibodies. Both the standard antibody-capture ELISA and the ToBI ELISA measure the induction of total toxin-specific antibodies without distinguishing between or measuring the relative abundance of neutralizing / non-neutralizing antibodies elicited by each toxoid.

The ToBI ELISA measures the ability of immune serum to compete with a standard polyclonal antibody [47-49]. In this assay, a non-guinea pig antibody reagent is allowed to bind to the toxin. Guinea pig immune serum is then added to compete with the first antibody preparation. The assay measures whether the guinea pig serum contains any variety or combination of antibodies that bind to or alter the regions of the toxin that are recognized by the non-guinea pig antibody reagent. An advantage of the ToBI ELISA is that it is typically easier to develop and qualify reagents for a biochemical *in vitro* method than for animal or cell culture-based methods. This is not necessarily the case for the diphtheria and tetanus reagents, however. Periodically replenishing the toxin standards with new toxin batches will introduce changes in antigenic content of the toxin, which can be substantial. Additionally, replacing the non-guinea pig polyclonal antibody test reagent will introduce an antibody preparation that recognizes different sets of epitopes on the test toxin. Thus, within several cycles of replacing reagents, a valid *in vitro* ELISA procedure may evolve to measuring antigenic qualities that do not reflect the potency of toxoid or effectiveness of the guinea pig immune serum.

ELISA methods share the same disadvantage first encountered with the Ramon-Glenny flocculation assay. Namely, toxoid-antibody interactions *in vitro* do not always correlate with *in vivo* potency, primarily because non-neutralizing antibody binding to toxin *in vitro* can yield confounding or misleading results. Whether an *in vitro* antigenicity test uses polyclonal antibodies to bind several epitopes to precipitate toxoid (Ramon flocculation) or uses the same polyclonal antibodies in an ELISA format, the common functional properties of each method introduce an element of uncertainty toward the final result because the physical consequences of antigen–antibody binding *in vitro* is not equivalent to toxin neutralization and biological protection.

Multiple epitopes exist on each toxoid. Some are critical for protection and some are not. Non-neutralizing antibody populations can antagonize neutralizing antibodies when binding to overlapping toxin epitopes. The diversity of the epitopes recognized and the abundance of each type of antibody within serum varies greatly upon immunization. The knowledge gap for understanding the molecular and biochemical qualities of neutralizing/non-neutralizing *antibodies* and neutralizing/non-neutralizing *toxoid epitopes* is wide. The interplay between these factors and how they influence the overall protective ability of each toxoid lot must be better understood before an *in vitro* antigenic alternative method is implemented. At present, there is no clear understanding of whether all toxoid lots from one manufacturer have a uniform distribution of the same neutralizing and non-neutralizing epitopes or whether the epitope distribution is random. If the distribution is random, it is uncertain that an ELISA or any *in vitro* antigen quantification assay can reliably distinguish between potent and subpotent toxoid lots.

4. Discussion

The mechanism of protection against both diphtheria and tetanus diseases is well known to be antibody-mediated neutralization of the toxins from each infection. In the United States, potency is currently measured as the ability of each toxoid lot to elicit a required threshold level of protective antibodies in laboratory animals. Protective antibody values are a combined measure of beneficial neutralizing antibodies offset by any antagonistic effects from non-neutralizing antibodies: diphtheria and tetanus immunization elicits both types of antibodies in varying abundance. The physical structure of each toxoid is a critical aspect affecting how well the vaccine lot will perform, but detailed knowledge of critical structural components of each toxoid remains unknown. Given that the overriding mechanism of protection is functional antibody titers, which are readily measurable with the current U.S. potency method, and, because the understanding of neutralizing and non-neutralizing antibodies and toxoid epitopes is greatly limited, a conservative regulatory approach would most likely opt for a potency assay that quantifies protective antibody levels.

To completely eliminate animal testing from these two U.S. potency tests a much deeper understanding of whether potent toxoid lots share universal qualities is required. The repertoire and variations of structural epitopes across the surface of the toxoid can vary significantly from the epitopes found on the parent toxin. The current state of science must elucidate the basis for why (or whether) various epitopes might constitute neutralizing and non-neutralizing epitopes and provide direct answers as to why (or whether) antibodies that bind to non-neutralizing epitopes can antagonize protective antibodies that bind to the same physical overlapping region of the antigen. The current state of science does not provide adequate knowledge regarding the diversity of epitopes within toxoid lots produced by one manufacturer and certainly not for similar toxoid products made by multiple manufacturers. Thus, although it is reasonable to expect that epitope content does not vary substantially lot to lot within vaccine from a single manufacturer, it is not known whether there are universally conserved protective epitopes among all the various diphtheria and tetanus toxoid products across all of the manufacturers. Therefore, the current state of science does not readily point towards an analytical method that can has the necessary qualities to serve as a universal potency test for each toxoid suitable for all companies making diphtheria and tetanus toxoid vaccines.

In a practical sense, diphtheria and tetanus immunization programs in the US have successfully eliminated two severe diseases. The assays for each toxoid were developed and implemented when the understanding of toxoid integrity was minimal. Currently the state of knowledge has not substantially improved to help understand the molecular basis for what constitutes a good toxoid batch or a poor one. Antibody-mediated neutralization of each toxin is well known to be the immunological basis of protection to each disease. Both potency assays continue to provide the critical ability to measure toxin neutralization which ensures that each lot of toxoid released into the U.S. market has the ability to elicit a defined level of protective antibodies.

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