THE ADSORPTION OF DIPHTHERIA TOXIN AND TOXOID ON COLLOIDAL GELS

F. A. MILLER, THOS. DEVRIES, and MARY ANN MILLER,
Eli Lilly Co. and Purdue University

Introduction

The work of Ramon[1] on the use of non-specific substances such as tapioca and alum in diphtheria toxin and toxoid for the purpose of increasing the degree of immunity produced in animals, has led to the investigation of other substances for this same use. The results of this early work, especially with alum, showed that while the addition of these substances definitely increased the rate of antitoxin production in most animals used, there were certain disadvantages to their repeated use on the same animal. In an effort to eliminate these disadvantages, and at the same time reduce the rate of elimination of the antigen by the animal, the use of colloidal substances was suggested. The increased rate of antitoxin production which occurs when these foreign substances are added to toxin is thought to be due to retarded adsorption and possibly also to increased irritation of the subcutaneous tissues. It is well known[2] that unmodified liquid toxin when injected into animals is rapidly lost by elimination. Only a small portion of the toxin is actually retained by the tissues and becomes effective as an immunizing agent. The adsorptive properties of various colloids for dyes, enzymes, and other similar substances would indicate their use for the adsorption of toxin from the original meat broth in which it is produced, with subsequent injection of the toxin-containing colloid into the animal. This toxin should be more slowly released and be effective for tissue stimulation over a period of time, thus giving greater immunity response with less antigen.

Experimental

a. Preparation of gels

As a first step, various gels were made up as follows: aluminum hydroxide from the mixing of solutions of aluminum nitrate and ammonium hydroxide; aluminum hydroxide from the hydrolysis of urea in aluminum nitrate solution; calcium phosphate from the mixing of solutions of calcium chloride and dibasic sodium phosphate; aluminum phosphate from solutions of aluminum nitrate and dibasic sodium phosphate; and silica gel from sodium silicate solution with acetic acid. The aluminum hydroxide gels were made from solutions with concentrations varying from 4 N to 0.001 N in order to determine the different types of gels

[1] Comptes Rendus, 93, 506 (1925).

[2] A. T. Glenny, G. A. H. Buttle, and F. Muriel. Diphtheria Toxoid Elimination. Jr. Path. and Bact., 34, 267 (1931).

that would be obtained. The relative rates of adsorption for the different kinds of gels (alumina gels from solutions of varying concentrations, calcia gels, etc.) were determined by the use of dyes and comparison in the colorimeter. Only slight differences in the rates of adsorption were indicated by this method.

b. Adsorption of toxin on gels

Experiments were then made using these gels in varying amounts with diphtheria toxin. Diphtheria toxin was chosen because of the greater accuracy of animal tests for its presence and quantity. The completeness of toxin adsorption was determined by the flocculation test and the minimum lethal dose test on guinea pigs. It was found that at least equal volumes of gel and toxin were necessary to secure the adsorption of from eighty to ninety per cent of the original toxin. These experiments were performed by adding to a quantity of toxin an equal volume of the colloid taken up in as small a volume of water as possible, and allowing the mixture to stand twelve to twenty-four hours with frequent agitation. Later experiments indicated that adsorption takes place almost immediately. The mixtures were then filtered and the gel washed with distilled water until the washings gave a negative biuret test. In this manner the meat proteids of the original toxin broth containing no essential constituents were removed. There is, then, a consequent purification of the toxin at this point. The washed gel was then resuspended in enough distilled water to bring the volume up to the original volume of the toxin in order to facilitate calculations for dosage to guinea pigs. A portion of the first filtrate was retained for tests for the presence of unadsorbed toxin. The tests on these filtrates from the alumina and calcia gels always indicated an adsorptive rate of from eighty to ninety per cent.

c. Toxicity of adsorbed toxins

The next problem was to discover to what extent the adsorbed toxin was retained by the gels when injected into guinea pigs. By calculating these tests on the original value of the toxin at an adsorptive rate of eighty per cent, it was found that in some instances as much as five minimum lethal doses of toxin could be injected without causing the immediate death of the animals. When larger doses were given the animals died, the time of death varying with the increase in the dose but nearly always occurring later than would have been expected had straight toxin been administered. This would indicate a relatively slow rate of release of the toxin from the gel. When the gel was given in amounts containing from one to five minimum lethal doses, the rate of release was such that the animal could develop sufficient immunity to protect itself. These animals lived from 18 to 32 days after injection of the doses. Large doses of the gels without adsorbed toxin caused no ill effects when injected into animals.

d. Adsorption of toxoid on gels and antigenic efficiency

These preliminary experiments were made with diphtheria toxin because of the more accurate available tests for checking both the rate

of adsorption and the rate of release of the adsorbed toxin when injected into live animals. When considered from the point of immunity response, however, it is known to be more difficult to immunize with the toxin than it is with the toxoid. The toxoid is the detoxified toxin, detoxification being obtained by the use of 0.4% formaldehyde and prolonged incubation. Since the gels so far tested seemed to possess the properties of adsorbing this toxin in considerable amounts and the rate of release when injected into animals was relatively slow, it would seem to offer advantages over the unmodified antigen in the production of antitoxin.

Further experiments were performed, therefore, for the purpose of studying the comparative degree of immunity developed when using adsorbed and unadsorbed toxoid. This test was made by injecting series of guinea pigs with one-half cubic centimeter amounts of the adsorbed and unadsorbed toxoids, allowing four to six weeks for immunity to develop and then giving varying doses of toxin. The pigs withstanding the largest doses of toxin would have developed the most antitoxin.

TABLE I

Immunity Test—Diphtheria Toxoid Adsorbed on Calcium Gel
Toxoid No. 872145

Guinea Pig. No.	Amount Injected	Date Inj.	Result	Toxin Inj. 9-11-33	Result	Toxin Inj. 9-22-33	Result
162	0.5 cc.	8-4-33	Lived	5 M.L.D.	Lived	500 M.L.D.	Lived
167	0.5 cc.	8-4-33	Lived	5 M.L.D.	Lived		
166	0.5 cc.	8-4-33	Lived	20 M.L.D.	Lived		
165	0.5 cc.	8-4-33	Lived	20 M.L.D.	Lived		
163	0.5 cc.	8-4-33	Lived	100 M.L.D.	Lived		
160	0.5 cc.	8-4-33	Lived	100 M.L.D.	Lived	300 M.L.D.	Lived

Immunity Test—Unmodified Diphtheria Toxoid No. 872145

Guinea Pig. No.	Amount Injected	Date Inj.	Result	Toxin Inj. 9-11-33	Result	Toxin Inj. 9-22-33	Result
164	0.5 cc.	8-4-33	Lived	5 M.L.D.	Lived	500 M.L.D.	Lived
161	0.5 cc.	8-4-33	Lived	5 M.L.D.	Lived		
168	0.5 cc.	8-4-33	Lived	20 M.L.D.	Lived		
171	0.5 cc.	8-4-33	Lived	20 M.L.D.	Lived		
174	0.5 cc.	8-4-33	Lived	20 M.L.D.	Lived		
169	0.5 cc.	8-4-33	Lived	100 M.L.D.	Lived		
172	0.5 cc.	8-4-33	Lived	100 M.L.D.	Lived		
170	0.5 cc.	8-4-33	Lived	100 M.L.D.	Lived	300 M.L.D.	Lived

Table I shows the result of this test. The doses of toxin given subsequent to the toxoid were not sufficient to kill any of the test animals, not even those receiving the unadsorbed toxoid although previous experience has shown that 100 M.L.D. of toxin should have killed all of these pigs. The only explanation for this fact is that the toxoid, as such, was better than had been expected. The subsequent injection of three hundred, then five hundred M.L.D. of toxin did not kill any of the test animals, showing a great deal of immunity was being developed.

The method of determining the degree of immunity developed by toxoid has now been modified.[3]

Instead of injecting increasing doses of toxin to find the degree of immunity developed in the animals, the guinea pigs are now bled three and four weeks after giving the toxoid and pooled samples of the blood serum are then tested for the actual number of antitoxic units produced.

Table II gives the results of an experiment using this modified method where diphtheria toxin was adsorbed on calcia and silica gels in comparison with unadsorbed toxoid. The toxoid was adsorbed by the calcia gels by two different methods. First (Number I), the prepared calcia gel was added to the toxoid as described; second (Number II), the gel was produced in direct contact with the toxoid as follows: the required amounts of the reagents, calcium chloride and dibasic sodium phosphate, were dissolved separately in the proper volumes of toxoid and then these two solutions combined. In this manner the gel was formed in direct contact with the toxoid. The results of this experiment as shown by rabbit skin test are as follows:

TABLE II

	Units	Serum samples taken three weeks after injection	Serum samples taken four weeks after injection
Control Standard Antitoxin + Standard Toxin	1	−+	−+
Unmodified Toxoid 0.5 cc.	0.5	+++	Not tested
	1.0	++++ Indurated	Not tested
	2.0	++++ Necrotic	++++ Necrotic
	4.0	++++ Necrotic	++++ Necrotic
	6.0		++++ Necrotic
Calcia Gel. Number I, 0.5 cc.	0.5	++++ Necrotic	
	1.0	++++ Necrotic	
	2.0	++++ Necrotic	Sample not obtained
	4.0	++++ Necrotic	
	6.0		
Calcia Gel. Number II, 0.5 cc.	0.5	++	
	1.0	+++	
	2.0	++++ Indurated	+++
	4.0	++++ Necrotic	++++ Indurated
	6.0		++++ Indurated
Silica Gel. Number III, 0.5 cc.	0.5	++++	
	1.0	++++ Necrotic	
	2.0	++++ Necrotic	++++ Necrotic
	4.0	++++ Necrotic	++++ Necrotic
	6.0		++++ Necrotic

[3] By National Institute of Health, Washington.

The actual degree of immunity (antitoxic units) developed from injecting guinea pigs with one-half cubic centimeter amounts of these adsorbed toxoids as compared with that resulting from the use of the same toxoid unmodified and used in the same amount, is indicated in the table by the use of the + sign. The control consists of standard toxin and antitoxin in such proportion as to give practically a neutral mixture and give a — + reaction. This reaction denotes the presence of one unit of antitoxin. Three weeks after injection of the adsorbed and unadsorbed toxoids and again after four weeks the guinea pigs were bled and the resulting serum tested for the presence of antitoxin in the above manner.

Discussion

While none of the adsorbed preparations or the unadsorbed toxoid produced as much as one-half unit of antitoxin, still it is clearly demonstrated that the calcia gel number II was superior to the other two gel preparations and to the unadsorbed toxoid. The number II calcia gel also shows an increased amount of antitoxin from the third to the fourth week while the others show no increase. The terms "indurated" and "necrotic" are indications of decreasing amounts of antitoxin.

The colloidal preparations used in these experiments were produced in the chemical laboratories at Purdue University, and the adsorption and animal tests were made at the Biological Laboratories of Eli Lilly and Company, at Greenfield, Indiana. This is a preliminary report on this work, and no claim is made for extreme accuracy or for any conclusive results. The nature of diphtheria toxin and toxoid, and especially the difficulty of making extremely accurate quantitive determinations upon its toxicity and antigenic properties, without the use of large numbers of animals, always imposes a considerable source of error. Caution is therefore always necessary in the interpretation of results. It is hoped that the investigations may be continued with different gels, as to degree of adsorption, rate of release in the living animal, and effect upon the development of immunity in animals.