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ANTIBACTERIAL IMMUNITY TO *CORYNEBACTERIUM DIPHTHERIAE*

By

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B. A., Whitman College, 1964

Presented in partial fulfillment of the requirements

for the degree of

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1966

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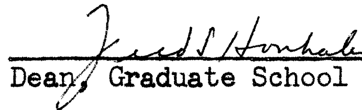

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Chapter I

INTRODUCTION

In 1888 Roux and Yersin discovered diphtheria toxin. They demonstrated that injection of sterile culture filtrates of the Klebs-Loeffler bacillus (Corynebacterium diphtheriae) into guinea pigs, rabbits, and pigeons produced symptoms analogous to diphtheria intoxication and late paralysis in man. This discovery laid the foundation for an era of search for means of eradicating diphtheria. Attempts were made to protect man by immunization with bacterial vaccines, but since these measures were largely unsuccessful investigations were carried out on different lines (Hartley, 1949). The outcome of research in this direction was the announcement by Ramon (1923) that formalin-treated toxin (anatoxine or toxoid) could be used successfully as an immunizing agent against diphtheria. Since then, toxoid has been employed in modified form for mass immunization against diphtheria in many countries.

Although vaccination with toxoid or treatment with antitoxin has generally proven effective (McLeod, 1943), there are two situations which raise doubt as to the efficacy of these materials. The first concerns the carrier problem, i.e. the inability of circulating antitoxin to eliminate the diphtheria bacilli from the nose and throat. This situation arises following infection with virulent corynebacteria. A susceptible individual responds by developing a subclinical or clinical disease. In either type of illness, the infectious process produces a Schick-negative state and the possibility of continuous infection of the

nasal-pharyngeal region with accompanying discharge of organisms (Wilson and Miles, 1964).

A significant proportion of diphtheria convalescents continue to harbor specific organisms in the nose and throat up to the tenth week after onset of illness (Russell, 1943) or even longer (Wilson and Miles, 1964). The importance of this is manifested in the data presented by Dolman and coworders (1948) in which they cite three separate outbreaks of diphtheria attributable to convalescent carriers. Similar evidence was reported by Morton (1941) and Turner (1942).

The second point raising doubts about the efficacy of antitoxic immunity is the occurrence of certain uncontrolled outbreaks of diphtheria in communities where the population was supposedly protected. The most outstanding of these was the Leed's epidemic during which many cases of malignant diphtheria, apparently refractory to antitoxic therapy, occurred (Anderson _et al_., 1931; McLeod, 1943). Morton (1941), reporting on a diphtheria epidemic in Halifax involving some 600 people, recorded 66 individuals hospitalized with diphtheria who had had some degree of immunity as measured by a previous negative Schick test or a history of immunization with toxoid. Twenty-two of these cases proved fatal, even when treated with antitoxin. In the United States an outbreak among 125 children occurred in a nutrition camp in Augusta County, Virginia, where 93 percent of the children had previous records of immunization with alum-precipitated toxoid (Turner, 1942). During a severe outbreak in Baltimore in 1944, Eller and Frobisher (1945) noted 18 cases of "bullneck" diphtheria among 141 school children, 64 percent of whom had county health records of immunization. Fourteen of these patients with "bullneck" diphtheria had

a history of prior inoculation. Other instances of diphtheria, sometimes of severe proportions, have been observed in persons who were thought to be reasonably protected on the basis of negative Schick tests or a history of immunization (Zinnemann and Zinnemann, 1939; Ipsen, 1946; Fanning, 1947; Dolman _et al_., 1948; Edsall, 1963). In the United States where herd immunity is one of the highest in the Western world, there were still 444 cases of diphtheria in 1962. In developing countries the figure is much higher (WHO, 1964), indicating the need for renewed interest in methods for controlling diphtheria.

Since most of the earlier outbreaks, particularly the one at Leeds, resulted from atypical strains of the _gravis_ type, Cooper and his coworkers (1936) suggested that some strains of the diphtheria bacillus might be more invasive than others. Further evidence was put forth by Zinnemann and Zinnemann (1939) that the _gravis_ strain possessed toxic or invasive propertiesother than the classical toxin, implying that these were associated with the existence of an endotoxin.

Investigations of the endotoxic activity of diphtheria bacilli have been few. Rist in 1903, after extracting toxigenic diphtheria bacilli with alcohol and ether, obtained a fraction that was lethal to guinea pigs and rabbits which had been protected with large doses of antitoxic serum. Cruveilhier (1909) found a heat-stable substance in the diphtheria organisms that produced death in guinea pigs when administered intracerebrally. The lethal effect of this material was not neutralized by addition of antitoxin prior to injection into animals. Similar findings were reported by Aviragnet _et al_. (1911) and Menard (1911).

Frobisher and Parsons (1940), in more recent investigations, showed

that intracerebral injections of atoxigenic strains of C. diphtheriae were lethal to mice. No difference was noted when the organisms were mixed with antitoxin before inoculation. Mice receiving virulent cultures plus antitoxin were affected in the same manner. No evidence for the presence of exotoxin could be observed in the avirulent strains, even though the method used was capable of detecting 0.00037 MLD of toxin (Frobisher and Parsons, 1943; Frobisher et al., 1947). This latter procedure involved testing sensitized animals (those inoculated with toxigenic corynebacteria) by subcutaneous injection of culture filtrates of the avirulent diphtheria bacillus in question. Since by this method as little as 0.00037 MLD toxin will stimulate an anemnestic response in sensitized animals, the authors were able to test for the presence of minute quantities of toxin in avirulent strains (Phair, 1942a).

A search of the literature for studies of production of antibacterial resistance in experimental animals employing endotoxins or some bacillary substance other than the classical exotoxin is only slightly rewarding. One of the first attempts was conducted by Cruveilhier (1911) who obtained antibacterial serum from goats injected intravenously first with autoclaved suspensions of virulent C. diphtheriae and then with living suspensions of virulent organisms. This serum proved more successful than ordinary antitoxic serum in preventing vaginal and vulval diphtheria in guinea pigs. Hewlett (1909) triturated diphtheria bacilli in the presence of liquid air and sand, filtered the ground material through a sterile Berkefeld filter, and used the extract obtained from the filtered solution to protect guinea pigs against infections with living virulent organisms (Hewlett, 1912). He emphasized the therapeutic value of this "endotoxin"

in treating chronic cases and carriers of diphtheria. In a subsequent study conducted at Croyden Hospital in London, Hewlett and Nankivell (1912) demonstrated definite improvement in patients receiving one or more injections of endotoxin. They also showed that a majority of cases so treated ceased to harbor diphtheria bacilli in their nose and throat. Serotherapy with antitoxin had previously failed to remove the bacilli even though the patient had passed convalescence. Durand, in a visit to Park and Williams in 1920, stated that "there was less paralysis and attendant mortality in cases treated with diphtheria serum produced by the inoculation of both toxin and bacilli; i.e., an antitoxic and antibacterial serum, than with serum produced by use of toxin alone."

Park and Zingher (1915), investigating the possible advantages of combining preparations of whole bacteria with toxin-antitoxin mixtures, were unable to show any increased proctection as measured by the Schick test. Rosenau and Bailey (1925) performed similar studies in large numbers of guinea pigs by giving repeated injections of live avirulent diphtheria bacilli for over a year. None of the animals developed a Schick negative state or increased immunity to a lethal dose of toxin. One would hardly expect, however, to detect antibacterial resistance in experimental subjects if they were challenged with diphtheria toxin.

Renewed interest concerning the value of antidiphtherial vaccines was stimulated in part by observations of Phair (1942b) of persons with low or deficient circulating antitoxin who were nevertheless resistant to diphtheria. Phair believed that antibacterial immunity was sufficient to suppress, or at least delay, infection until an adequate antitoxin level was reached.

Another stimulating factor was a series of reports concerning the association of avirulent strains of C. diphtheriae with the carrier state. Rosenau and Bailey (1925) recorded a greater number of isolates of avirulent diphtheria bacilli than of virulent organisms in a community in which there was a high incidence of naturally acquired resistance. Dudley, in 1932, showed in a study of carrier rates and latent infection that carriers of virulent diphtheria bacilli were 3 times greater than expected and were more frequently found in persons having negative Schick tests. Chason (1936), working in a community in which 92 percent of the population was Schick negative (all with negative clinical and immunization histories), observed a greater proportion of carriers with avirulent strains of C. diphtheria than with virulent strains. According to Gill (1940), there were 0.9 percent carriers with virulent bacilli and 3.0 percent carriers of avirulent organisms in a group of white school children that had a high percentage of immunity, as measured by the Schick reaction, regardless of whether the child had received an artificial immunizing agent or not. In another study Stebbins (1940) isolated from 3,223 individuals, of whom 33 percent were Schick negative, 42 cultures of C. diphtheriae, only one of which proved to be virulent when tested in rabbits.

These investigations prompted Frobisher and Parsons (1943) to study the antigenic properties of avirulent diphtheria bacilli and the possibility of producing antibacterial immunity against infections with virulent organisms. They injected relatively large doses of broth cultures containing live avirulent, nontoxigenic diphtheria bacilli intracutaneously, subcutaneously, and intravenously into 21 rabbits. When these rabbits

were inoculated, i.e., with a live virulent culture, 4 survived and 17 died after an average of 7 days. Control animals receiving the same challenge dose died in an average of 3.6 days. This degree of resistance, although slight, was attributed to a heightened local tissue reactivity which held the virulent organisms and toxin at the inoculation site. A subsequent investigation with a larger number of animals yielded essentially the same results (Frobisher and Updyke, 1947).

In 1950 Frobisher and Parsons immunized guinea pigs with "Rist-type antigens" made from virulent and avirulent strains of _gravis_ and _mitis_ types of _C_. _diphtheriae_ and from a virulent _minimus_ (_intermedius_) strain. The antigens were prepared by extracting the bacilli with equal volumes of alcohol and ether and then shaking them with "glow beads." Guinea pigs immunized with these antigens were resistant to challenge with toxigenic organisms. The virulent _mitis_ antigen afforded the greatest degree of protection. Tests performed on the Rist-type antigens with methods capable of detecting very small amounts of toxin (Phair, 1942a) were invariably negative. A followup experiment by Parsons and Frobisher in 1950 revealed that macerated antigens, whether extracted or not, were better immunizing antigens than extracted intact bacteria.

A further study on the role of bacterial vaccines in diphtheria prophylaxis was undertaken by Scheibel at the State Serum Institute in Copenhagen (1950). Utilizing guinea pigs, she compared the immunogenic properties of formalin-killed virulent corynebacteria and diphtheria toxoid. Approximately one week after the last dose of antigen had been administered, all animals were challenged by placing a small quantity of a virulent culture in broth onto a lightly scarified area of the ventral

wall of the abdomen. The guinea pigs protected with toxoid survived without symptoms of disease. Those receiving bacterial vaccines suffered the same mortality rate as the controls, but survived a greater length of time.

Another attempt to test the value of cellular antigens was reported by Bowen, Wyman, and McComb (1954). They injected heat- or chemically-killed suspensions of _C. diphtheriae_ or standard diphtheria toxoid subcutaneously into different groups of guinea pigs and then challenged them intradermally with both homologous and heterologous strains of virulent diphtheria bacilli. For the most part, their results with whole cell antigens confirmed the earlier studies of Frobisher. Guinea pigs were partially protected against challenge with the homologous strain of organism. However, animals immunized with toxoid were almost always completely resistant to infection with any of the virulent strains of _C. diphtheriae_.

Aside from the work of these 3 groups of investigators, little has been reported in the literature concerning the protective antigens of _C. diphtheriae_. Alcohol-soluble lipoid substances have been extracted from washed cultures of organisms (Freund, 1927; Hoyle, 1942). Lipoid-polysaccharide and protein fractions have been described by Wong and T'ung (1939,1940). Oeding (1950), Lautrop (1950), and Cummins (1954) demonstrated a superficial, thermolabile, specific protein and a deeper, thermostable group antigen which was probably a polysaccharide. According to Holdsworth (1952), the cell wall of _C. diphtheriae_ consists mainly of a protein-polysaccharide complex with small amounts of lipids. None of these investigators studied the prophylactic value of these antigens.

Instead the data was compiled in order to study the serological classification and antigenic structure of the diphtheria bacillus.

That protective antigens reside in the cell walls of some bacteria was demonstrated by the work of several investigators. A review by Munoz (1963) pointed out that a saline extract, prepared from acetone extracted and disrupted *Bordetella pertussis* cells, contained the functional protective antigen (PA). This antigen is known to be a cell wall component (Munoz *et al.*, 1959; Billaudelle *et al.*, 1960) and capable of protecting mice against challenge with virulent *B. pertussis*. Krause (1963) showed that hemolytic streptococci disrupted by Mickle's method yielded cell walls which possessed the immunogenic characteristics of the cell. Investigations with *Histoplasma capsulatum* (Salvin and Ribi, 1955), *Bacterium tularense* (Shepard *et al.*, 1955; Ormsbee *et al.*, 1955), and species of *Salmonella* (Ribi and coworkers, 1958; Carey and Baron, 1958; Ribi *et al.*, 1959) indicated that cell walls contained the immunogenic antigens of the cell and protected against experimental infection. Foster and Ribi (1962) demonstrated that brucellosis could be controlled in mice with cell-wall antigens. Recent studies by Ribi and coworkers (1966) have shown that pure cell walls obtained from BCG by disruption in a refrigerated pressure cell (Ribi *et al.*, 1959) produced better resistance in mice than the standard living BCG vaccine.

CHAPTER II

STATEMENT OF PROBLEM

This research was conducted in an attempt to demonstrate that cell walls of *Corynebacterium diphtheriae* could be used to induce active antibacterial resistance to diphtheria in guinea pigs and rabbits. Data are presented to show that resistance can be developed to infections with virulent bacilli when one or two MLD's of organisms are used and that this resistance is not due to soluble toxin in the antigen or antitoxin in the serum of immunized rabbits. Reasons for the failure of antibacterial immunity are discussed.

CHAPTER III

METHODS AND MATERIALS

(1) Organisms Employed

Four strains of *Corynebacterium diphtheriae* were used throughout this research. They were obtained from Dr. Joseph N. Schubert, Chief, General Bacteriological Unit, Communicable Disease Center, U. S. P. H. S., Atlanta, Georgia, and designated as follows:

C. diphtheriae gravis 6061--toxigenic
" *mitis* 6823--toxigenic
" *mitis* 6795--nontoxigenic
" *intermedius* 6796--toxigenic

Since the pathogenicity of the organism is primarily dependent upon its ability to produce toxin, the diphtheria bacillus is more properly referred to as toxigenic or nontoxigenic. However, virulent and avirulent are occasionally used interchangeably with toxigenic and nontoxigenic throughout this paper.

(2) Special Materials

Diphtheria toxin (deep culture), 240 L_f per ml, Lot No. 963454, and Formol Toxoid (deep culture concentrate), 1000 L_f per ml, Lot No. 025746-1, were obtained from Parke, Davis and Company through the courtesy of Dr. H. B. Devlin. In one phase of these studies, diphtheria antitoxin (equine), purchased from Davis Brothers, Missoula, Montana, was used.

(3) Buffers, Saline Solutions, and Diluents

A. Moloney Buffer

The buffer solution Moloney and Taylor (1932) was used as a diluent for diphtheria toxin and toxoid and was prepared as follows: a liter of 0.10 N Sorenson's borate buffer (Hawk *et al.*, 1962) was adjusted to pH 8.0 with 0.10 N HCl. Sodium chloride and gelatin were then added to a final concentration of 0.85% and 0.02% respectively. After autoclaving at 121 C for 15 minutes, the solution was diluted 1:4.

B. Tween-saline (TSS)

A 0.85% NaCl and 0.02% Tween 80 solution was warmed gently to dissolve the Tween 80 prior to autoclaving. The final pH was 6.2.

This solution without the Tween 80 was also employed and designated as sterile physiological saline or SS.

C. Casamino acid diluent

This solution was prepared by dissolving 30g Casamino Acids (Difco) in 1 liter of distilled water and autoclaving at 121 C for 15 minutes.

(4) Culture Media

Pai's medium (McGuigan and Frobisher, 1936) was prepared by beating 375 ml whole fresh eggs and 1.06 g NaCl in 125 ml distilled water. Glycerol was added in a final concentration of 8% (Frobisher and Parsons, 1950). The mixture was transferred to a beaker and thoroughly stirred, filtered through three layers of sterile gauze, tubed, slanted, and autoclaved according to the method used for heat-coagulable media (Difco, 1963).

Loeffler's Blood Serum slants were prepared according to the Difco Manual (1963).

Both of these media were used for routine maintenance of stock cultures.

The chemically defined medium developed by Drew and Mueller (1952) and abbreviated M&M was used as the primary medium for growth of all strains.

(5) Basic Solutions

A. Amino acids and ammonium:

1.	Glycine, anhydride (Nutritional Biochemicals Corp.)	-0.24 g
2.	L-glutamic, hydrochloride "	-8.0 g
3.	DL-methionine "	-0.27 g
4.	L-cystine "	-0.4 g
5.	DL-tryptophan "	-0.12 g
6.	DL-valine (Eastman Organic Chemicals)	-1.2 g
7.	DL-leucine "	-0.8 g
8.	L-proline "	-0.16 g
9.	$(NH_4)_2$ SO_4 (Reagent Grade)	-2.4 g
10.	Distilled H_2O to make	-1000 ml

L-cystine was dissolved separately in 3 ml of concentrated HCl.

B. Accessories and Metals:

1.	-alanine (Eastman Organic Chemicals)	-0.115 g
2.	Pimelic acid "	-7.5 mg
3.	Nicotinic acid (Nutritional Biochemicals Corp.)	-0.115 g
4.	$CuSO_4$ $5H_2O$ (1% solution) (Analytical Reagent)	-5.0 ml
5.	$ZnSO_4$ $7H_2O$ " "	-4.0 ml
6.	$MnCl_2$ $4H_2O$ " "	-1.5 ml
7.	$MgSO_4$ $7H_2O$ (Reagent Grade)	-22.5 g
8.	HCl (conc)	-3.0 ml
9.	Distilled H_2O to make	-100 ml

Nicotinic acid was dissolved separately in 3 ml of concentrated HCl.

C. Cystine:

1.	L-cystine (Nutritional Biochemicals Corp.)	-20 g
2.	HCl (conc)	-20 ml
3.	Distilled H_2O to make	-100 ml

D. Maltose-Calcium Chloride Solution:

1.	Maltose (Difco)	-50 g
2.	Calcium chloride $2H_2O$ (Reagent)	-0.5 g
3.	Distilled H_2O to make	-100 ml

Fifty grams of maltose were added to 60 ml of H_2O and solid

$CaCl_2$. This was stirred, warmed gently until dissolution occurred, and then diluted to 100 ml with distilled H_2O.

E. Iron:

1. $FeSO_4 \cdot 7H_2O$ (Reagent)	-1.0 g
2. HCl (conc)	-1.0 ml
3. Distilled H_2O to make	-100 ml

Since Pappenheimer and Johnson (1936) reported on the significance of adjusting the iron concentration of the medium, all solutions except Solution E were tested for their iron content by the method of Mueller and Miller (1941). A sample of each solution was boiled with HNO_3 and the reaction mixture was then tested with KSCN and sufficient alcohol against Fe standards. Analysis revealed that only Solutions A and D contained an excess of iron. Removal of iron from these 2 solutions was accomplished by precipitation on calcium phosphate (Mueller and Miller, 1943).

To prepare the medium 2.0 ml of Solution B and 1.0 ml of Solution C were added for every liter of Solution A. The medium was stirred thoroughly after each addition in order to avoid precipitation of any salts. A sufficient volume of Solution E was added to bring the total iron content to 500 gamma. This concentration allowed for satisfactory growth with little toxin production. Since there was a definite tendency towards clumping, Tween 80 (Nutritional Biochemicals Corp.) was added in a final concentration of 0.05% (Ward, 1948).

The media was then dispensed in 100 ml amounts in 1 liter Erlenmeyer flasks, stoppered with absorbent cotton, and sterilized for 10 minutes at 10 lbs in a Steroclave. When cooled, 4 ml of maltose solution, previously sterilized by Seitz filtration, was added. The pH of the final media was 7.6.

The Mueller-Tellurite medium (Difco, 1963) described by Mueller (1946) was used for plating out suspensions of diphtheria bacilli.

Agar Base

Casamino Acids, Technical	-20 g
Casein	-5 g
L-tryptophane	-0.05 g
KH_2PO_4	-0.3 g
$MgSO_4 \cdot 7H_2O$	-0.1 g
Agar	-20 g
Distilled water	-1000 ml

Serum Tellurite

Sodium lactate (60% Merc)	-40 ml
Ethyl alcohol	-10 ml
Calcium pantothenate	-0.2 mg
Sterile beef serum	-50 ml

The agar base was sterilized by autoclaving and cooled to 50 C. Just before plating, 5 ml of sterile serum tellurite was added for every 100 ml of base.

Beef-heart infusion agar (BHIA) was prepared according to the Difco Manual (1963), except that sterile rabbit serum was added to a final concentration of 2%.

(6) Maintenance of Cultures

All the strains of *Corynebacterium* were maintained on slants of Pai's medium or Loeffler's serum and stored at 4 C. Transfers were made monthly. In addition, triplicate sets of vials of each strain were lyophilized and kept at -20 C. Periodically the cultures were examined for contamination by staining with Loeffler's alkaline methylene blue (LAB).

(7) Counting Methods

Serial 10-fold dilutions of organisms in TSS were plated after the method of Fenner *et al*. (1951). Three 0.1 ml drops of each dilution were placed on duplicate plates of serum tellurite medium. The plates were

incubated at 34 C in an inverted position after the drops had dried. After 48 hours the colonies were enumerated with a Quebec Darkfield colony counter (Spencer).

(8) Preparation of Cell Suspensions

Cells for experiments were grown on Loeffler's serum slants, incubated for 18-24 hours at 34 C, and transferred in TSS to 2-liter Erlenmeyer flasks, each containing 100ml of M&M media. The flasks were then incubated for 3 days at 34 C. After checking the purity of the cultures with LAB, a 5 ml inocula was transferred to the desired number of fresh flasks of M&M media. These were incubated for 48 hours in the 34 C incubator prior to the harvest of the bacilli.

For experimental procedures, cells grown in M&M media for 48 hours were collected in 250 ml Nalgene antigen bottles and centrifuged at 22,000 G for 30 minutes in a Sorval Refrigerated Centrifuge (RC-2). The packed cell mass was resuspended in TSS and filtered through Whatman #4 filter paper in a Büchner funnel. The suspension of cells was diluted with TSS to an appropriate concentration on the basis of their turbidity in a Klett Summerson photoelectric colorimeter fitted with a #42 blue filter.

(9) Disintegration of Bacteria and Preparation of Cell Wall Material

The procedure described by Cummins (1954) was used to disrupt *Corynebacterium* in the Mickle Tissue Disintegrator. Cells harvested from M&M media were initially stained for purity, then poured into 250 ml antigen bottles and centrifuged for 30 minutes at 22,000 G in the RC-2. The supernatant was discarded but the cells were washed twice with sterile distilled water. Approximately 500 mg wet weight of washed bacilli were

deposited in 8 ml of cold distilled water. This suspension was added to a cold Mickle tube containing 4 g of #12 Cataphote glass beads. A single layer of celophane was placed around the rubber stopper to prevent contamination with small particles of rubber. Disruption was carried out in the cold for 2 hours, at which time only an occasional Gram positive cell was visible in a smear. A 10 C rise in temperature was noted.

When shaking was completed, the supernatant was pipetted from the glass beads and placed into a 40 ml plastic centrifuge tube. The beads were washed twice with 1-2 ml volumes of sterile distilled water. These washings were added to the supernatant, and the combined cell wall suspension was centrifuged for 2 hours at 27,000 G in the RC-2. Following this initial centrifugation, the supernatant, or protoplasm, was decanted from the packed cell walls and stored at 4 C for later use. The cell walls were washed 4 times in sterile distilled water with centrifugation at 27,000 G after each washing. The final suspension of cell walls was lyophilized and stored at -20 C.

Prior to use, the cell walls were weighed on an analytical balance and the desired amount suspended in 0.4% formalin. The suspension was refrigerated overnight at 4 C, then checked for sterility by plating of undiluted and 1:10 dilutions of the suspension on serum tellurite medium.

(10) In Vitro tests for Antigen-Antibody Determinations

Agglutination reactions: Twenty-four hour cultures of organisms grown on BHIA-2% serum slants were suspended with TSS and centrifuged at 22,000 G for 10 minutes. The packed cells were resuspended in a few mls of TSS and heated at 60 C for one hour. Since diphtheria bacilli had a tendency to clump spontaneously, the heat-killed cells were filtered

through Whatman #4 filter paper just prior to use. The filtrate was diluted to the Francis Standard of approximately 3 mg dry weight/ml.

The agglutination reactions were carried out on Perspex agglutination trays (Instruments Association Inc.). Six-tenth ml of a 1:5 dilution of the serum under test was placed in the first cup of the row and 0.3 ml sterile saline in each of the remaining cups. A serial 2-fold dilution was made by transferring 0.3 ml of the serum dilution with 0.5 ml pipettes. Saline and normal rabbit sera were used as controls. To each dilution an equal volume (0.3 ml) of cell suspension was added. The trays were covered with Saran Wrap to prevent evaporation and placed in a 37 C incubator for 2 hours followed by overnight refrigeration at 4 C. The highest dilution showing a positive reaction was considered as the titer of that serum.

Precipitin reactions: Protoplasm was titrated in the same manner as the antiserum in the agglutination test, except that the initial dilution was undiluted (10 mg/ml, dry weight). A capillary precipitation test was performed by layering the diluted antigens (protoplasm) on top of the antiserum. A positive reaction was recorded by a precipitation at the interface of antigen and antibody after incubating at 37 C for 1 hour and 4 C overnight.

Ramon flocculation experiments were also conducted. Serial 2-fold titrations of antiserum (0.5 ml) were mixed with a constant amount of protoplasm (5 mg/ml, dry weight) or diphtheria toxin (1, 2, 5, 10, or 20 L_f/ml). The reaction mixtures were placed in a 50 C water bath and observed at frequent intervals to determine the tube containing an optimal proportion of antigen and antibody.

(11) Schick Testing

Diphtheria toxin was diluted in Moloney buffer to 0.00004 L_f per 0.2 ml. This constituted 1 Schick test dose (STD). Animals to be tested received 0.2 ml of an unheated and a heated (70 C for 15 minutes) STD intradermally. Reactions were measured at the end of 48 hours with a metric ruler and a Schnelltester (System Kröplin).

(12) Test for Presence of 0.001 Unit of Antitoxin

This procedure was modified from the method developed by Fraser (Frobisher, 1950). Dilutions of test serum in 1 ml amounts were mixed with an equal volume of standardized toxin, incubated at 37 C for 1 hour, and then stored overnight at 4 C. Normal rabbit serum (NRS) and antitoxin diluted to contain 0.001 unit were substituted for the test serum in the controls. Two-tenth ml of each dilution was injected intracutaneously into the shaven side of normal guinea pigs. After 48 hours the reactions were measured and recorded in mm. For purposes of this research, a reaction greater than 9x9 mm in diameter produced with test serum and toxin mixture was rated as negative for 0.001 unit of antitoxin.

(13) Animals

Guinea pigs of the Hartley strain purchased from R. C. Rosencranz, Hamilton, Montana, were used. These animals weighed 275 to 350 g when immunized and were 4 to 5 weeks old. Albino rabbits of both sexes, purchased from L. VanGilder, Missoula, Montana or John's Rabbitry, Spokane, Washington, and weighing 1.8-2.7 kg, were employed.

CHAPTER IV

EXPERIMENTAL PROCEDURES AND RESULTS

Experiment 1. Titration of toxigenic *Corynebacterium* in the skin of rabbits. The procedure for titrating the virulent strains of *Corynebacterium* was modelled after the method of Larson *et al.* (1960). Toxigenic strains were harvested from 3-day cultures of M&M media and prepared as previously described. After filtering, the cell suspension was adjusted to various turbidity readings on the colorimeter and plated on serum tellurite medium. For preliminary titrations the adjusted suspensions were diluted in 10-fold increments, but in the final titration, 2-fold serial dilutions were used. Two-tenth ml of each dilution was injected intracutaneously into the shaven, lateral side of 3 rabbits. All dilutions were made in TSS. The course of the skin reactions was followed at intervals of 24, 48, 72, 96 hours. The measurements of the lesions were recorded in mm; the degree of edema being determined with a Schnelltester. Any reaction less than 5x5 mm in diameter and 0.3 mm in thickness was termed a flush (fl).

When selected 10-fold dilutions of the toxigenic strains were titrated in the skin of normal rabbits, it appeared that between 500 and 5,000 organisms were required to produce a lesion. Therefore, 2-fold serial dilutions of organisms were made and adjusted to contain approximately 500 viable units in the final dilution. Tables I, II, and III show the skin reactions observed during a period of 4 days following

Table I: Dermal reactivity in rabbits produced by intradermal inoculation of toxigenic Corynebacterium diphtheriae mitis strain 6823.

Rabbit No.	Time of reading (hrs.)	Cutaneous reaction in mm at indicated dose, x10^3 viable units					
		32	16	8	4	2	1
A	24	16x16* 1.3**	15x13 0.6	14x12 0.3	10x12 0.2	fl.	--
	48	16x17 1.4	14x13 0.8	11x10 0.7	8x6 0.5	fl.	--
	72	16x17 1.1	13x12 0.7	10x10 0.7	7x6 0.4	fl.	--
	96	15x14 1.2	11x11 0.7	9x8 0.6	5x4 0.2	fl.	--
B	24	18x17 1.5	14x13 0.7	13x12 0.4	11x10 0.5	fl.	--
	48	17x16 2.0	12x11 1.1	11x9 0.8	7x7 0.5	fl.	--
	72	16x14 1.6	12x11 0.8	10x9 0.7	8x7 0.4	fl.	--
	96	15x14 1.5	10x10 0.8	9x9 0.7	6x6 0.3	fl.	--
C	24	16x14 1.4	10x11 0.5	2x8 0.3	6x7 0.2	fl.	--
	48	16x15 1.9	12x13 1.1	8x7 0.6	5x6 0.4	fl.	--
	72	14x13 1.5	11x11 0.9	7x7 0.5	5x4 0.2	fl.	--
	96	13x13 1.5	9x9 0.8	5x6 0.4	fl.	fl.	--

* diameter of lesion
** thickness of lesion

Table II: Dermal reactivity in rabbits produced by intradermal inoculation of toxigenic <u>Corynebacterium</u> <u>diphtheriae</u> <u>gravis</u> strain 6061.

Rabbit No.	Time of reading (hrs.)	Cutaneous reaction in mm at indicated dose, x10^3 viable units						
		40	20	10	5	2.5	1.25	0.625
A	24	17x18* 1.9**	16x14 1.5	12x11 0.8	8x9 0.3	7x7 0.2	fl.	--
	48	20x18 3.1	16x15 2.1	13x12 1.3	9x11 0.8	6x7 0.5	fl.	--
	72	16x15 3.0	14x13 1.6	11x11 1.3	9x8 0.6	8x7 0.3	fl.	--
	96	14x14 2.2	13x14 1.2	9x10 0.9	8x8 0.5	6x7 0.3	fl.	--
B	24	21x19 2.0	18x14 1.5	16x13 1.1	9x10 0.3	7x8 0.2	fl.	--
	48	23x20 3.4	21x13 2.3	16x12 1.5	10x10 1.1	7x9 0.5	fl.	--
	72	18x16 2.7	15x12 1.4	13x11 1.3	8x8 0.8	8x7 0.6	fl.	--
	96	17x16 2.8	13x13 1.3	13x12 1.0	7x9 0.7	7x7 0.4	fl.	--
C	24	22x22 2.6	19x15 2.0	15x13 0.7	10x9 0.6	8x9 0.2	fl.	--
	48	28x25 3.3	20x17 2.4	15x15 1.3	11x10 0.7	8x9 0.5	fl.	--
	72	23x32 3.1	21x18 2.1	14x12 1.4	10x8 0.8	8x7 0.4	fl.	--
	96	20x18 2.6	20x15 1.7	12x12 1.3	7x8 0.7	7x5 0.4	fl.	--

* diameter of lesion
** thickness of lesion

Table III: Dermal reactivity in rabbits produced by intradermal inoculation of toxigenic <u>Corynebacterium</u> <u>diphtheriae</u> <u>intermedius</u> strain 6796.

Rabbit No.	Time of reading (hrs.)	Cutaneous reactions in mm at indicated dose, x10^3 viable units						
		40	20	10	5	2.5	1.25	0.625
A	24	24x18* 1.9**	16x15 1.2	14x11 0.7	10x10 0.5	6x9 0.2	fl.	--
	48	22x20 2.4	17x13 1.6	15x13 1.1	10x8 0.7	7x9 0.5	fl.	--
	72	21x19 2.0	16x13 1.2	14x12 1.1	8x7 0.6	6x7 0.4	fl.	--
	96	20x19 1.8	15x11 1.1	12x12 0.7	6x6 0.4	5x4 0.3	fl.	--
B	24	17x18 1.2	14x9 0.8	10x9 0.5	7x7 0.3	4x7 0.2	fl.	--
	48	20x16 1.7	16x10 1.3	14x12 1.2	8x10 0.7	5x5 0.4	fl.	--
	72	17x15 1.5	15x11 1.2	13x12 0.9	6x7 0.5	5x6 0.2	fl.	--
	96	16x16 1.4	15x10 1.0	11x11 0.8	6x6 0.5	fl.	fl.	--
C	24	12x13 1.0	12x12 0.9	9x9 0.7	6x6 0.2	8x5 0.2	fl.	--
	48	12x15 1.4	11x11 1.2	10x8 1.0	8x8 0.7	7x5 0.4	fl.	--
	72	11x12 1.3	10x8 0.7	9x9 0.6	7x6 0.4	fl.	fl.	--
	96	11x9 1.0	10x7 0.8	9x8 0.6	5x5 0.2	fl.	fl.	--

* diameter of lesion
** thickness of lesion

injection of a series of 2-fold dilutions of toxigenic _mitis_, _gravis_, and _intermedius_ organisms into the skin of 3 rabbits. At 24 hours the lesion was represented by a diffuse erythema and edema, which was not easily measured with the Schnelltester due to the softness of the lesion. Much more defined lesions were apparent at 48 hours, and the degree of edema was read without difficulty. The reactions had progressed no further when measured at 72 hours and were showing evidence of healing. The lesions were dark or purplish. At the site where the highest concentration of bacilli had been injected, central necrosis was evident. During the next few days there was a decrease in the size of the lesions, and by the seventh or eighth day only slight indications of dermal reaction remained.

These results indicate that the height of the inflammatory response occurred 48 hours after injection of toxigenic organisms. Moreover, the number of organisms required to produce a dermal reaction was about the same for the 3 strains.

Experiment 2. _Immunization with cell walls incorporated in complete Freund's adjuvant_. This preliminary experiment was designed to determine the ability of _C. diphtheriae_ cell walls in complete Freund's adjuvant to evoke an immunogenic response in rabbits. The adjuvant was prepared by grinding simultaneously Arlacel A (Atlas Chemical Company) and sufficient tubercle bacilli (acetone-dried BCG) to give 1 mg per dose. To this was added nine parts light mineral oil (Pharmaceutical Laboratories). An equal volume of formalin-sterilized cell wall material was then slowly added to the oil mixture to form a fine emulsion. Eight rabbits were

divided into 2 groups of 4 each; group I received the cell wall antigen and group II served as controls. Prior to injection of the antigen, a 20 ml sample of blood was collected from each animal. The rabbits in group I each received 4 subcutaneous injections of 1 ml cell wall-adjuvant mixture, 1 in each inguinal and axillary region. This amounted to 1 mg of tubercle bacilli and 0.5 mg of cell walls per injection site, or a total of 2 mg cell wall material per animal. Booster injections were given subcutaneously in the nuchal region at the end of the fifth and sixth weeks. Each dose contained 0.5 mg of cell walls diluted in 0.5 ml saline.

Starting on the 14th day after the initial immunizing dose, weekly samples of blood were collected from the marginal ear vein of each rabbit. In addition to determining the level of antitoxin, the serum agglutinin and precipitin titers were determined. During the ninth week, all rabbits were Schick tested and challenged by intracutaneous injection of a series of dilutions of toxigenic C. diphtheriae mitis strain 6823 and a suspension of heat-killed organisms (70 C for 15 minutes). All skin reactions were recorded as described in the initial experiment.

The ability of cell walls to stimulate the formation of agglutinating antibodies against whole cells of toxigenic mitis is shown in Fig. 1. Although the serum titers varied in the different animals, there was a progressive increase until the sixth week when the antibody titer began to decrease. The agglutinin titers against heterologous toxigenic strains were somewhat lower (Fig. 2).

None of the sera contained precipitins against homologous or heterologous protoplasm at any dilution. Ramon tests were negative. In

Figure 1: Serum agglutination titers to heat-killed Corynebacterium diphtheriae mitis strain 6823 in rabbits immunized with cell walls of the same strain.

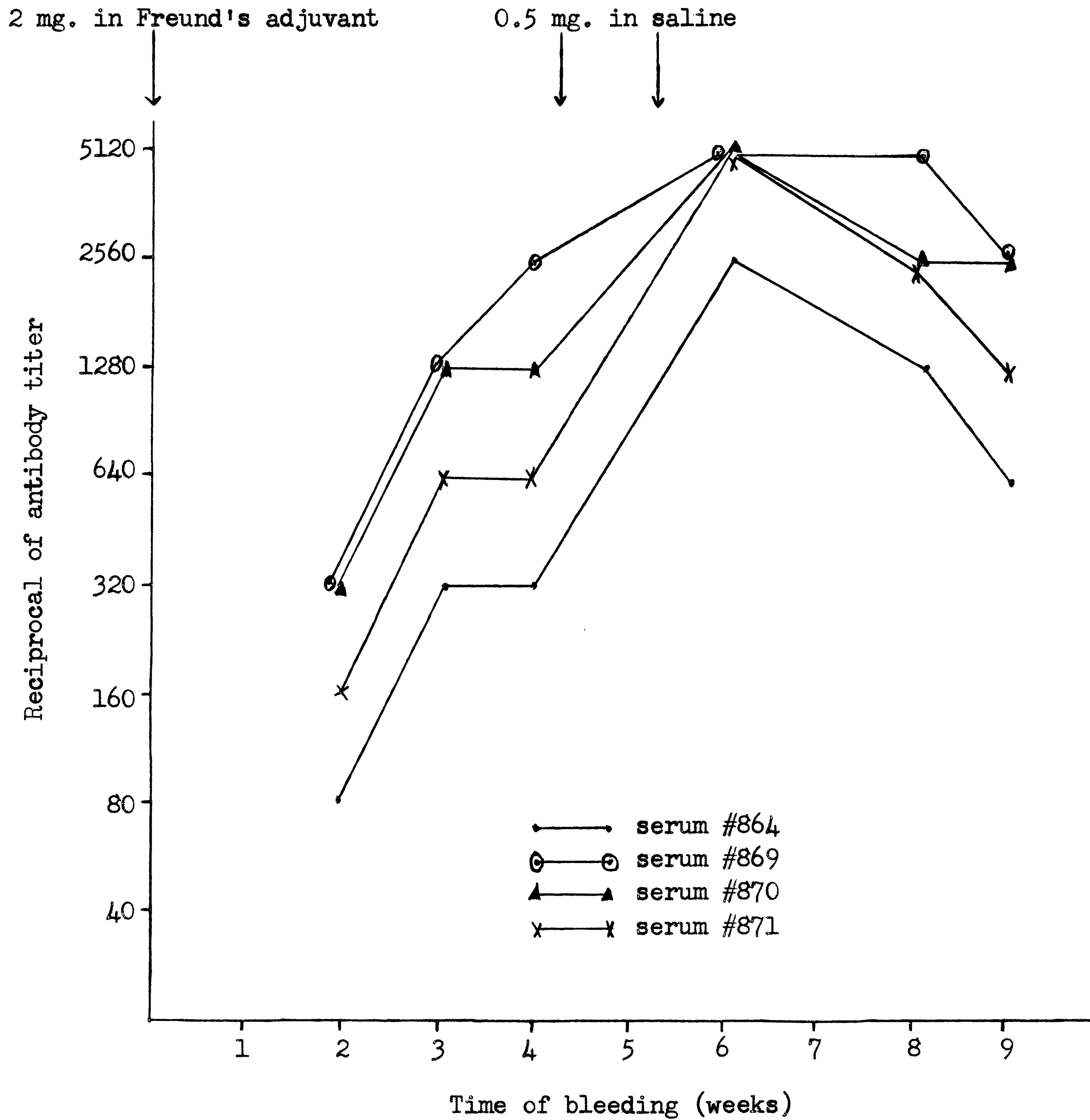
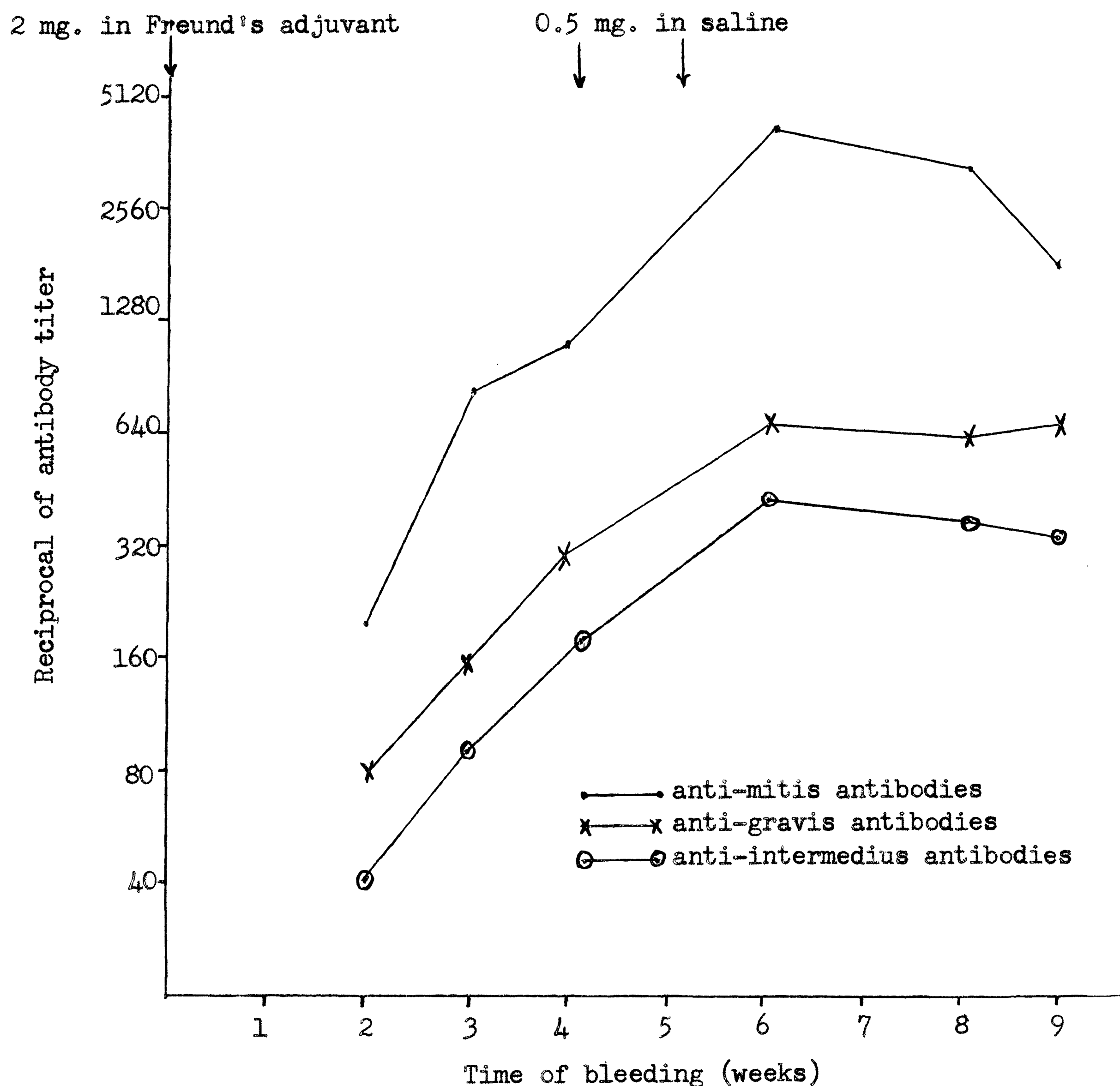


Figure 2: Mean serum agglutinin titers to heat-killed *mitis* 6823, *gravis* 6061, and *intermedius* 6796 strains of *Corynebacterium diphtheriae* in rabbits immunized subcutaneously with cell walls of *C. diphtheriae mitis*.



addition, no antitoxin was detected at the 0.001 level or by the Schick test (Table V). These data, therefore, suggest that cell walls contain very little, if any, toxin, and that the greater part of any resistance would have to be of an antibacterial nature.

The results of the intradermal challenge appear in Table IV. The development of dermal reactions in the immunized animals follows the same pattern as in the control rabbit (see also Table I) with the height of inflammatory response occurring at 48 hours. However, the intensity of the inflammatory responses in the immunized animals was considerably less than in the control rabbit. Whereas the control animal reacted to 3.0×10^3 viable units, none of the immunized animals showed a reaction at this dilution (a 4-fold difference).

The delayed reactions to heat-killed organisms are listed in Table V. All the experimental animals demonstrated some delayed response, presumably directed toward the cells since heated toxin elicited no response. The results of Tables IV and V suggest that the reactions to live organisms might in great part be delayed reactions against cell walls.

Experiment 3. Neutralizing ability of specific immune serum. In order to determine if delayed hypersensitivity had masked the immune response of the animals in Experiment 2, immune serums from 2 of the animals were tested for their ability to neutralize toxigenic *mitis* organisms prior to injection into animals. A suspension of toxigenic *C. diphtheriae mitis* was prepared in the usual manner. Sera employed were from rabbits in Experiment 2 and consisted of normal serum from rabbit 867; immune sera from rabbits 869 and 870 bled 6 weeks after

Table IV: Skin reactions in rabbits immunized with cell walls of *Corynebacterium diphtheriae mitis* strain 6823 in Freund's complete adjuvant and challenged 9 weeks later by intradermal inoculation of toxigenic *Corynebacterium diphtheriae mitis* strain 6823.

Rabbit No.	Immunizing dose	Time measured (days)	Cutaneous reactions in mm at indicated dose, x10^3 viable units			
			48	12	3	0.75
864	3 mg, subcu*	1	7x5x0.6	fl.	-	-
		2	9x8x0.9	4x5x0.5	-	-
		3	9x8x0.8	5x5x0.3	-	-
		4	8x8x0.4	4x5x0.2	-	-
869	"	1	10x9x0.8	5x5x0.2	-	-
		2	9x8x0.9	5x5x0.4	-	-
		3	8x8x0.9	5x5x0.4	-	-
		4	7x7x0.5	5x4x0.3	-	-
870	"	1	5x6x0.3	fl.	-	-
		2	7x7x0.7	fl.	-	-
		3	4x5x0.4	fl.	-	-
		4	fl.	fl.	-	-
871	"	1	5x5x0.4	fl.	-	-
		2	8x8x0.9	5x6x0.4	-	-
		3	8x8x0.9	5x6x0.4	-	-
		4	6x7x0.7	5x6x0.3	-	-
867	Control**	1	10x11x1.8	7x6x0.5	fl.	-
		2	17x28x3.5	13x15x1.7	fl.	-
		3	13x13x2.0	9x9x1.2	fl.	-
		4	12x13x1.6	9x9x0.8	fl.	-

* Immunizing dose, 2 mg cell wall material (dry weight) in Freund's complete adjuvant and 1 mg in 2 booster doses separated by one week.

** 3 other control rabbits died of an intercurrent infection, but compare with Table I.

Table V: Delayed reactions to heat-killed Corynebacterium diphtheriae mitis strain 6823 or diphtheria toxin in rabbits immunized with cell walls of Corynebacterium diphtheriae mitis strain 6823 in Freund's complete adjuvant.

Rabbit No.	Immunizing dose	48-hour cutaneous reactions in mm						
		Heat-killed org.x10^{4}					Schick test (toxin)	
		19.2	4.8	1.2	0.3	0.075	Unheated	Heated
864	3 mg, subcu	5.5 0.5	5.4 0.3	fl.	-	-	15x11 0.2	-
869	"	10x10 1.1	7x8 0.7	6x7 0.6	3x4 0.4	fl.	8x9 0.2	-
870	"	5x5 0.3	fl.	fl.	-	-	12x11 0.2	-
871	"	6x5 0.4	5x6 0.4	4x4 0.3	fl.	-	13x13 0.4	-
867	control*	-	-	-	-	-	12x13 0.3	-

* 3 other rabbits succumbed to an intercurrent infection

initial injection of antigen. Each immune serum had an agglutination titer of 5120 against toxigenic mitis. Serum HF-1 was obtained from a rabbit that had received 2 mg of cell walls in Freund's adjuvant subcutaneously and 3 weekly injections of 0.5 mg cell walls intravenously. This serum had an agglutination titer of 20,480 against toxigenic mitis. After equal volumes of diluted serums and serial 2-fold dilutions of mitis suspension were mixed and incubated at room temperature for 1 hour, 0.2 ml of each dilution was injected intradermally into the sides of 2 normal rabbits. The skin reactions observed at 48 hours are shown in Table VI. The data show that specific immune sera, 869 and 870, neutralized the toxigenic mitis strain approximately 4-fold in comparison with the normal serum. The HF-1 serum neutralized the toxigenic strain to a greater extent. By comparing the ability of sera from rabbits 869 and 870 to neutralize *in vitro* the effects of intradermal injection of toxigenic mitis organisms and the ability of these rabbits to neutralize the same organisms *in vivo* (Table IV), it appeared that the results of the *in vivo* test represented neutralization of toxigenic activity and were not influenced entirely by delayed hypersensitivity. It is difficult, however, in view of Frobisher's studies (1943), to minimize the value of a heightened tissue response against the mitis organisms.

Experiment 4. Comparative protection against toxigenic mitis, gravis, and intermedius strains. In this experiment 24 rabbits were divided into 4 groups of 6 each. The animals in groups I and II were immunized with cell wall antigens; the former received cell walls of the atoxigenic mitis strain, the latter, cell walls of the toxigenic mitis strain. These were

Table VI: Ability of specific immune serum to neutralize <u>Corynebacterium diphtheriae</u> <u>mitis</u> strain 6823.

Serum No.	Agglutination titer against *mitis* 6823	48-hour skin reactions in mm at indicated dose, x10^3						
		54	27	13.5	6.8	3.4	1.7	0.85
		Rabbit no. D						
NRS	negative	20x22 2.5	18x14 2.0	15x10 1.1	9x7 0.8	6x7 0.5	fl.	-
869	5120	16x14 1.8	11x12 0.9	6x7 0.6	fl.	-	-	-
870	5120	17x13 1.8	9x10 1.0	7x7 0.6	fl.	-	-	-
HF-1	20,480	12x14 1.0	7x8 0.7	fl.	-	-	-	-
		Rabbit no. E						
NRS	negative	20x18 2.1	15x14 1.8	13x11 1.2	9x10 0.7	5x5 0.4	fl.	-
869	5120	14x15 1.3	10x10 0.9	8x6 0.5	fl.	-	-	-
870	5120	13x12 1.5	10x11 0.8	5x6 0.4	fl.	-	-	-
HF-1	20,480	11x12 0.9	7x7 0.6	fl.	-	-	-	-

administered intravenously in the marginal ear vein according to the following schedule:

Week	Dose/0.5 ml and day injected	
	Monday	Friday
1	0.1 mg	0.2 mg
2	0.2	0.3
3	0.3	0.4

Total of 1.5 mg/rabbit

The rabbits in group III were given a human dose of Formol Toxoid (FT). This consisted of 2 subcutaneous injections of 25 L_f in 0.5 ml each, 1 during Monday of the first week and the other on Monday of the third week. The last group of animals served as the normal controls (group IV). All the experimental and control animals were bled the same day (7 days after receiving the last immunizing injection). The level of antitoxin and serum agglutinin and precipitin titers were determined.

One week after the last immunizing injection, each of the rabbits was Schick tested and challenged intradermally with titrated toxigenic _mitis_, _gravis_, and _intermedius_. Since they could not all be conveniently tested on the same day, the rabbits were challenged over a period of 3 days. The results appear in Tables VII through XIV, and each table contains results following challenge with a single strain of organism.* The rabbits immunized with toxoid demonstrated a greater degree of resistance against all 3 challenge strains than did animals of either of the groups immunized with cell walls. In the latter groups (with the exception

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* The last group of rabbits lacked suitable skin area for challenge with _C. diphtheriae intermedius_ strain 6796.

Table VII: Skin reactions in rabbits protected with cell walls of *Corynebacterium diphtheriae mitis* strain 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid, and challenged 28 days after the primary injection by intradermal inoculation of toxigenic *Corynebacterium diphtheriae mitis* strain 6823.

Rabbit No.	Immunizing antigen	48-hour cutaneous reaction in mm at indicated dose, x10^3 v.u./dose					
		44	22	11	5.5	2.75	1.38
1631	6823 c.w.*	6x6 0.3	fl.	fl.	-	-	-
1624	6795 c.w.*	12x12 0.9	8x6 0.5	7x5 0.3	fl.	-	-
1588	Toxoid**	fl.	-	-	-	-	-
1662	Control	14x15 1.6	12x10 1.0	6x6 0.4	4x5 0.3	fl.	-

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f Formol Toxoid given subcutaneously.

Table VIII: Skin reactions in rabbits protected with cell walls of Corynebacterium diphtheriae mitis strains 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid, and challenged 28 days after the primary injection by intradermal inoculation of toxigenic Corynebacterium diphtheriae gravis strain 6061.

Rabbit No.	Immunizing antigen	48-hour cutaneous reactions in mm at indicated dose, $x10^3$ v.u./dose						
		52	26	13	6.5	3.3	1.6	0.8
1631	6823 c.w.*	7x7 0.8	6x5 0.8	5x6 0.7	5x5 0.2	5x3 0.2	fl.	-
1624	6795 c.w.*	9x10 1.3	9x8 0.8	7x7 0.5	5x4 0.3	fl.	-	-
1588	Toxoid**	fl.	fl.	fl.	fl.	-	-	-
1662	Control	20x21 2.0	15x13 1.5	11x11 1.1	11x9 0.9	8x8 0.4	6x7 0.2	fl.

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f given subcutaneously.

Table IX: Skin reactions in rabbits protected with cell walls of *Corynebacterium diphtheriae mitis* strain 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid, and challenged 28 days after the primary injection by intradermal inoculation of toxigenic *Corynebacterium diphtheriae intermedius* strain 6796.

Rabbit No.	Immunizing antigen	48-hour cutaneous reaction in mm at indicated dose, x10^3 v.u.					
		50	25	12.5	6.25	3.13	1.6
1631	6823 c.w.*	fl.	fl.	-	-	-	-
1624	6795 c.w.*	5x5 0.3	5x6 0.3	5x4 0.2	fl.	-	-
1588	Toxoid**	-	-	-	-	-	-
1662	Control	9x9 0.6	8x9 0.4	8x7 0.4	5x6 0.2	fl.	-

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 Lf Formol Toxoid given subcutaneously.

Table X: Skin reactions in rabbits protected with cell walls of <u>Corynebacterium</u> <u>diphtheriae</u> <u>mitis</u> strains 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid and challenged 29 days after the primary injection by intradermal inoculation of toxigenic <u>Corynebacterium</u> <u>diphtheriae</u> <u>mitis</u> strain 6823.

Rabbit No.	Immunizing antigen	48-hour cutaneous reactions in mm at indicated dose, x10^3 v.u./dose						
		50	25	12.5	6.25	3.1	1.6	0.8
1633	6823 c.w.*	7x7 0.5	6x6 0.4	5x5 0.3	fl.	-	-	-
1634	"	12x11 0.8	8x8 0.6	5x5 0.3	fl.	-	-	-
1627	6795 c.w.*	13x15 1.2	11x12 1.0	9x10 0.4	fl.	-	-	-
1629	"	11x10 0.7	7x7 0.4	6x5 0.4	5x5 0.3	fl.	-	-
1589	Toxoid**	fl.	fl.	-	-	-	-	-
1590	"	fl.	fl.	fl.	fl.	-	-	-
1594	Control	13x13 1.3	9x9 0.8	7x7 0.6	6x7 0.5	5x5 0.4	fl.	-
1660	"	15x14 1.0	9x9 0.8	7x6 0.6	6x5 0.4	4x5 0.3	fl.	-

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f Formol Toxoid given subcutaneously.

Table XI: Skin reactions in rabbits protected with cell walls of <u>Corynebacterium diphtheriae mitis</u> strains 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid and challenged 29 days after the primary injection by intradermal inoculation of toxigenic <u>Corynebacterium diphtheriae gravis</u> strain 6061.

Rabbit No.	Immunizing antigen	48-hour cutaneous reactions in mm at indicated dose, x10^3 v.u./dose						
		48	24	12	6	3	1.5	0.75
1633	6823 c.w.*	20x20 1.5	12x12 1.0	10x10 0.9	9x10 0.7	5x5 0.3	fl.	-
1634	"	14x15 1.0	13x14 0.9	11x10 0.8	10x10 0.6	5x5 0.3	fl.	-
1627	6795 c.w.*	18x17 2.1	16x15 1.6	9x8 0.8	8x8 0.3	fl.	-	-
1629	"	20x21 4.0	15x13 3.0	14x12 1.3	11x11 1.1	11x13 1.0	8x8 0.4	fl.
1589	Toxoid**	9x9 0.3	5x6 0.2	fl.	-	-	-	-
1590	"	fl.	fl.	fl.	fl.	-	-	-
1594	Control	25x30 3.0	14x14 1.7	12x11 1.2	11x10 0.9	9x8 0.4	7x5 0.3	fl.
1660	"	22x22 2.3	15x12 1.3	11x11 1.0	10x10 0.8	6x7 0.5	6x6 0.3	fl.

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f Formol Toxoid given subcutaneously.

Table XII: Skin reaction in rabbits protected with cell walls of Corynebacterium diphtheriae mitis strain 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid and challenged 29 days after the primary injection by intradermal inoculation of toxigenic Corynebacterium diphtheriae intermedius strain 6796.

Rabbit No.	Immunizing antigen	48-hour cutaneous reactions in mm at indicated dose, x10^3 v.u./dose						
		38	19	9.5	4.75	2.38	1.19	0.599
1633	6823 c.w.*	11x11 1.1	8x7 0.6	6x6 0.4	fl.	-	-	-
1634	"	13x14 1.1	12x12 0.7	10x9 0.4	7x6 0.3	fl.	-	-
1627	6795 c.w.*	16x18 2.0	14x16 1.2	10x9 0.4	fl.	-	-	-
1629	"	12x11 1.2	8x8 1.0	6x6 0.6	5x6 0.4	fl.	-	-
1589	Toxoid**	fl.	fl.	-	-	-	-	-
1590	"	fl.	fl.	fl.	-	-	-	-
1594	Control	14x13 1.4	12x12 1.2	10x7 0.9	6x8 0.5	5x6 0.3	fl.	-
1660	"	17x21 1.7	13x15 1.0	10x9 0.8	7x8 0.4	5x4 0.3	fl.	-

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f Formol Toxoid given subcutaneously.

Table XIII: Skin reactions in rabbits protected with cell walls of _Corynebacterium diphtheriae mitis_ strains 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid, and challenged 30 days after the primary injection by intradermal inoculation of toxigenic _Corynebacterium diphtheriae mitis_ strain 6823.

Rabbit No.	Immunizing antigen	48-hour cutaneous reactions in mm at indicated dose, $x10^3$ v.u.						
		46	23	11.5	5.75	2.88	1.44	0.72
1630	6823 c.w.*	5x6 0.4	5x4 0.2	fl.	-	-	-	-
1632	"	9x10 0.8	8x8 0.7	5x6 0.3	fl.	-	-	-
1635	"	7x7 0.5	5x5 0.3	fl.	-	-	-	-
1625	6795 c.w.*	11x11 0.9	8x9 0.7	6x6 0.4	fl.	-	-	-
1626	"	8x8 0.6	6x7 0.3	fl.	-	-	-	-
1628	"	8x7 0.5	7x7 0.5	6x7 0.4	6x5 0.3	fl.	-	-
1591	Toxoid**	fl.	fl.	-	-	-	-	-
1592	"	fl.	fl.	fl.	fl.	-	-	-
1593	"	fl.	-	-	-	-	-	-
1595	Control	15x18 2.0	13x15 1.4	9x10 0.9	7x7 0.7	6x5 0.4	fl.	-
1596	"	16x17 1.7	12x14 1.1	10x12 0.9	8x9 0.6	6x7 0.5	fl.	-
1597	"	18x20 1.8	15x16 1.4	14x11 1.0	8x7 0.7	5x4 0.4	fl.	-

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f Formol Toxoid given subcutaneously.

Table XIV: Skin reactions in rabbits protected with cell walls of Corynebacterium diphtheriae mitis strains 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid and challenged 30 days after the primary injection by intradermal inoculation of toxigenic Corynebacterium diphtheriae gravis strain 6061.

Rabbit No.	Immunizing antigen	48-hour cutaneous reactions in mm at indicated dose, $x10^3$ viable units						
		54	27	13.5	6.8	3.4	1.7	0.85
1630	6823 c.w.*	14x15 1.4	11x13 1.1	8x10 0.8	7x6 0.4	4x5 0.3	fl.	-
1632	"	15x13 1.3	14x11 0.9	10x10 0.6	6x5 0.4	fl.	-	-
1635	"	10x11 0.7	8x9 0.5	6x7 0.4	5x5 0.2	fl.	-	-
1625	6795 c.w.*	21x17 2.4	16x16 1.8	13x10 1.1	8x9 0.7	6x7 0.5	fl.	-
1626	"	15x18 1.6	12x13 1.1	8x11 0.8	7x7 0.5	fl.	-	-
1628	"	14x16 1.7	12x13 1.4	9x11 1.2	6x8 0.7	4x5 0.3	fl.	-
1591	Toxoid**	fl.	fl.	fl.	-	-	-	-
1592	"	8x7 0.3	6x5 0.2	fl.	fl.	-	-	-
1593	"	fl.	fl.	-	-	-	-	-
1595	Controls	25x27 3.2	17x19 2.1	15x16 1.5	12x11 0.9	9x9 0.5	5x7 0.4	fl.
1596	"	29x21 2.7	21x17 1.8	12x13 1.2	11x9 0.9	8x7 0.5	6x7 0.4	fl.
1597	"	22x19 2.3	17x14 1.6	15x11 1.1	10x10 0.9	9x6 0.7	5x4 0.3	fl.

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f Formol Toxoid given subcutaneously.

of rabbit 1629) 2 to 16 times more organisms were required to produce a lesion than in the control animals. The immunized animals were able to control the infection more satisfactorily since the intensity of the reaction to the same dose was more pronounced in the controls. Of the two groups, those immunized with cell walls of the toxigenic mitis strain were protected more favorably than the animals receiving nontoxigenic mitis cell walls. The data also show that the mitis cell wall antigens immunize somewhat more effectively against the mitis challenge than against the gravis or intermedius challenge.

That the resistance afforded rabbits immunized with cell walls was not due to the presence of detectable antitoxin is illustrated by positive Schick tests (Table XV), negative flocculation reactions, and negative tests for determination of antitoxin at the 0.001 level. Moreover, none of the rabbits immunized with cell walls or with FT reacted to $50x10^4$ heat-killed toxigenic mitis (Table XV) indicating that delayed reactions probably had little to do with the test.

Examination of the serum agglutinin titers (Table XVI) shows that cell wall antigens administered in increasing doses over a short time can effectively induce the formation of antibodies against all 3 toxigenic strains of the diphtheria bacilli.

Experiment 5. Minimal lethal dose (MLD) in guinea pigs of toxigenic mitis. A suspension of viable C. diphtheriae mitis strain 6823 was prepared in the usual manner. This suspension was diluted over a wide range of turbidity readings on the colorimeter and plated on serum tellurite medium. For each dilution, a single 8 to 10-week-old guinea pig was

Table XV: Skin reactions to Schick Test dose of diphtheria toxin and heat-killed *Corynebacterium diphtheriae mitis* strain 6823 in rabbits immunized with cell walls of *Corynebacterium diphtheriae mitis* strains 6823 (toxigenic) or 6795 (nontoxigenic) or with Formol Toxoid.

Rabbit No.	Immunizing antigen	48-hour skin reactions in mm		
		Schick Test Dose, 0.00004 L_f		Heat-killed organisms 50×10^4
		unheated	heated	
1630	6823 c.w.*	9x10x0.2	-	-
1631	"	12x12x0.3	-	-
1632	"	11x10x0.4	-	-
1633	"	9x11x0.3	-	-
1634	"	9x10x0.4	-	-
1635	"	8x9x0.2	-	-
1624	6795 c.w.*	7x8x0.2	-	-
1625	"	12x11x0.3	-	-
1626	"	9x10x0.3	-	-
1627	"	8x11x0.2	-	-
1628	"	13x11x0.4	-	-
1629	"	11x10x0.4	-	-
1588	Toxoid**	-	-	-
1589	"	-	-	-
1590	"	-	-	-
1591	"	-	-	-
1592	"	-	-	-
1593	"	-	-	-
1594	Control	9x8x0.2	-	-
1595	"	11x10x0.4	-	-
1596	"	9x9x0.2	-	-
1597	"	11x9x0.3	-	-
1660	"	10x10x0.3	-	-
1662	"	10x12x0.2	-	-

* Immunizing dose was 1.5 mg cell wall material (dry weight) given intravenously.

** Human dose, 50 L_f Formol Toxoid given subcutaneously.

Table XVI: Serum agglutinin titers to heat-killed *mitis* 6823, *gravis* 6061, and *intermedius* 6796 strains of *Corynebacterium diphtheriae* in rabbits immunized intravenously with cell walls of *C. diphtheriae mitis* 6823 (toxigenic) and 6795 (nontoxigenic).

Serum No.	Immunizing antigen	Agglutinin titer to organisms diluted to Francis Std.			
		mitis 6795	*mitis* 6823	*gravis* 6061	*intermedius* 6796
1630	6823 c.w.	1280	5120	1280	640
1631	"	640	2560	640	640
1632	"	320	2560	640	320
1633	"	320	2560	640	160
1634	"	640	5120	1280	320
1635	"	640	2560	640	640
1624	6795 c.w.	1280	2560	640	160
1625	"	5120	2560	640	320
1626	"	5120	2560	1280	640
1627	"	1280	1280	640	320
1628	"	2560	1280	640	320
1629	"	640	160	80	80

injected intradermally with 0.2 ml of the suspension. Deaths were recorded daily. The data indicated that between $10x10^6$ and $30x10^6$ viable organisms were required to kill a guinea pig in 4 days (1 MLD). When suspensions of organisms within this range were injected into guinea pigs on 3 separate occasions the results showed that 1 MLD was approximately $21x10^6$ organisms (Table XVII). This concentration, the lethality of which was verified on 5 additional guinea pigs (Table XVII, group D), was employed as 1 guinea pig MLD.

Guinea pigs receiving doses of organisms above one MLD succumbed with characteristic symptoms of diphtheritic intoxication. Onset of illness was sudden followed by lethargy and death within minutes from what appeared to be acute respiratory or myocardial failure. The reactions at the site of inoculation of the toxigenic organisms were characteristically slight, with no marked edema. Guinea pigs injected with smaller concentrations of organisms were affected in a different manner. They became cachectic on the second or third day and remained in a quiescent condition toward the rear of the cage. On the third or fourth day paralysis of the hindquarters developed. If death supervened, intense dyspnea and convulsions were apparent, possibly being provoked by paralysis of the diaphragm. At this stage death was imminent. The local lesions in these animals were quite large and much more pronounced than in guinea pigs which expired rapidly.

Experiment 6. Resistance of guinea pigs immunized with cell walls of toxigenic and nontoxigenic mitis. This experiment involved determining the efficacy of using cell walls as immunizing antigens in guinea

Table XVII: Determination of the minimal lethal dose in guinea pigs to intradermal challenge with toxigenic <u>Corynebacterium diphtheriae mitis</u> strain 6823.

Group	Challenge dose* (0.2 ml)	No. of guinea pigs	Day of death					Viable count, x10^6
A	KS 150	1	2					27.2
	KS 125	1	3					22.6
	KS 100	1	9					18.1
	KS 75	1	-					13.6
	KS 50	1	-					9.1
B	KS 150	1	3					26.6
	KS 125	1	4					22.2
	KS 100	1	10					17.7
	KS 75	1	-					13.3
	KS 50	1	-					8.7
C	KS 150	1	2					24.4
	KS 125	1	4					20.3
	KS 100	1	7					16.3
	KS 75	1	-					12.2
	KS 50	1	-					8.1
			No. dead on indicated day					
			1	2	3	4	5	
D	KS 125	5	-	-	2	3	-	20.0

* Adjusted with Klett-Summerson photoelectric colorimeter (KS)

pigs. For this purpose the cell walls of the toxigenic and nontoxigenic strains of mitis were incorporated in Freund's complete adjuvant. The adjuvant was prepared as described in Experiment 2. Guinea pigs, 4 weeks old, were divided into 3 groups of 6 each. Group I received the cell walls of the toxigenic strain and group II received cell wall antigen of the nontoxigenic strain. The third group served as normal controls. Each animal in groups I and II received 4 subcutaneous injections of the respective antigen, one in each inguinal and axillary region. This amounted to one mg tubercle bacilli per injection site and a total of 2 mg cell walls per animal. Booster injections were given subcutaneously in the nuchal region at the end of the third and fourth week. Each dose contained 0.5 mg of cell walls (dry weight) suspended in 0.5 ml saline.

One week after the final immunizing dose, all animals were Schick tested and challenged intradermally with 1.5 MLD's of toxigenic C. diphtheriae mitis strain 6823. The results are shown in Table XVIII and seem to support the data obtained from the previous studies with rabbits. Cell walls derived from either toxigenic or nontoxigenic mitis can serve as immunizing antigens against challenge with toxigenic mitis. A comparison of the effectiveness of the cell wall antigens is given in Fig. 3. The results taken when 50% of the animals in each group were dead show that guinea pigs immunized with cell walls of the nontoxigenic strain were approximately 2 times more resistant than the controls. The animals protected with cell walls of the toxigenic strain were about 4 times more resistant. Moreover, one-half of the guinea pigs in the latter group survived the total effects of the challenge dose and appeared normal at

Table XVIII: Protection of guinea pigs against intradermal challenge with toxigenic *Corynebacterium diphtheriae mitis* 6823 by subcutaneous immunization with cell walls of *Corynebacterium diphtheriae mitis* strains 6823 (toxigenic) or 6795 (nontoxigenic).

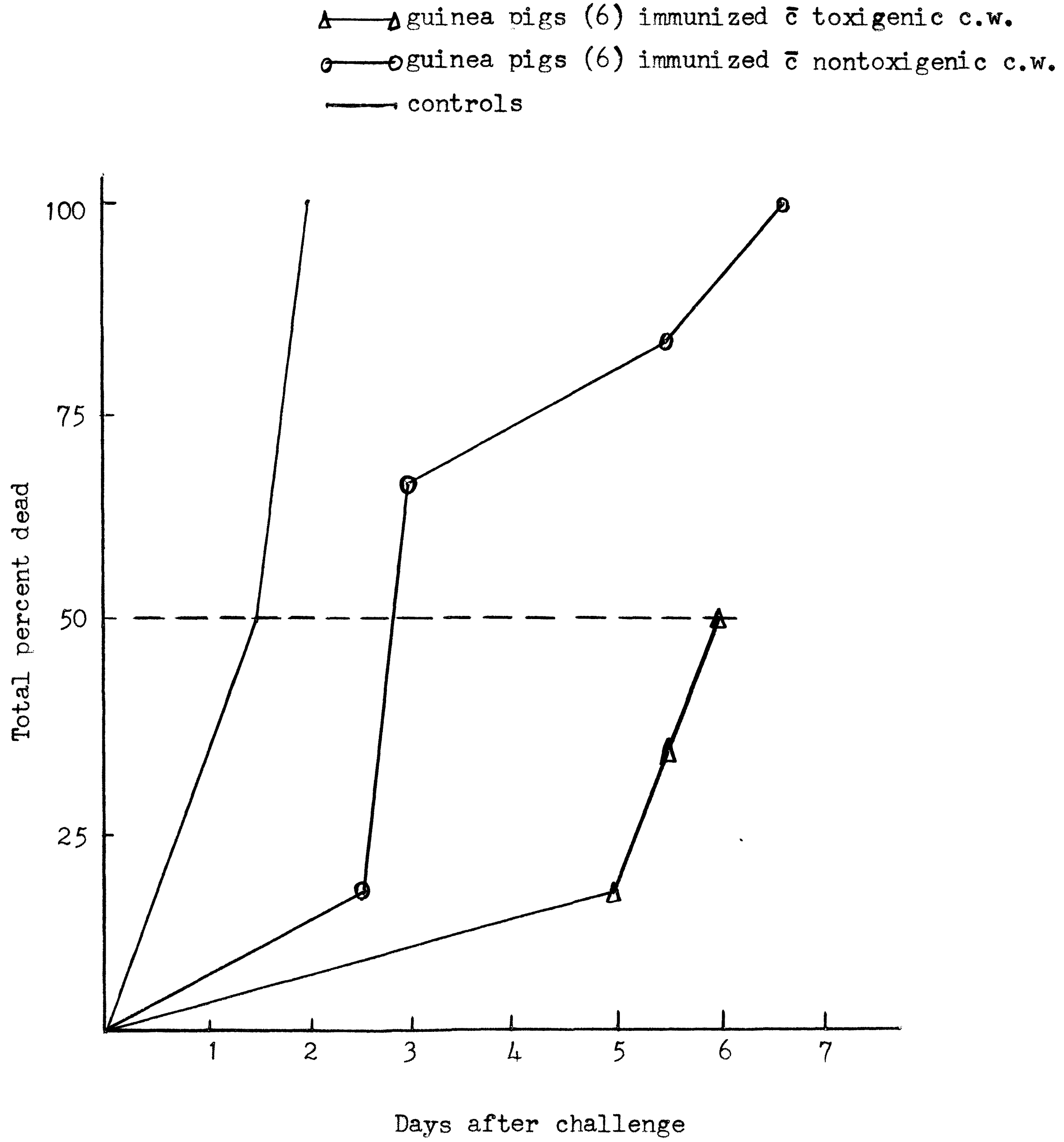
Group	Immunizing antigen	No. of guinea pigs	Challenge dose (MLD)	No. dying on indicated day									Total survivors/ Total dead
				1	2	3	4	5	6	7	8	14	
I	6823 c.w.*	6	1.5**	-	-	-	-	0/1§	1/1	-	-	-	3/3
II	6795 c.w.	6	1.5	-	-	1/3	-	-	1/0	1/0			0/6
III	Control	6	1.5	-	3/3								0/6

* Immunizing dose was 3 mg cell wall material (dry weight) given subcutaneously.

** Challenge dose (0.2cc) consisted of 3.0×10^7 viable units.

§ No. of guinea pigs dying in first 12 hrs./no of guinea pigs dying in second 12 hrs.

Figure 3: Immunizing effect of toxigenic and nontoxigenic *mitis* cell walls against challenge with 1.5 MLD's.



14 days. No sign of postdiphtheritic paralysis was observed up to the thirtieth day when the animals were discarded.

The inoculation site in the control animals was characterized by a nonedematous, diffuse reaction. In sharp contrast, both groups of immunized guinea pigs developed large, well-demarcated lesions indicating an attempt by the animal's defense mechanism to counteract the infective process.

All the guinea pigs were Schick test positive on the day after challenge, indicating that resistance was not due to the presence of detectable antitoxin.

Experiment 7. Protective effect of toxigenic mitis cell wall antigen. The degree of resistance to various lethal doses of toxigenic organisms was examined. Twenty-four guinea pigs were divided equally into 4 groups. The first 3 groups were immunized with cell walls of the toxigenic *mitis* strain in Freund's complete adjuvant as described in the previous experiment. Each animal received 2 mg cell walls in adjuvant as the primary stimulus and 1 mg as the secondary stimulus. Group IV received a human dose of FT (50 L_f).

One week after receiving the last immunizing dose of antigen, the animals were Schick tested and challenged with the toxigenic strain of *mitis* according to the protocol in Table XIX. The results indicate that cell wall immunization completely protected against 1 MLD (group I). Although the immunized guinea pigs in this group developed large necrotic lesions at the site of the inoculation of the toxigenic culture, there was no evidence of intoxication or cachexia, and all animals remained

Table XIX: Protection of guinea pigs against intradermal challenge with 1, 2, and 4 MLD's of toxigenic *Corynebacterium diphtheriae mitis* strain 6823 by subcutaneous immunization with cell walls of *C. diphtheriae mitis* strain 6823 or with Formol Toxoid.

Group	Immunizing antigen	No. of guinea pigs	Challenge dose (MLD)	No. dying on indicated day									Survivors/ Total dead
				1	2	3	4	5	6	7	8	14	
I	6823 c.w.*	6	1	-	-	-	-	-	-	-	-	-	6/6
II	"	6	2	-	-	-	0/3§	1/1	-	1/0			0/6
III	"	6	4	-	0/5	-	1/0						0/6
IV	Toxoid**	6	2	-	-	-	-	-	-	-	-	-	6/6
V	Control	6	1	-	-	0/2	2/2						0/6
VI	"	6	2	-	0/5	1/0							0/6
VII	"	5	4	-	5/0								0/6

* Immunizing dose was 3 mg cell wall material (dry weight) given subcutaneously.

** Human dose, 50 L_f given subcutaneously.

§ No. of guinea pigs dying in first 12 hrs./no. dying in second 12 hrs.

healthy. The challenge dose produced only local reactions comparable to those in normal guinea pigs infected with a sublethal dose of C. diphtheriae. The protection against 2 MLD's of organisms (group II) was considerably less since none of the experimental animals survived the challenge. A comparison of the 50% survival times (Fig. 4) shows that the immunized guinea pigs survived 4 days while the controls (group VI) only lived 1.25 days, indicating that the experimental animals were about 3 times more resistant. Moreover, the experimental animals did not succumb from rapid intoxication as did the controls, but from slow paralysis indicating some ability on behalf of the immunized guinea pigs to control the infection. It is interesting to note that guinea pigs immunized with a human dose of FT (group IV) survived 2 MLD's of the toxigenic mitis strain, suffering no ill effects other than a moderate reaction at the site of inoculation.

Immunized guinea pigs were not protected against challenge with 4 lethal doses of organisms (group III). None of the immunized animals survived beyond the third day, although all of them lived longer than the controls challenged with the same dose. At most, the animals protected with cell wall antigens were 1.6 times more resistant than the controls (Fig. 4).

With the exception of the guinea pigs vaccinated with FT, all the experimental and control animals were Schick positive on the day of challenge, indicating that resistance was not due to the presence of detectable antitoxin.

Experiment 8. Immunogenicity of cell walls suspended in adjuvant

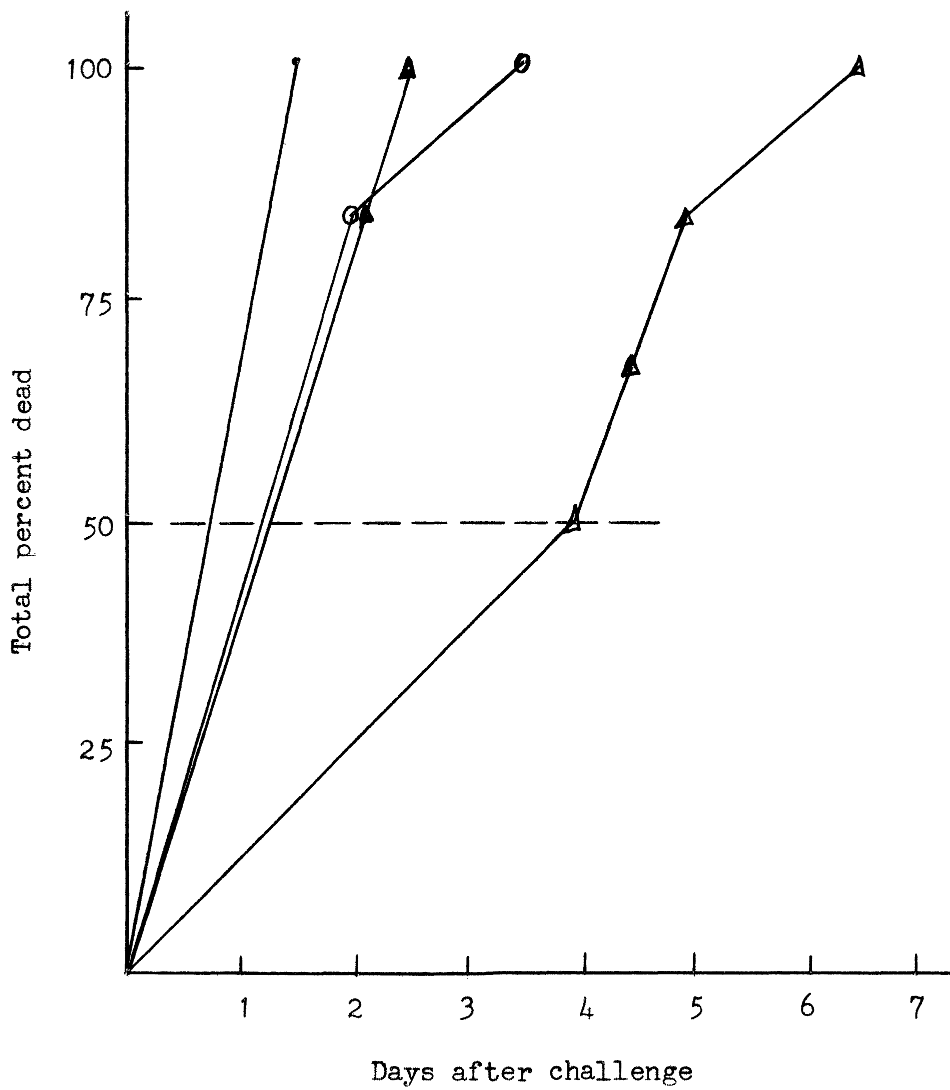
Figure 4: Immunizing effect of toxigenic *mitis* cell walls against challenge with 2 and 4 MLD's.

guinea pigs (5) control, challenged c̄ 4 MLD's

guinea pigs (6) immunized, challenged c̄ 4 MLD's

guinea pigs (6) control, challenged c̄ 2 MLD's

guinea pigs (6) immunized, challenged c̄ 2 MLD's



or saline. Normal guinea pigs, 3- to 4-weeks-old, were divided into 4 groups of 6 each and immunized with cell walls prepared from *C. diphtheriae mitis* strain 6823. The animals in group I were each immunized with 3 mg of cell wall material incorporated in Freund's adjuvant (Experiment 2). Group II received a similar preparation except the adjuvant was void of tubercle bacilli (Freund's incomplete adjuvant). Group III was administered a single dose of 3 mg of cell walls suspended in sterile physiological saline (SS). The animals in group IV were immunized with 2 doses of cell walls (1.5 mg each) in SS spaced 3 weeks apart. All the antigen mixtures were injected subcutaneously and distributed equally in each inguinal and axillary lymph node region. In addition to the guinea pigs immunized with cell walls, 2 other groups were used. The 6 guinea pigs comprising group V were given a human dose of FT (50 L_f). The sixth group served as normal controls.

Data presented in Table XX show that the immunogenicity of the cell wall antigen was enhanced by the presence of the oil in Freund's adjuvant. However, comparable protection was obtained when oil was not used and the antigen was inoculated in two separate doses (group IV) indicating that resistance to 1 MLD of *C. diphtheriae* can be obtained without the use of an adjuvant. The degree of non-specific resistance due to the tubercle bacilli cannot be determined from this experiment, although other experiments may reveal the participation of non-specific factors.

Experiment 9. Survival of *C. diphtheriae* in spleens of immunized guinea pigs. During one phase of the guinea pig protection experiments, spleens of immunized animals that had been challenged intravenously with

Table XX: Protection of guinea pigs immunized subcutaneously with cell walls of <u>Corynebacterium diphtheriae mitis</u> strain 6823 and challenged intradermally 28 days after primary injection with 1 MLD of toxigenic <u>C. diphtheriae mitis</u> strain 6823.

Group	No. of guinea pigs	Immunizing antigen	Immunizing dose (subcut.)	No. dying on indicated day												Survivors/ total tested
				1	2	3	4	5	6	7	8	9	10	11	20	
I	6	cell walls in Freund's complete adjuvant	3 mg.	-	-	-	-	-	-	-	-	-	-	-	-	6/6
II	6	cell walls in Freund's incomplete adjuvant	3 mg.	-	-	-	-	-	-	-	-	-	-	-	-	6/6
III	6	cell walls in saline	3 mg.	-	-	-	-	-	-	1	-	1	1	-	-	3/6
IV	6	cell walls in saline	1.5 mg. 1.5 mg.*	-	-	-	-	-	-	-	-	-	-	1	-	5/6
V	6	Toxoid	50 L_f	-	-	-	-	-	-	-	-	-	-	-	-	6/6
VI	6	Control	-	-	-	2	3	1								0/6

* Separated by 3 weeks.

toxigenic organisms were cultured (Larson and Wicht, 1962). Subsequently 5 guinea pigs were immunized in the conventional manner with cell walls of the toxigenic mitis strain of C. diphtheriae emulsified in Freund's complete adjuvant. Ten days after immunization, the experimental and 4 control animals were inoculated intravenously with $80x10^5$ toxigenic C. diphtheriae mitis. After 24 hours the animals were sacrificed and autopsied. Each spleen was removed aseptically, weighed, and diluted 1:10 in casamino acid diluent. The organ was then ground for 1 minute with a Teflon grinding apparatus and serial 10-fold dilutions were made in the same diluent. Selected dilutions in 0.1 ml quantities were plated in duplicate and allowed to incubate for 2 to 3 days at 34 C, after which time the colonies were counted. A comparison of the number of viable organisms recovered from immunized and normal animals is shown in Table XXI. Guinea pigs protected by cell wall immunization demonstrated marked ability to control the multiplication of toxigenic mitis in the spleen. The data show that an average of 1573 viable organisms were recovered from spleens of the control animals, whereas not a single spleen culture from the immunized group was positive, indicating greater than a 1000-fold difference. This result implies that the protection afforded guinea pigs immunized with cell walls might be greater than that indicated by the data obtained from the previous experiments.

Experiment 10. Lethality of protoplasmic fraction. During the course of the guinea pig experiments, normal animals were injected with protoplasm obtained from 1, 4, and 10 MLD's of toxigenic organisms in order to determine if the lethal effect of $20x10^6$ organisms (1 MLD) was

Table XXI: Survival of toxigenic *Corynebacterium diphtheriae mitis* strain 6823 in the spleens of guinea pigs immunized with cell walls of *Corynebacterium diphtheriae mitis* strain 6823.

Animal No.	Immunizing antigen	Viable cell count** in spleens
1	6823 c.w.*	0
2	"	0
3	"	0
4	"	0
5	"	0
6	Control	1230
7	"	530
8	"	2600 (average 1573)
9	"	1930

* Immunizing dose was 3 mg cell wall material given subcutaneously.

** Results obtained 24 hours after intravenous challenge with 80×10^5 v.u./dose.

due to the toxin contained in the protoplasm of that number of organisms or was due to proliferation of organisms and subsequent release of toxin. Accordingly C. diphtheriae mitis strain 6823 was prepared in the usual manner for procuring challenge doses. The equivalent of 100 MLD's (2000×10^6 organisms) was suspended in Moloney buffer and disrupted in the Mickle tissue disintegrator. The protoplasm (supernatant) was separated from the cell walls by high-speed differential centrifugation. Preparations containing any visible cell fragments were discarded. Since the protoplasm was obtained from 2000×10^6 organisms, and 20×10^6 organisms constitutes 1 MLD, it was possible to estimate the amount of protoplasm contained in 20×10^6 bacilli by diluting the supernatant 100-fold. Similarly the protoplasm from 80×10^6 organisms (4 MLD's) was obtained by diluting the original supernatant 25-fold. All dilutions were made in Moloney's buffer. Each dilution was injected intradermally into 4 normal guinea pigs and the deaths were recorded daily.

Data presented in Table XXII show that only those guinea pigs receiving protoplasm obtained from 4 and 10 MLD's of toxigenic mitis organisms suffered a mortality rate not too different from the animals injected with live organisms. Guinea pigs inoculated with protoplasm from 1 MLD of organisms lived, although all manifested symptoms of diphtheritic intoxication. When heat-sterilized cell walls were added to 2 of the protoplasmic fractions, the mortality rate of the guinea pigs receiving the equivalent of 4 MLD's of protoplasm and cell walls was nearly the same as the group challenged with 1 MLD of virulent organisms. Those receiving protoplasm from 1 MLD of organisms and cell walls lived, albeit with large necrotic dermal lesions at the injection site. A

Table XXII: Susceptibility of guinea pigs to the protoplasmic fraction obtained from toxigenic *Corynebacterium diphtheriae mitis* strain 6823.

Group	No. of guinea pigs	Protoplasm from (0.2 ml)	No. dying on indicated day									Survivors/ total tested
			1	2	3	4	5	6	7	8	9	
I	4	10 MLD's	-	-	-	-	1	2	1			0/4
II	4	4 MLD's	-	-	-	-	-	-	1	1	2	0/4
III	4	1 MLD's	-	-	-	-	-	-	-	-	-	4/4
IV*	3	4 MLD's + cell walls	-	-	-	1	1	-	1			0/3
V*	3	1 MLD + cell walls	-	-	-	-	-	-	-	-	-	3/3
VI	6	1 MLD live organisms	-	-	2	3	1					0/6

* Same as groups II and III except the suspension also contained heat sterilized cell walls from 4 and 1 MLD, respectively, of *C. diphtheriae mitis* strain 6823.

similar study involving heat-killed (58 C for 10 min) whole virulent C. diphtheriae mitis strain 6823 in equivalent challenge doses of 1, 2, and 4 MLD's proved even less successful. Although all the test animals developed dermal necrotic lesions, they were not as large as the reactions on the guinea pigs in Table XXII and none of the doses were fatal. However, both of these methods for examining the toxin production by a certain number of cells only consider the toxin content of the cells at a given time. In the actual challenge with living organisms, toxin production is a dynamic process, rather than a static one, and is much more marked during induced lysis (Barksdale, Garmise, and Rivera, 1961).

Experiment 11. Production of "natural" diphtheria in guinea pigs. Since the data in Table XXII indicated that death by intradermal challenge did not involve an infectious process like that occurring in man, other methods of challenge were tried. Repeated attempts (Roux and Yersin, 1888; Loeffler, 1884) to produce a diphtheritic membrane in guinea pigs by first excoriating the superficial epithelium of the eye or trachea and then daubing these areas with a pure culture of toxigenic organisms were of little success. Occasionally, guinea pigs receiving a massive quantity of organisms ($>10^8$ toxigenic organisms) suffered clinical syndromes of diphtheritic intoxication terminating in death. A similar procedure involving a few rabbits yielded the same results. Another technique used by Scheibel (1950) to establish a cutaneous focus of infection on the ventral wall of the animals also failed to demonstrate a pattern of pathogenesis similar to diphtheria in man. Partial success was achieved, however, when the method of Loeffler (1884) and Cruveilhier

(1911) was used. By applying a virulent broth culture onto a mechanically ruptured hymen, most of the guinea pigs could be shown to develop vulval diphtheria with typical membrane formation and a slight amount of inflammation. Although, none of the guinea pigs succumbed as a result of the disease, which usually cleared up in 4 to 6 days, immunization studies could be performed using the development of vulval diphtheria as a test for resistance. However, this would necessitate the use of large numbers or experimental animals, a situation impractical in this study.

CHAPTER V

DISCUSSION

Previous studies on development of resistance to diphtheria have centered around the use of classical diphtheria toxoid rather than bacterial vaccines as immunizing agents. In view of the success of Larson and his associates (Larson *et al.*, 1963; Ribi *et al.*, 1966) in protecting mice by immunization with cell wall antigens against infection with *Mycobacterium tuberculosis*, it was thought possible to actively immunize guinea pigs and rabbits against experimental diphtheria with cell walls of *Corynebacterium diphtheriae*.

The concept was that by inducing only an antibacterial response, the immune animals would upon subsequent contact with virulent organisms react by eliminating the organisms, preventing an infectious process from becoming established, and thus make it impossible for sufficient toxin to be produced to lead to illness. On a broader scope, children protected by antibacterial resistance and later infected with a virulent strain of the diphtheria bacillus would either destroy the organisms immediately or would enjoy exceedingly low morbidity rates in the process of becoming increasingly resistant. The latter implies a minor clinical infection developing in the nasal-pharyngeal region due to a slow anemnestic response on the part of the host or because of a delayed migration of antibody-producing cells to the infection focus. As a result, a small number of organisms would multiply and secrete toxin into the surrounding

epithelial tissue until such time that sufficient irritation caused the stimulation of a minor inflammatory reaction. The subsequent release of humoral and cellular resistance factors from the blood vascular system would provide the necessary defense to destroy further proliferation of the organisms. In the instance of a slowly developing inflammatory response, the continued release of toxin into the neighboring tissue by a small number of organisms could initiate an antitoxic response leading to a Schick negative condition in the host.

To test the general hypothesis of antibacterial resistance to diphtheria, rabbits and guinea pigs were immunized with cell walls prepared from the *mitis* strain of *C. diphtheriae*. This strain was chosen since it is reportedly the least virulent of the *C. diphtheriae* strains (Cooper, Happold, and McLeod, 1936; McLeod, 1943) and would thus be the strain least likely to cause a reaction in the host during immunization. Dubos (1949) has questioned whether or not a virulent strain of organisms is preferable to a less virulent one for production of non-living vaccines.

Preliminary studies in this investigation show that rabbits immunized with cell walls of virulent and avirulent diphtheria bacilli develop resistance to intradermal infection with virulent diphtheria bacilli. This resistance is demonstrated by the ability of immunized animals to tolerate from 2 to 16 times more organisms than non-immunized controls and by the increased capacity of immune animals to control the infectious process caused by larger numbers of virulent organisms. The immune response is probably not associated with delayed hypersensitivity or dependent upon the ability of the host to localize or bind toxin at

the initial focus of infection (Frobisher and Parsons, 1943; Frobisher and Updyke, 1947), but is due to an enhanced humoral response directed specifically against the bacterial cells themselves. This is supported by the appearance of agglutinating antibodies in the blood stream, by the correlation between the *in vivo* and *in vitro* neutralization to toxigenic activity, by the inability of toxigenic organisms to survive in the spleen, and by the lack of any measurable antitoxic response in rabbits injected with cell wall antigens, a fact also noted by Frobisher (Frobisher and Parsons, 1950) and Scheibel (1950).

The route of inoculation does not appear to be as critical as the administration of a second dose of cell wall antigens. Both the intravenous and subcutaneous routes serve about equally well as routes for injection of the antigen, although the agglutination titers are somewhat higher in rabbits inoculated subcutaneously. In the latter case the immune response is undoubtedly enhanced by the adjuvant effect of mineral oil and tubercle bacilli in Freund's complete adjuvant. These substances are known to stimulate increased antibody response by permitting the slow release of antigen and permitting a focus for antibody release (Humphrey and White, 1964).

Resistance afforded rabbits immunized with cell walls of the *mitis* strains of *C. diphtheriae* is more pronounced against homologous challenge than against heterologous challenge with the toxigenic *gravis* and *intermedius* strains. Similar observations were made by Frobisher and Parsons (1950) and by Bowan, Wyman, and McComb (1954). This correlates with the humoral response against the three different strains (Figure 2 and Table XVII) and reflects their antigenic differences. It also shows that the

immune response in animals receiving cell wall preparations is directed toward the organisms *per se* and not against the exotoxin as Frobisher has proposed (Frobisher and Parsons, 1950). If the latter were the case, all animals receiving cellular vaccines from one strain would react equally against challenge by any one of the strains. Moreover, one would be inclined to assume that the toxin elaborated by the 3 strains would be antigenically different, a point refuted by existing evidence (for references, see Zinnemann, 1943).

The fact that immunization with cell walls of the toxigenic *mitis* strain confers greater protection than cell walls of the nontoxigenic strain of *mitis* can be explained by antigenic dissimilarities within the strain. Similar data has been shown with smooth (virulent) and rough (avirulent) strains of *Salmonella typhi* which have been shown to possess different antigenic properties when examined by agar gel diffusion techniques (Digeon *et al.*, 1965).

The current studies with guinea pigs show that they can be immunized with cell wall antigens of *C. diphtheriae mitis* strain 6823. Complete protection is afforded against the lethal effect of 1 MLD (20×10^6 viable units) of the toxigenic *mitis*. Immunized guinea pigs develop partial resistance to 1.5 and 2 MLD's and almost no immunity to 4 MLD's. The absence of a detectable antitoxic response is inferred from the data presented in the experiments on rabbits (the cell walls were prepared in the same fashion) and shown by positive Schick reactions in guinea pigs at the time of challenge.

The inability of cell wall vaccines prepared from the nontoxigenic *mitis* to elicit an immune response as efficient as that from cell walls

of the toxigenic *mitis* strains appears to reflect differences in antigenic composition between the strains. Since neither group of experimental animals, and especially those receiving toxigenic *mitis* cell walls, demonstrate an anti-toxic response, the mechanism of resistance cannot be associated with toxin neutralization.

The addition of adjuvants to the cell walls enhances the immunogenicity of these preparations. Adjuvants of this type seem to provide a prolonged antigenic stimulus, a depot in the tissues in the form of a granulomatous lesion which permits the slow and continuous release of antigen, and a stimulus to the antibody-producing cells in the vicinity (Wilson and Miles, 1964). Guinea pigs receiving Freund's adjuvant (complete or incomplete) are able to withstand the lethal effects of 1 MLD of viable organisms whereas guinea pigs receiving cell walls suspended in saline are not protected to the same extent. However, it is noteworthy that cell walls suspended in saline and introduced subcutaneously induce a favorable immune response in guinea pigs. This means that cell walls themselves, divorced from an adjuvant, can serve as effective immunizing agents.

The absence of virulent *mitis* organisms in the spleens of guinea pigs immunized with cell walls lends further support to the existing data. It is regretable that a third group consisting of animals vaccinated with diphtheria toxoid was not included, as this would aid in giving a comparison between the relative merits of toxoid and cell wall antigens. Hypothetically, at least, the number of organisms recovered from spleens of toxoid immunized animals would vary little from the number recovered from the controls, since the response induced by the toxoid

would be directed against elaborated toxin and not against the organism. That toxoid immunization evokes a purely antitoxic response is substantiated by the work of Huang (1942).

During this phase of the research the importance of the protoplasmic fraction of toxigenic C. diphtheriae mitis was fully realized. The realization was that such large numbers of toxigenic organisms were required to produce a lethal infection in guinea pigs that antibacterial immunity could play only a very restricted role in prevention of deaths due to toxin. If the lethal effect due to 1 MLD of toxin is contained in fewer than 8 to 16 times the number of viable units required to infect guinea pigs, it is difficult to visualize how antibacterial immunity could be effective. The effect of antibacterial immunity in prevention of death among mice infected with plague bacilli is evident from Larson's studies (unpublished data). Mice succumb to plague toxin elaborated during the course of infection, but infection can be initiated by only a few plague bacilli which then proliferate through many generations to produce sufficient toxin. With systems represented by C. diphtheriae and guinea pigs it is evident that proliferation of organisms was not too great a factor in production of lethal disease. These ideas were strengthened by belated examination of a report by Roux and Yersin (1888) in which they noted the failure of toxigenic diphtheria to proliferate to any extent when injected into the cutaneous tissues of guinea pigs.

Our tests of the lethal effect of protoplasm in guinea pigs show that the protoplasm of 4 MLD's of viable toxigenic organisms is approximately equal to 1 MLD of living organisms in producing lethal effects. This small discrepancy between results obtained with protoplasm and with

viable bacilli may result from detoxification during cell disruption and the time required to harvest and inject protoplasm into the guinea pigs. However, as mentioned in the previous section, toxinogenesis is a dynamic process; at a given time cells do not contain all the toxin they are capable of producing (Barksdale et al., 1961). Moreover, Pappenheimer and Yoneda (1957) have shown that $20x10^6$ toxigenic mitis organisms (a lysogenic mitis) produce on the order of 0.03 L_f toxin protein, and there is evidence that differences in toxinogenicity between individual virulent strains is not very wide (Hewlitt, 1948). Since approximately 0.020 L_f toxin protein constitutes 1 MLD of organisms (Wilson and Miles, 1964), the infective dose of $20x10^6$ toxigenic mitis organisms, or 1 MLD as used in this study, is capable of producing sufficient toxin to kill normal guinea pigs in 3 to 4 days. Even though this is an a priori argument, the evidence indicates that the intradermal challenge system used in this study was adapted primarily to studies of antitoxic resistance and could be expected to show antibacterial immunity only if resistance was of high magnitude. It appears from these studies that the antibacterial resistance produced with cell walls limited growth of the infective dose of toxigenic organisms since 1 MLD of toxigenic bacilli did not produce death. In spite of the artificiality of the challenge system, it is interesting to note that we were able to produce resistance to 1 and 2 MLD's of toxigenic mitis organisms. Previous workers were unable to achieve protection of this type. This suggests that with a natural route of infection in a natural host where proliferation of organisms occurs, the efficacy of cell wall immunization might be much more pronounced than is indicated in this study.

Attempts to discover a natural route of infection in guinea pigs (and to a limited extent in rabbits) for which relatively few organisms would produce a clinical disease were largely unsuccessful. The work of Loeffler (1884) and Roux and Yersin (1888) indicated that slight trauma to certain mucous membranes or the cornea in guinea pigs, pigeons, cats, dogs, rabbits, and monkeys followed by inoculation of the area with large numbers of organisms in a broth culture sometimes produced a typical diphtheritic membrane and death. The only method found to be slightly successful was inoculation of the vulval area of guinea pigs after the hymen had been ruptured mechanically (Loeffler, 1884). Results were inconsistent and variable. Whether the _mitis_ strain loses its infectivity after primary isolation as does _Bordetella pertussis_ (Leslie and Gardner, 1931) was not determined. The most likely answer is that guinea pigs and rabbits, and probably the other animals tested by Loeffler and Roux and Yerson, are unnatural hosts, and that _C. diphtheriae_ is a natural pathogen only for man. If one accepts this conclusion, it seems hardly likely that one could accurately test the efficacy of an immunizing antigen in an artificial host. Therefore, in order to determine the actual value of cell wall antigens of _C. diphtheriae_, it would be necessary to test the preparations in man.

CHAPTER VI

SUMMARY

1. Attempts were made to induce active antibacterial resistance in rabbits and guinea pigs against diphtheria by immunization with cell walls of the *mitis* strain of *Corynebacterium diphtheriae*.

2. Rabbits immunized with cell walls of either the toxigenic or atoxigenic *mitis* strain of *C. diphtheriae* were shown to be protected against subsequent intradermal infection with sublethal doses of toxigenic diphtheria bacilli.

3. Resistance afforded rabbits immunized with cell walls of the *mitis* strains is more pronounced against homologous challenge than against heterologous challenge with the toxigenic *gravis* and *intermedius* strain.

4. Antibacterial sera prepared in rabbits immunized with cell walls of the toxigenic *mitis* is capable of neutralizing *in vitro* the toxinogenicity of *C. diphtheriae mitis*.

5. Guinea pigs immunized with cell wall antigens of the toxigenic *mitis* in Freund's complete adjuvant demonstrated complete resistance to the lethal effects of 1 MLD, partial resistance to 1.5 and 2 MLD's, and no protection to 4 MLD's of toxigenic *mitis* organism.

6. Cell walls emulsified in Freund's adjuvant (complete or incomplete) offered greater protection than cell walls suspended in saline, when both preparations were administered in a single dose. A saline

suspension of cell walls injected in 2 separate doses provided immunity comparable to that obtained with a single dose of cell walls in Freund's adjuvant.

7. The resistance to infection with toxigenic diphtheria bacilli in guinea pigs and rabbits immunized with cell wall antigens is shown not to be associated with the presence of demonstrable antitoxin.
8. The failure of inducing antibacterial immunity in laboratory animals is discussed in terms of the inability of toxigenic organisms to proliferate in the cutaneous tissues of the host.

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