INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.
FREDERICK, Grover Thomas, 1946-

IMMUNOLOGICAL RESPONSES TO GLYCEROL-TEICHOIC
ACIDS.

The Ohio State University, Ph.D., 1972
Health Sciences, immunology

University Microfilms, A XEROX Company, Ann Arbor, Michigan

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED.
IMMUNOLOGICAL RESPONSES TO GLYCEROL-TEICOHIC ACIDS

DISSertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Grover Thomas Frederick, B.Sc., M.Sc.

The Ohio State University
1972

Approved by

Frank W. Hoppenrung
Adviser
Department of Microbiology
PLEASE NOTE:

Some pages may have
indistinct print.
Filmed as received.

University Microfilms, A Xerox Education Company
ACKNOWLEDGEMENTS

I wish to thank Dr. Frank W. Chorpenning for his help and guidance during the preparation of this dissertation and especially for his thought provoking questioning of the data and its interpretations.

I wish to thank Mr. R.A. Holmes for his help with the MIF testing of guinea pigs and Dr. R.A. Feller for his assistance with the Baird-Atomic Radiochromatogram Scanner.

Finally, I wish to thank my wife, Geri, for her encouragement and understanding during the investigations reported in this dissertation.
VITA

September 16, 1946..............  Born - Cleveland, Ohio
1968..............................  B.Sc., The Ohio State University
           Columbus, Ohio
1969..............................  M.Sc., The Ohio State University
           Columbus, Ohio
1968-1969.........................  Teaching Assistant, Department
           of Microbiology, The Ohio State
           University, Columbus, Ohio
1969-1972.........................  Teaching Associate, Department
           of Microbiology, The Ohio State
           University, Columbus, Ohio
May-June, 1971....................  Research Associate, 4th U.S. Army
           Medical Laboratory, Fort Sam Houston,
           Texas

PUBLICATIONS


FIELDS OF STUDY

Major Field: Microbiology

Studies in Immunology. Professors F.W. Chorpenning and M.C. Dodd

Studies in Pathogenic Microbiology. Professor M.S. Rheins

Studies in Microbial Physiology. Professor C.I. Randles

Studies in Electron Microscopy. Professor R.M. Pfister
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................. i

VITA ............................................................................................ iii

LIST OF TABLES ................................................................. vii

LIST OF FIGURES ............................................................. viii

INTRODUCTION ....................................................................... 1

A REVIEW OF THE LITERATURE .............................................. 3

MATERIALS AND METHODS ........................................ 16

- Animals ............................................................................. 16
- Antigens ........................................................................... 17
- Passive Hemagglutination .............................................. 19
- Antisera ............................................................................. 20
- Immunoelectrophoresis ............................................... 22
- Immunodiffusion ............................................................ 22

Distribution of Anti-teichoic Acid Antibodies in
  Normal Sera ....................................................................... 23
- Radial Immunodiffusion ................................................. 23
- Characterization of Anti-teichoic Acid Antibodies .......... 23
- Determination of Natural Cell-mediated Immunity to
  Glycerol-teichoic Acid in Guinea Pigs ............................. 25
- Injection of Normal Guinea Pigs with Four Forms of
  Glycerol-teichoic Acid ..................................................... 27
- Equilibrium Dialysis ....................................................... 28
- Macrophage Cytophilic Antibodies ................................. 34
- Bactericidal Activity of Normal Guinea Pig Anti-
  teichoic Acid Antibodies ............................................... 34

RESULTS ............................................................................. 36

- Characterization and Specificity of Immunoochemical
  Reagents ........................................................................... 36
- Distribution of Normal Anti-teichoic Acid Antibodies ..... 39
- Characterization of Anti-teichoic Acid Antibodies ......... 39
- Ontogeny of Natural Anti-teichoic Acid Antibodies in
  Germfree and Conventional Guinea Pigs ....................... 45
- Ontogeny of Natural Cell-mediated Immunity to
  Glycerol-teichoic Acid in Guinea Pigs ............................ 50
<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effects of Artificial Immunization on the Humoral and Cell-mediated Responses to Teichoic Acid</td>
<td>54</td>
</tr>
<tr>
<td>Chemistry and Labeling of Synthetic Polyglycerophosphate</td>
<td>57</td>
</tr>
<tr>
<td>Affinity of Natural ( \gamma )-G Anti-teichoic Acid Antibodies in Conventional Guinea Pigs</td>
<td>61</td>
</tr>
<tr>
<td>Macrophage Cytophilic Antibodies</td>
<td>61</td>
</tr>
<tr>
<td>Bactericidal Properties of Normal Guinea Pig Anti-teichoic Acid Antibodies</td>
<td>61</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>70</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>86</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>90</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

| Table 1. Distribution of normal anti-teichoic acid antibodies in adult sera of selected mammalian species | Page 44 |
| Table 2. Characterization of anti-teichoic acid antibodies in adult sera of selected mammalian species | 45 |
| Table 3. Cell-mediated immunity to glycerol-teichoic acid in normal guinea pigs as demonstrated by delayed skin reactions or by the inhibition of macrophage migration | 53 |
| Table 4. Skin tests of guinea pigs artificially immunized with various forms of glycerol-teichoic acid | 60 |
| Table 5. Concentration and average affinity of \( \gamma \)G anti-teichoic acid antibodies in normal adult guinea pig sera | 69 |
| Table 6. Bactericidal properties of normal adult guinea pig serum | 69 |
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Plate I</th>
<th>Immuno-electrophoresis of purified guinea pig IgM and various rabbit anti-guinea pig IgM antisera</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate II</td>
<td>Immunodiffusion and immunoelectrophoresis of guinea pig $\gamma_1$G and $\gamma_2$G, unabsorbed rabbit anti-guinea pig $\gamma_1$G and absorbed rabbit anti-guinea pig $\gamma_1$G and anti-$\gamma_2$G</td>
<td>41</td>
</tr>
<tr>
<td>Figure 1</td>
<td>Immuno-electrophoresis of two guinea pig whole sera vs. rabbit anti-guinea pig whole serum prepared by the author and commercial rabbit anti-guinea pig whole serum</td>
<td>43</td>
</tr>
<tr>
<td>Plate III</td>
<td>Radial immunodiffusion patterns of guinea pig whole serum vs. monospecific rabbit anti-guinea pig IgM and IgG</td>
<td>47</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Relative levels of total IgG and IgM in germ-free and conventional whole sera</td>
<td>49</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Average titers of specific anti-teichoic acid antibodies in IgM and IgG classes of immunoglobulins as measured by passive hemagglutination</td>
<td>52</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Average whole serum titers of four groups of guinea pigs on days 0, 4, 7 and 14 after intraperitoneal injection with whole bacilli, acid-precipitated teichoic acid, purified teichoic acid and synthetic polyglycerophosphate</td>
<td>56</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Average whole serum titers of four groups of rats on days 0, 4, 7 and 14 after intraperitoneal injection with whole bacilli, acid-precipitated teichoic acid, purified teichoic acid and synthetic polyglycerophosphate</td>
<td>59</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Fractionation of synthetic polyglycerophosphate on Sephadex LH-20</td>
<td>64</td>
</tr>
</tbody>
</table>
Figure 7. Fractionation of PGP-1-$^{14}$C-glycine on Sephadex LH-20

Plate IV. Plots of moles of PGP-1-$^{14}$C-glycine bound per mole of anti-teichoic acid antibody/free concentration of PGP-1-$^{14}$C-glycine vs. moles of PGP-1-$^{14}$C-glycine bound per mole of anti-teichoic acid antibody.
INTRODUCTION

Because of the interest shown by many immunologists in the possible roles of antigenic stimuli and genetic and physiologic factors in the immunologic development of an animal, investigations of the ontogeny of cell-mediated immunity and humoral antibody responses during maturation of the host have become increasingly valuable because they provide the necessary basis from which to begin studies in these areas. Two issues of current interest are the factors determining activation of delayed hypersensitivity versus antibody formation and the rules governing which immunoglobulin class is to be synthesized (Nossal, 1972). In order to study the development of the immune responses, a system must be employed which is well defined and which meets certain criteria. To reduce the complexities inherent in cross-reactions and to insure unity of all in vitro and in vivo observations, the system should involve a single antigenic specificity. It should include the development of cell-mediated and humoral immunity to natural as well as to artificial immunization. The techniques and concepts developed during the investigation of the system should be easily transferrable to similar models in other animal species.

This dissertation describes the investigation of the ontogeny of natural humoral and cell-mediated immune responses to polyglycerophosphate (PGP) in guinea pigs. The classes of anti-PGP antibodies in germfree and conventional guinea pigs are characterized and their
occurrence during maturation of both groups of animals was determined. The development of a natural cell-mediated immunity to PGP, demonstrated in vivo by a delayed skin reaction and in vitro by the inhibition of macrophage migration, was investigated. The effects of immunization with various forms of PGP on the cell-mediated response in guinea pigs and the humoral antibody response in guinea pigs and rats were determined. Included is a brief study of natural anti-PGP antibodies in other species as well as certain important biological characteristics of anti-PGP antibodies.

The results of these studies provide a detailed description of the natural development of immune responses in guinea pigs to a single antigenic specificity and the effect of artificial immunization on these responses. Comparisons are made of the classes of natural anti-PGP antibodies present in adult guinea pigs, rabbits, rats and humans and of the humoral antibody responses in guinea pigs and rats to artificial immunization. These results are discussed with regard to their implications concerning basic immunological concepts about the development of the immune responses.
A REVIEW OF THE LITERATURE

The ontogeny of the cellular and humoral stages of the immune response has come under close scrutiny during the last ten years. Two areas of current interest are: (1) the development and interrelationships of antibody formation and cell-mediated immunity during maturation of the host and (2) the role(s) of antigen in the induction and elicitation of these two types of immune responses.

Investigations of the development of antibody formation have been concentrated in two areas. The first, the sequential appearance of the classes of immunoglobulins during an immune response, has been well established. Kim et al. (1966, 1967, 1968), working with germfree swine, a species which does not have a placental transfer of antibody (Brambell, 1958), have demonstrated that the first antibody to MSP-2 actinophage developing in swine was a 2-mercaptoethanol (2-me) sensitive 19S/6 immunoglobulin with subunits antigenically identical to the 2-me resistant 7S/6 antibody of the same specificity which appeared later and was present in normal adult sow sera. The 19S/6 was transient and was followed by the appearance of 19S/4 antibody. They have also shown a similar pattern in mice (Kim et al., 1964). Sell (1965) demonstrated a sequential development of 19S to 7S antibody in germfree mice and guinea pigs. Uhr and Finkelstein (1963) have clearly shown that the antibody response in adult rabbits and guinea pigs to 0X 17k virus was characterized by the early appearance of 19S/4 antibodies.
followed by the formation of 7S\(\gamma\)G antibodies. Stelos and Taliaferro
(1959) observed the change of 19S antibody to the 7S type during im-
munization of rabbits with sheep erythrocytes. Fink et al. (1961)
demonstrated the formation of 19S/M antibody followed by 7S\(\gamma\)G antibody
in immunized infants. Kincade and Cooper (1970) reported a similar
sequence in the chicken. The typical 19S to 7S change need not occur,
however. In certain species (e.g., pig, chicken) 19S antibodies to
some antigens are predominantly formed even after repeated immunization
(Franek et al., 1962; Dreesman et al., 1965). Michael and Rosen (1963)
and Smith (1960) have shown that many natural anti-bacterial antibodies
are detected only in the 19S/M class of immunoglobulins.

The second area of the development of antibody formation which
has received considerable attention is the time during maturation from
fetus to adult at which the various immunoglobulin classes appear in
response to an antigenic stimulus. Silverstein et al. (1963) have shown
that the developing fetus responds to different antigens at different
periods of time during maturation, the typical 19S to 7S sequential
development being even more pronounced than in the neonate or adult.
Adler et al. (1967) demonstrated the appearance of 19S/M antibody for
Salmonella paratyphi B in 2-day old rabbits immunized at birth, fol-
lowed by the appearance of 7S\(\gamma\)G soon thereafter. By day 10, the 7S\(\gamma\)G
was the predominant immunoglobulin. It has clearly been shown that most
fetal and neonatal animals, except the rat, are capable of producing
both 19S/M and 7S\(\gamma\)G antibodies soon after immunization and that the
sequential development of normal immunoglobulins, without regard to
antibody specificity, follows the same pattern (Thorbecke and van Furth,
1967). There have been no complete investigations, however, of the sequential development, to include the time of appearance, of the different classes of natural antibodies during maturation of the host from neonate to adult.

Studies of the ontogeny of cell-mediated immune responses have been undertaken in the areas of homograft rejection, delayed hypersensitivity (skin reactions), the graft-versus-host reaction and the mixed lymphocyte reaction. Most of the reports concerning homograft rejection have compared the capabilities of the neonate and adult members of a species to reject tissue homografts. Rawles (1955) and Medawar and Woodruff (1958) have shown the neonatal rat to be incapable of rejecting a tissue homograft. On the other hand, Fowler et al. (1960) found the human newborn quite capable of homograft rejection. Similar observations of the immunocompetence of neonates were made by Howard and Michie (1962) in the mouse, Solomon (1963, 1964) in the chick embryo and Silverstein and Kraner (1965) in the fetal lamb.

Appreciably less has been learned about the ontogenesis of delayed hypersensitivity as manifested by skin reactions. This is probably due in great measure to the repeated demonstrations by Freund (1927), Sterzl and Hrubesova (1959) and Salvin et al. (1962) that the skin of certain newborn mammalian species (e.g., guinea pigs, rabbits) is, for some apparently non-specific reason, an unsatisfactory site for the elicitation of delayed inflammatory reactions. Despite this difficulty, a few investigators have been able to achieve success in this area. Uhr (1960) and Weiss (1958) were able to induce delayed-type hypersensitivity to a variety of antigens in fetal and neonatal
guinea pigs and to elicit a delayed skin reaction soon thereafter. The newborn human infant was shown to develop contact hypersensitivity to poison ivy by Strauss (1931) and to dinitrofluorobenzene by Uhr et al. (1960). In contrast, Beachey et al. (1969) were not able to demonstrate delayed hypersensitivity (skin reaction) to M proteins in young guinea pigs, while adult animals were consistently positive. The young guinea pigs did develop a delayed skin reaction to intradermal injections of the antigen when previously immunized with M protein.

The capability of spleen cells to elicit graft-versus-host reactions is indicative of the maturation of cell-mediated immunity (Playfair, 1971). Bortin et al. (1969) have shown that spleen cells from mice develop immunologic competence between the 6th and 8th day postnatally. Goldstein et al. (1971) have shown that the graft-versus-host reactivity of thymocytes from mice develops within 6 hours after birth and the spleen cells of these same animals become competent 3-4 days postnatally. Similar results have been reported by Cohen et al. (1963) in mice.

Recent work by Knight and Thorbecke (1971) has shown that the ability of thymocytes from neonatal rats to function in the mixed lymphocyte reaction is considerably less than the ability of thymocytes from 7-day old rats or from adult rats. Both neonatal and adult thymocytes could produce a graft-versus-host reaction, however.

Of the four correlates of cell-mediated immunity discussed above, only the delayed skin reaction does not involve a response to histocompatibility antigens. Because of the complex nature of these antigens, the specificity of responses to them are not clearly defined.
The skin test for delayed hypersensitivity can be induced and elicited with chemically defined antigens (Burger et al., 1971; Howe and Battisto, 1971) and is one of the methods of choice for demonstrating specific cell-mediated immunity (Fick and Turk, 1972). A second method which can be employed using purified antigens is the inhibition of macrophage migration. George and Vaughan (1962) and David et al. (1964) were the first to show that peritoneal exudate cells, obtained from sensitized animals, were inhibited from migrating out of capillary tubes by the sensitizing antigen. Bloom and Bennett (1966) and David (1966) have shown that this inhibition of migration was due to a soluble material, migration inhibition factor, which is elaborated by sensitized lymphocytes in contact with the appropriate antigen. When a purified antigen is employed, the specificity of the delayed skin reaction and the inhibition of macrophage migration is known and these two reactions can then be used to study the ontogeny of a specific cell-mediated reaction during maturation of the host.

The work reported in this dissertation employed a purified and chemically defined antigen in all phases of testing in which it was required. The antigen was a glycerol-teichoic acid whose specificity has been shown to reside completely in its polyglycerophosphate backbone (Decker et al., 1972). This antigen is similar to one first reported by Rantz et al. (1952, 1956) in the culture filtrates of a number of gram-positive organisms and was associated with a bacteriogenic transfusion reaction by Chorpenning and Dodd (1965, 1966). Neter et al. (1960) were the first to show these early crude antigens to be capable of modifying erythrocytes, a property which has been confirmed in our
laboratory for the purified antigen (Decker et al., 1972; Chorpenning and Stamper, 1972). Erythrocytes modified with this purified antigen were then used to detect anti-teichoic acid antibodies by the method of passive hemagglutination. This author has shown the purified antigen to be capable of eliciting a delayed skin reaction in guinea pigs and of causing the inhibition of migration of peritoneal cells (Frederick et al., 1972).

Hand in hand with the investigations of the ontogeny of antibody formation and cell-mediated immunity has developed an intense interest in the interrelationships between these two types of responses. Since Miller's demonstration that neonatal thymectomy in mice prevented the proper development of all cell-mediated and some antibody responses (Miller, 1961), the concept of two separate populations of lymphocytes, one for each type of response, has been well established. Good et al. (1962) demonstrated that in human congenital immunological deficiency, two systems of cells could be independently affected: (1) a predominant-ly plasma cell population—deficient in sex-linked (Bruton's) agamma-globulinemia and (2) a population of lymphocytes associated with the thymus—totally absent in the DiGeorge syndrome. An even more clear cut distinction between these two populations emerged in the chicken. Warner and Szenberg (1964) demonstrated that removal of the bursa or thymus had quite separate consequences, bursectomy reducing the antibody response and depleting plasma cells and immunoglobulins, but not homograft immunity or delayed hypersensitivity, and thymectomy having exactly the reverse effect. The terms "T-cell" and "B-cell" have been coined to refer to "thymus-dependent" lymphocytes (lymphocytes which
fail to develop after neonatal thymectomy) and "bursal-equivalent" or "bone-marrow derived" lymphocytes, independent of direct thymus control.

Even though these two subpopulations of lymphocytes have been shown, in a number of cases, to be independent of each other (Leskowitz, 1970; Liew and Parish, 1972; Gotoff and Vizral, 1972; Gell and Benacerraf, 1960; Pachman and Fox, 1970; Borel and David, 1970), there is considerable evidence that T and B cells act synergistically during the antibody responses to certain antigens. Diener and Mallory (1932), Sell and Weigle (1959) and Salvini and Smith (1959) reported that injection of soluble proteins resulted in a delayed skin reaction followed by antibody formation. They hypothesized that delayed hypersensitivity was a prerequisite for antibody formation to these antigens. Benacerraf et al. (1967) demonstrated that guinea pigs of one strain could not respond, by antibody formation or delayed hypersensitivity, to a dinitrophenyl-polysine complex, yet if the complex was coupled to bovine serum albumin, normal responses were found. Rajewsky et al. (1969) found in normal rabbits that a secondary response to a hapten-carrier complex could be elicited only if the hapten was attached to the same carrier for both the primary and secondary challenges. Mitchison (1967) reported the inability to make anti-hapten antibody in X-irradiated mice who had received lymphoid cells from mice primed to a hapten-carrier complex if that carrier was different from the one used to challenge the recipient mice. Responsiveness could be restored by the addition of cells from another mouse primed with the same carrier-hapten complex as the one used to challenge the recipient. The phenomenon described in these last three studies is termed the "carrier
effect" and is interpreted to mean that different populations of lymphocytes were responding to the carrier and to the hapten, both responses being needed for anti-hapten antibody to be formed.

A very simple demonstration of the need for two cell types in the antibody response was an experiment by Claman et al. (1966) in which cells were injected into irradiated mice together with sheep erythrocytes. Thymus cells alone gave no antibody response, marrow cells alone very little, but the mixture of thymus and marrow cells resulted in a significant antibody response. Dutton (1971) has been able to demonstrate this thymus-bone marrow cell cooperation phenomenon in vitro.

Although the antibody response to sheep erythrocytes (and some other antigens, e.g., heterologous serum albumins) requires both a T and B cell population, antibody responses to certain other antigens apparently do not require the involvement of an active T cell population. For example, keyhole-limpet hemocyanin, pneumococcal polysaccharide (Humphrey et al., 1964) and flagellin (Armstrong et al., 1969) have been shown to be thymus-independent antigens.

From these data arises a very intriguing question of primary importance to the present work. During maturation of the host and, presumably, of the immune system, is the development of a cell-mediated response to teichoic acid related in any way to the immunoglobulin class of antibody produced to this antigen? Arnason et al. (1964), Curtis et al. (1970) and Playfair and Purves (1971) have all reported data which suggests that IgM antibody formation is independent of T cell function. On the other hand, Daguillard et al. (1970), Barth et al.
(1971) and Alm (1971) have shown that suppression of T cell function affects IgM antibody production to keyhole-limpet hemocyanin, sheep erythrocytes and pneumococcal polysaccharide, respectively. Pneumococcal polysaccharide has been shown to be thymus-independent (Humphrey et al., 1964), which raises doubts about the validity of statements regarding the thymus dependency of some antigens. Most investigators do agree, however, that the elicitation of a secondary (IgG) response is dependent on the thymus cell, probably for a memory function (Bosman and Feldman, 1970; Miller, 1971; Anderson et al., 1972; Mitchell et al., 1972; Henry and Kodlin, 1971).

One of the questions concerning the development of immunity which is still controversial is the role of antigen in the maturation, induction and elicitation of the humoral and cellular stages of the immune response. Silverstein and Kraner (1965) have shown in the fetal lamb that development of antibody formation is not achieved simultaneously to all antigens, but that the ability to respond to some is evident early in gestation, to others later in gestation and to several only some time after birth. This maturation was independent of exposure to antigen. On the other hand, Silverstein and Auerbach (1971), in reviewing recent data, concluded that the steps involved in lymphocyte differentiation during maturation of the immune response are entirely antigen dependent.

One of the topics of importance to this dissertation, which is related to the effect of antigen on the development of the immune response, is the occurrence of natural antibodies. The term "natural antibodies" has been employed to describe substances with antibody
activity that are detected in small amounts in the sera of animals before any controlled immunization or apparent infection. This definition covers not only antibodies which may be formed spontaneously on the basis of genetic determination and without antigenic stimuli, but also antibodies which are formed as a result of nonovert immunization with natural microflora, with antigens of food, with inhaled antigens, or as a result of inapparent infection. Many theories of antibody formation, most notably those of Ehrlich and Morgenroth (1900), Burnet and Fenner (1949), Jerne (1955), Burnet (1959, 1964) and Kerman et al. (1970), require some type of preformed recognition factors, either humoral or cell-bound, and most require that these factors be specific globulins (i.e., antibodies). Thus the question of the origin and development of natural antibodies is becoming increasingly more important, and it is imperative that it be answered if any real progress is to be made concerning the processes of antibody formation.

The question as to the origin of such natural antibodies was already receiving much attention in the 1920's. The argument for genetic control of their formation was first offered by Hirzfeld (1926) as he was investigating the isoagglutinins and autoagglutinins of normal human sera. He concluded that natural antibodies were normal serum proteins which had a strong affinity for the genetically controlled blood cell antigens. Manresa (1932) hypothesized that resistance in rabbits to infection by Brucella abortus was inherited. However, injections of minute amounts of organism into susceptible animals stimulated immunity identical to that found in the resistant animals, which tended to refute his hypothesis. Abdoosh (1936), investigating
bactericidins for gonococcus, found that eleven mammalian species had such antibodies in their normal sera but that all normal human sera lacked such bactericidins even though the gonococcus was claimed to be a strict human pathogen. He concluded that since the one species which harbored the organism most frequently (i.e., human) lacked the antibody, the production of this antibody was genetically controlled. Recently, Gorzynski et al. (1970), Rathbun and Hildemann (1970) and Paul et al. (1970) have reported strain differences in mice with regard to specificity and levels of antibody present in normal sera which reacted with dinitrophenyl and trinitrophenyl haptens.

Some authors take the view that all natural antibodies in vertebrate sera are present as a consequence of previous antigen stimulation with either the ubiquitous test antigen or some cross-reacting antigen (e.g., Forssman antigen, Forssman, 1911; Buchbinder, 1935; Boyd, 1956; Jenkin, 1963). A system which has received special attention is the appearance of hemagglutinins in normal sera for foreign erythrocytes. Herman (1936) found such agglutinins for horse red cells in the sera of normal humans. Finland and Curnen (1938) attributed deaths in humans from the prophylactic administration of horse antipneumococcal serum to high titers of agglutinins for A, B, AB and 0 (H) erythrocytes in the immune serum, which suggested a heterogenetic stimulation of these hemagglutinins by the pneumococcus. Weiner (1951) supported the hypothesis that antigens shared by bacteria and similar to red cell A and B antigens played a role in the formation of natural hemagglutinins. Kirshbom and Hoecker (1963) attributed the increase in the hemagglutinating ability of mouse sera for human erythrocytes
to a constant subclinical infection with organisms having antigens similar to those of the test red cells. One may note that most of the organisms believed to act as stimuli for these hemagglutinins are ubiquitous gram-negative flora of the gut. Substances with blood group A activity appear in type XIV specific pneumococcal polysaccharide (MacDuffie and Kabat, 1956) and various gram-negative bacteria (Springer et al., 1961). Substances cross-reacting with group B substances are also found in many bacteria (MacDuffie and Kabat, 1956; Springer et al., 1961).

One of the most important advances in the investigation of natural antibodies which attempted to answer the question concerning the role of bacteria in the stimulation of these antibodies was made by Wagner (1955) when he used the germfree animal to study the immune response. In the germfree environment, many natural antigenic stimuli can be completely eliminated. Wagner found no hemagglutinins for rabbit erythrocytes in the sera of germfree chicks although they were present in the sera of conventional chicks of the same age. The agglutinins did appear, however, in the sera of the germfree chicks after 30 days of age, which indicated that microorganisms, if they did play a role in the stimulation of these antibodies, need not be viable. Germfree chicks likewise showed a lack of bacterial agglutinins for Paracolobactrum aerogenoides up to one year when no viable organisms could be demonstrated in the diet prior to autoclaving. He also found that germfree and conventional chicks responded identically to intravenous injections of Salmonella pullorum, which indicated that the germfree animal was as immunocompetent as its conventional counterpart.
Therefore, a lack of antibody could not be attributed to a deficiency of the immune system caused by the germfree state. Later work by Springer et al. (1959) supported Wagner. They fed germfree chicks a semi-synthetic diet free of demonstrable blood group activity. These chicks were free of B agglutinins up to 60 days of age while conventional chicks fed the same diet showed such agglutinins at four weeks. When the germfree chicks were fed Escherichia coli strain 086, they developed high titers of B agglutinins.

What is lacking in the literature, however, is a study of the effect of artificial immunization with antigen on the maturation of natural antibodies as well as natural cell-mediated immune responses. Because natural immunity to teichoic acid develops more slowly than the responses to injection of the antigen (Frederick, 1969), the steps involved in maturation and the effects of single injections of antigen can be more easily studied. The use of chemically defined antigen leaves no doubt as to the specificity being investigated. The results of this work are a detailed description of the development of cell-mediated and humoral antibody responses to glycerol-teichoic acid and the effect of injection of this antigen on both types of responses.
MATERIALS AND METHODS

Animals Animals used in this study were: random-bred albino rabbits raised on Purina Rabbit Chow (Ralston-Purina Co., St. Louis, Mo.); conventional random-bred albino mice raised on Purina Rat Chow; germfree random-bred albino mice raised on autoclaved Purina Rat Chow, supplemented with heat-labile vitamins; random-bred albino rats raised on Purina Rat Chow; conventional random-bred guinea pigs raised on Purina Guinea Pig Chow; and germfree guinea pigs raised on a diet (No. 010999, General Biochemicals, Chagrin Falls, Ohio) nutritionally identical to the Purina Guinea Pig Chow. Germfree mice were received through the courtesy of Children's Hospital, Columbus, Ohio. Germfree guinea pigs were derived by hysterectomy into flexible plastic isolators previously sterilized by spraying with 2.5% peracetic acid. Sterility of the isolator, feed, water, bedding, cages and animals was checked by daily culturing with moistened swabs. The swabs were placed in thioglycollate broth and incubated at 37°C for two weeks. The absence of growth during incubation was taken to indicate that the isolator and its contents were sterile at the time of culturing. More extensive culturing on Sabouraud's agar, brain-heart infusion agar and blood agar at 20, 25 and 37°C under aerobic and anaerobic conditions was carried out once a week. Attempts to raise selected litters on synthetic diets prepared by the author met with little success, as the longest any animal was kept alive on any of the diets was 15 days.
Antigens  Four forms of glycerol-teichoic acid, representing four stages of purity, were used in this study. Whole bacilli (Bacillus sp. O.S.U. 372) were grown in 3% Trypticase Soy Broth (BBL, Cockeysville, Md.) at 37 C for 48 hours in a 1-Liter Microfern Fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) with constant agitation and aeration. The organisms were collected by centrifugation, washed three times with phosphate-buffered saline (PBS), pH 7.3 and resuspended in PBS to the appropriate concentration.

Acid-precipitated teichoic acid (AP) was prepared by the method of Schmidt (1968). Whole bacilli were suspended to a 5% concentration in PBS, pH 6.7 and boiled for 30 minutes. The supernate was clarified by centrifugation and mixed with eleven volumes of cold acetone. The resulting precipitate was collected by centrifugation, dried in air and dissolved in PBS, pH 6.7. The pH of this solution was reduced to 4 with the dropwise addition of 0.01M acetate buffer, pH 2.7. The precipitate formed was collected by centrifugation and dissolved in PBS, pH 6.7. The acid precipitation was repeated twice and the final precipitate dissolved in PBS, pH 6.7, dialyzed against 4 changes of double-distilled, demineralized (DDD) water (100 volumes) and lyophilized.

Purified teichoic acid (PCE) was prepared by the method of Decker et al. (1972) using a lyophilized phenol extract graciously supplied by Mr. G.P. Decker. Three hundred milligrams of the extract were treated with 10mg bovine pancreatic ribonuclease (Sigma Chemical Co., St. Louis, Mo.) at 37 C for 48 hours. Following RNA hydrolysis, the mixture was cooled to 5 C, treated with crystalline trichloroacetic
acid added to a 10% concentration and stirred for 2 hours at 4°C. Five volumes of cold 95% ethanol were added and the resulting precipitate collected by centrifugation, redissolved in PBS, pH 7.3 and chromatographed on a 2.5cm x 45cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The fractions having erythrocyte modifying activity, determined by the method of passive hemagglutination described below, were pooled, dialyzed against DDD water and further purified on a 2.5cm x 45cm column of Dowex 50Wx12 (H form) cation exchange resin (Bio-Rad Laboratories, Richmond, Calif.) with 0.1M acetate buffer, pH 2.7. The teichoic acid-containing fractions were pooled, dialyzed against DDD water and lyophilized.

Polyglycerophosphate (PGP) was prepared by the method of McGarty (1959). A 10% solution of sodium-α-glycerophosphate (Nutritional Biochemicals Corp., Cleveland, Ohio) was passed over a column of Dowex 50Wx12 (H form). Consecutive 1ml fractions of the effluent were assayed for organic phosphorus by the method of Ma and McKinley (1953). One-tenth milliliter aliquots of each fraction were placed in separate Kjeldahl boiling flasks. To each flask were added 1.5ml of sulphuric acid and 0.2ml of nitric acid. A smooth glass bead was placed in each flask to prevent bumping and the solutions were boiled until clear and sulphur trioxide fumes were emitted. The flasks were cooled and an additional 0.1ml of HNO₃ was added. The solutions were boiled for an additional 30 minutes, cooled to room temperature and chilled in ice. The samples were transferred quantitatively to 100ml volumetric flasks, diluted to 65ml with DDD water and treated with 1.0ml sulphuric acid. Ten milliliters of a 0.20% ammonium vanadate solution in dilute
sulphuric acid (0.85%) was added slowly, with continuous swirling, to each sample, followed by 10ml of a 10% ammonium molybdate solution. The flasks were filled to the mark with DDD water and allowed to stand at room temperature for 30 minutes. The yellow color which developed was compared with a blank containing no phosphorus. The optical density of each sample at 410nm was measured on a Coleman Model 130 Perkin-Elmer Spectrophotometer (Arthur H. Thomas Co., Philadelphia, Pa.). The amount of phosphorus was determined from a linear plot of concentration of phosphorus versus O.D. at 410, using a standard KH₂PO₄ solution. The fractions containing PGP (organic phosphorus) were pooled and dried in a vacuum over P₂O₅.

Passive Hemagglutination Anti-teichoic acid antibodies were detected by the method of passive hemagglutination (PHA). A 5% suspension of erythrocytes from the appropriate species, thrice-washed in PBS, pH 7.3, was mixed with an optimal concentration of glycerol-teichoic acid (250μg AP/ml or 1μg PCE/ml as determined by a checkerboard titration) and incubated at 35°C for 30 minutes. The modified cells were washed three times with cold PBS and resuspended to a 1% concentration in the same buffer. The modified cells were used immediately. Five-hundredths milliliter of the cell suspension was mixed with 0.05ml of the appropriate dilution of serum or serum fraction to be tested and the cell-serum mixtures incubated at 4°C for 30 minutes. The mixtures were centrifuged for 15 seconds at 2500 x g and the patterns of agglutination read microscopically and scored as follows:

4+ a single clump of cells
3+ a few large clumps of cells
2+ many small clumps of cells
1+ many small clumps and a few free cells
0 no agglutination

As a control for erythrocyte modification, a standard rabbit antiserum, prepared as described below, was tested in parallel. If the titer of this antiserum agreed with the maximum titer determined during the checkerboard titration, the cells were considered to be optimally modified.

Antisera  Standard anti-teichoic acid antisera were prepared by injecting 500mg of AP in 1ml PBS, pH 7.3, mixed with an equal volume of Freund's Complete Adjuvant (Difco Laboratories, Ann Arbor, Mich.), into the hind footpads of 3.5kg rabbits. On day 14 after injection, the animals were bled by cardiac puncture. After the clot had formed and retracted, the serum was removed, clarified by centrifugation and stored at -20 C. Selected sera were absorbed with equal volumes of the appropriate packed erythrocytes prior to use as standard antisera.

Antisera used in immunodiffusion, immunoelectrophoresis or radial immunodiffusion were commercial antisera or were prepared as follows. Rabbit anti-guinea pig whole serum was prepared by injecting 1ml of fresh guinea pig whole serum, mixed with an equal volume of Freund's Complete Adjuvant, into the hind footpads and intradermally over the thigh muscles of a 3.5kg rabbit. Seven to fourteen days after the injections, the serum was collected as described above and tested for activity against guinea pig whole serum by immunoelectrophoresis. Rabbit anti-guinea pig IgM was prepared in the same manner using purified guinea pig IgM as the immunogen. A 2ml aliquot of fresh
guinea pig whole serum was fractionated on Sephadex G-200 in PBS, pH 7.3. The first UV absorbing peak (FI) was concentrated to 2ml using a DIAFLO Ultrafiltration Apparatus (Amicon Corp., Lexington, Mass.) with an XM100A membrane. Agar-gel electrophoresis of FI was carried out in 0.5% Special Agar-Noble (Difco Laboratories, Ann Arbor, Mich.) using barbital buffer, pH 8.6, µ = 0.1. An agar slab, 1cm x 4cm x 30cm, was poured into a plastic mold. A well, 1cm x 3cm, was cut in the center of the slab and filled with FI. Paper wicks, placed at each end of the agar, were immersed in tanks of barbital buffer and the sample was electrophoresed at 200 volts for 4 hours on an LKB Electrophoresis Apparatus (Gelman Instrument Co., Ann Arbor, Mich.). The purified IgM was recovered from the cathodic portion of the slab by freezing that portion (in glass tubes) in dry ice and methanol for 30 minutes, followed by thawing overnight at room temperature. The gel was filtered 3 times through glass wool and the clarified liquid portion concentrated to 0.5ml by ultrafiltration. The purity of this preparation was tested by immunoelectrophoresis versus rabbit anti-guinea pig whole serum.

Rabbit anti-guinea pig \(\gamma_1\)G, anti-\(\gamma_2\)G and anti-IgG were prepared as above using \(\gamma_1\)G alone, \(\gamma_2\)G alone and a mixture of \(\gamma_1\)G and \(\gamma_2\)G, respectively, as the immunogens. Fresh guinea pig whole serum was fractionated on Sephadex G-200 as described above. The second UV absorbing peak (FIT) was concentrated by ultrafiltration and fractionated by ion-exchange chromatography on Sephadex DEAE-A50 (Pharmacia Fine Chemicals, Piscataway, N.J.) using tris buffer, pH 8.0, in stepwise increasing molarities (0.01, 0.05, 0.1, 0.3) to elute the
proteins. The first UV absorbing peak was \( \gamma_2 \)G, the second UV absorbing peak was \( \gamma_1 \)G. These preparations were tested for purity as described above.

Rabbit anti-IgM was absorbed with Sephadex FII and the anodic portion of agar-gel electrophoresis of Sephadex FI to render it monospecific. Anti-\( \gamma_1 \)G was absorbed with \( \gamma_2 \)G. Anti-\( \gamma_2 \)G, when absorbed with \( \gamma_1 \)G, no longer gave any line of precipitation with \( \gamma_2 \)G or with whole serum. The specificities of these antisera were checked by immunoelectrophoresis and immunodiffusion versus guinea pig whole serum, IgM, IgG, \( \gamma_1 \)G, \( \gamma_2 \)G, Sephadex FI and Sephadex FII.

**Immunoelectrophoresis**  Immunoelectrophoresis was carried out on an LKB Immunoelectrophoresis Apparatus using 1% Special Agar-Noble in barbital buffer, pH 8.6, \( \mu = 0.1 \). Slides were poured to a depth of 2-3mm. Patterns were cut using an LKB Immunoelectrophoresis Punch Kit (Gelman Instrument Co., Ann Arbor, Mich.) and the wells filled with 10\( \mu \)l of the sample. Electrophoresis was carried out at 250 volts for 1 hour. At the end of the run the troughs in the agar were filled with the appropriate antiserum and the slides placed in a humid chamber for 2 hours at room temperature. Lines of precipitation were viewed with the naked eye.

**Immunodiffusion**  Immunodiffusion was carried out on microscope slides covered with 1\( \mu \)l of 1% Special Agar-Noble in barbital buffer, pH 8.6, \( \mu = 0.1 \). Patterns were cut in the agar using an LKB Immunodiffusion Punch Kit (Gelman Instrument Co., Ann Arbor, Mich.) and the wells filled with the appropriate antigen(s) and antiserum(a). The slides were placed in a humid chamber at room temperature and checked for
lines of precipitation at 24, 48 and 120 hours. Tests were considered negative if no precipitation could be noted during the 5 day incubation period.

**Distribution of Anti-teichoic Acid Antibodies in Normal Sera**

Normal sera from 112 adult humans, 12 adult rabbits, 111 adult rats, 108 adult mice and 127 adult guinea pigs were tested for anti-teichoic acid antibodies by PHA using the appropriate erythrocytes modified with purified teichoic acid.

**Radial Immunodiffusion**

Levels of IgM and IgG classes of immunoglobulins in normal guinea pig sera were determined by radial immunodiffusion (RID) using rabbit anti-guinea pig IgM and anti-IgG. Microscope slides were uniformly covered with exactly 1ml of 1% Special Agar-Noble in barbital buffer, pH 8.6, μ = 0.1, containing 5% of the appropriate antiserum. Patterns were cut in the agar using an LKB Immunodiffusion Punch Kit. The wells were filled with exactly 10μl of the serum to be tested and the slides placed in a humid chamber at room temperature for exactly 24 hours. The RID patterns were photographed with a Cordis Immunodiffusion Camera (Cordis Instruments, Miami, Florida). The diameters of the patterns were measured from the photographs using calipers.

**Characterization of Anti-teichoic Acid Antibodies**

Antibodies present in randomly selected normal sera from humans, rabbits and rats, normal sera from consecutive weekly bleedings of conventional and germfree guinea pigs, and immune sera from rabbits and conventional and germfree guinea pigs were characterized. The methods employed were Sephadex fractionation, PHA, 2-mercaptoethanol (2-me) susceptibility,
immunoelectrophoresis and immunodiffusion. Each whole serum was fractionated on Sephadex G-200 as described above. Sephadex FI and FII were tested for anti-teichoic acid antibodies by PHA. Susceptibility of the antibodies in either fraction to reduction was determined by 2-me treatment. Equal volumes of the fraction in PBS, pH 7.3, and of a 1:4 dilution of 2-me (K & K Laboratories, Plainview, N.Y.) in PBS, pH 7.3 were mixed and incubated at 37 C for 30 minutes. The mixtures were concentrated to near dryness by ultrafiltration, washed three times with PBS to remove the 2-me and diluted to the original fraction volume with PBS, pH 7.3. These 2-me treated serum fractions were tested by PHA in parallel with the untreated fractions. Complete and incomplete anti-Rh0(D) antisera (Dade Reagents, Inc., Miami, Florida) were treated by the same method as a control.

Further fractionation of guinea pig FII was carried out by ion-exchange chromatography as described above. The first and second ion-exchange peaks, $\gamma_2$G and $\gamma_1$G respectively, and the pooled remaining peaks (containing IgA), were tested for the presence of anti-teichoic acid antibodies by PHA.

The proteins in each whole serum and corresponding fractions were identified by immunoelectrophoresis as described above. Antisera used were: goat anti-human whole serum, goat anti-human IgM, goat anti-human IgG (Hyland Laboratories, Los Angeles, Calif.); goat anti-rabbit whole serum, goat anti-rabbit IgM, goat anti-rabbit IgG (Miles Laboratories, Elkhart, Indiana); and rabbit anti-guinea pig whole serum, rabbit anti-guinea pig IgM, rabbit anti-guinea pig IgG, rabbit anti-guinea pig $\gamma_1$G, prepared as described above.
To confirm the identity of the anti-teichoic acid antibodies in guinea pig, human and rabbit sera, the appropriate monospecific antiserum was used to precipitate all of the antibodies of the desired immunoglobulin class from the serum fractions tested. Equal volumes of the serum fraction and of an optimal concentration of the monospecific antiserum were mixed and incubated at 37°C for 30 minutes, followed by incubation overnight at 4°C. The absorbed serum fraction was then tested for the presence of any remaining anti-teichoic acid antibodies.

**Determination of Natural Cell-mediated Immunity to Glycerol-teichoic Acid in Guinea Pigs**

Forty-two conventional guinea pigs, ranging in age from 7 to greater than 40 weeks, were each tested for naturally-occurring cell-mediated immunity to teichoic acid by one of two methods: a delayed-type hypersensitive reaction to intradermal injection of the antigen (skin test) or by the inhibition of macrophage migration.

Eighteen guinea pigs were skin tested in the right flank by the intradermal injection of 50μg of purified teichoic acid in 0.05ml PBS, pH 7.3. As a control, intradermal injections of PBS alone were made in the left flank. The sites of injection were inspected at \( \frac{1}{2} \) hour intervals up to 4 hours post-injection and then again at 8, 12 and 24 hours. Indurated areas were excised and vertically sliced through the skin layers. Impression smears were made, stained with Wright's stain and examined microscopically for mononuclear cell infiltration. Skin tests were considered positive if the diameter of induration at 24 hours was greater than 5mm and there was evidence of considerable mononuclear cell infiltration.

The remaining 24 animals were each injected intraperitoneally
with 30ml of thioglycollate. Forty-eight hours later each animal was injected intraperitoneally with 20ml Hank's Balanced Salt Solution (HBSS) containing 5 units of heparin/ml. The animals were then sacrificed and the peritoneal exudate (PE) removed aseptically. The cells from each animal were treated with 0.83% ammonium chloride to lyse erythrocytes, washed three times with HBSS and resuspended to a concentration of 1 x 10^7 cells/ml. Capillary tubes (1.4mm O.D.) were filled with the suspensions, plugged with Seal-Ease (Clay-Adams, Inc., New York, N.Y.) and centrifuged for 5 minutes at 1000 x g. The tubes were then scored and broken below the cell-fluid interface at identical heights above the Seal-Ease. For each animal, seven tubes were placed in each of two migration chambers. The chambers were constructed from two glass slides separated by capillary tubes (1.6mm O.D.) and sealed with silicone glue as described by Scheetz et al. (1972). The tubes containing the packed cells were inserted into the chamber through holes molded in the silicone and the chambers were sealed with paraffin. One chamber was filled with HBSS containing 50µg purified teichoic acid/ml and the other with HBSS alone. All chambers were incubated for 24 hours at 37°C in 5% CO₂. Migration patterns were traced on a Nikon Profile Projector Model 6C (Nikon Kogako K.K., Japan). The areas of migration were determined planimetrically. The percentage inhibition of migration was calculated as follows:

\[
% \text{ inhibition} = \left( 100 - \frac{\text{average migration area with antigen}}{\text{average migration area without antigen}} \right) \times 100.
\]

Inhibition of migration greater than 20% was considered positive as suggested by Pick and Turk (1972). PE cell suspensions were checked
for viability by trypan blue dye exclusion. Viability of PE cells incubated in HBSS alone and PE cells incubated in HBSS containing 50μg of purified teichoic acid/ml remained above 85%, indicating that the antigen was not toxic for the cells at this concentration.

Injection of Normal Guinea Pigs with Four Forms of Glycerol-teichoic Acid

To determine the effect of artificial immunization on the cell-mediated and humoral antibody responses, four groups of 11 guinea pigs 11-13 weeks of age, representing an age group not displaying natural cell-mediated immunity to teichoic acid nor IgG anti-teichoic acid antibodies, and four groups of eight adult rats, were each injected intraperitoneally with one of four forms of teichoic acid. A suspension of whole bacilli (Bac) and solutions of acid-precipitated teichoic acid (AP), purified teichoic acid (PCE) and synthetic polyglycerophosphate (PGP) were prepared so that each immunogen had identical amounts of polyglycerophosphate/ml. Twenty-five milligrams of AP was chosen as an arbitrary amount. From five extractions of whole bacillus, it was determined that 62.4 grams Bac (wet weight) yielded 3.33 grams AP. Since the wet weight of Bacillus sp. O.S.U. 372, as prepared for these studies, was approximately $2.1 \times 10^{-11}$ grams/organism (Stamper, H.B., personal communications), an identical dose of Bac was calculated to be $2.23 \times 10^{10}$ organisms. The amount of PGP in 25mg AP was determined by performing a total organic phosphorus assay, described above, subtracting the amount of phosphorus calculated to be in the RNA contaminant and calculating the weight of PGP representing the amount of phosphorus remaining. Total RNA was determined by the orcinol-FeCl₃ method (Williams and Chase, 1968). Two-milliliter samples of AP and varying
amounts of standard RNA (Nutritional Biochemicals Corp., Cleveland, Ohio) were each mixed with 2ml of concentrated HCl containing 0.5ml of a 10% FeCl₃ solution/ml. After addition of 0.2ml of a 10% alcoholic orcinol solution, the mixtures were heated for 3 minutes in boiling water. The color intensities were measured at 610nm and the amount of RNA in the AP was determined from a calibration curve of O.D.₆₁₀ versus micrograms standard RNA. The amount of PCE used for immunization was equal in weight to the calculated weight of polyglycerophosphate in 25mg of AP. Because of the difficulty encountered in weighing, a dosage of PGP was used which contained an amount of organic phosphorus equal to that in the polyglycerophosphate backbone of 25mg of AP.

All animals were bled just prior to injection (day 0) and again on days 4, 7 and 14. On day 14 all of the guinea pigs were injected with 30ml thioglycollate and their PE cells tested for inhibition of macrophage migration as described above except that the cells were aspirated from the peritoneal cavity of living animals under anesthesia using a syringe and a blunt 18 gauge needle. These same animals were then skin tested immediately thereafter. Guinea pig serum fractions FI and FII and whole rat sera were tested for anti-teichoic acid antibodies by PHA.

Equilibrium Dialysis To ascertain if the components of normal sera which reacted with teichoic acid antigen were antibody molecules and not non-specific serum proteins, Sephadex FII serum fractions from four different adult normal guinea pig sera were tested by equilibrium dialysis for their ability to bind radiolabeled PGP, prepared as follows. Two hundred fifty milligrams PGP in PBS, pH 7.3, 1.25μC ¹⁴C-glycine
in DDD water and 50mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (K & K Laboratories, Plainview, N.Y.) in PBS were mixed and allowed to react at room temperature for one hour. Disodium dihydrogen ethylene diaminetetraacetate dihydrate (Smith Chemical Co., Columbus, Ohio) was added to stop the reaction. This mixture was fractionated by upward flow column chromatography on Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N.J.) and the effluent collected in consecutive ml samples. One-tenth milliliter from each was assayed for organic phosphorus as described above. One-tenth milliliter of each sample was added to separate liquid scintillation vials containing 9.9ml Scintisol-Complete (Isolab, Inc., Akron, Ohio). The vials were monitored for radioactivity on a Packard Tricarb Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) at 5.7% gain with discriminators set at 50 and 1000. The effluent peaks of organic phosphorus and radioactivity corresponded, which gave preliminary indication that the PGP was successfully radiolabeled. For confirmation, thin-layer chromatography of the tagged PGP was carried out in three solvent systems: propanol-NH$_4$OH-H$_2$O 6:3:1 (v/v/v); propanol-acetic acid-H$_2$O 6:1:3 (v/v/v); and butanol-acetic acid-H$_2$O 4:1:5 (v/v/v), organic phase. Two 25 lambda samples each of PGP-1-$^{14}$C-glycine, PGP alone and 1-$^{14}$C-glycine alone were spotted separately on each of three strips of Gelman Chromatography Media I.T.I.C. type 3G (Gelman Instrument Co., Ann Arbor, Mich.). The strips were placed in three tanks, each saturated with one of the solvent systems, and chromatographed until the solvent fronts reached 15cm. The strips were removed, dried in a hot air oven at 105°C and cut in half. One
half was sprayed with a sulphuric acid-potassium permanganate solution (0.47gm KMnO$_4$, 10ml H$_2$O, 10ml H$_2$SO$_4$) to detect polyols. The other half was scanned for radioactivity using a Baird-Atomic Thin-Layer Radio-chromatogram Scanner Model RSC-363 (Baird-Atomic Instruments Co., Cambridge, Mass.). In all three solvent systems, the PGP spot detected by the spray had an $R_f$ value equal to that of the radioactive peak, confirming that the polymer was labeled.

The average chain length of the PGP-1-$^{14}$C-glycine was defined as the ratio of the moles of total phosphorus in a carefully measured sample of the tagged polymer, to the moles of formaldehyde released from an equal volume of sample by periodate oxidation. Two milliliters of 0.1M sodium periodate was added to each of two 100ml volumetric flasks. An aliquot of the PGP-1-$^{14}$C-glycine was added to one flask and an equal volume of DDD water to the other. Both sample and blank were diluted to 20ml with DDD water and allowed to react at room temperature for one hour. Two milliliters of 0.1M sodium sulfite were pipetted into each flask. The samples were diluted to 100ml with DDD water and 10ml aliquots of each sample transferred to clean 50ml volumetric flasks. Five-hundreths gram of sodium 1,8-di-hydroxynaphthalene-3,5-disulfonate (Eastman Organic Chemicals, Rochester, N.Y.) was added to each flask. The flasks were filled to the mark with 95% sulphuric acid and the normal heat rise allowed to occur. When the contents had cooled, the absorbance of the sample versus the blank was measured at 570nm. The concentration of 1,2-glycol (PGP) was read from a calibration curve prepared using Na$_2$,$\beta$-glycerophosphate as the standard.
The equations representing the interaction of this polymer and specific antibody at equilibrium were derived as follows. The quantitative relationship of the interaction between PGP-1-$^{12}$C-glycine and anti-teichoic acid antibody at equilibrium is represented by the equation:

$$\frac{ka}{kd} = \frac{Ab + Ag}{AbAg}$$

where Ab = free antibody

Ag = free antigen

AbAg = antibody-antigen complex

From the law of mass action, the affinity of the antibody is represented by the equilibrium constant K:

$$K = \frac{ka}{kd} = \frac{(AbAg)}{(Ag)(Ab)}$$

K was determined by measurement of antibody-bound antigen (AbAg) and free antigen (Ag) at equilibrium at several antigen concentrations using the following calculations:

If $c = \text{free concentration of hapten at equilibrium and}$

$r = \text{the ratio of the number of moles of hapten bound per mole of antibody at equilibrium,}$

then $\frac{r}{c} = nK - rK$

where $n = \text{the number of antibody combining sites.}$

The data is plotted as $\frac{r}{c}$ vs. $r$. The average association constant ($K_0$) is taken as equal to the reciprocal of the concentration $c$ at $r = 1$ ($\frac{1}{2}$ of the antibody combining sites occupied for IgG), and is obtained by reading from the graph the value of $\frac{r}{c}$ at $r = 1$.

The calculation of $K$ from this equation required a knowledge
of total antibody concentration (Ab). Attempts by this author to purify anti-teichoic acid antibody met with little success. Precipitation of anti-teichoic acid from normal serum failed, in most cases, due to the low levels of this antibody in normal sera. Certain high-titered normal sera contained precipitating antibody, but measurement of the affinity of this precipitin would not have been representative of most of the normal anti-teichoic acid antibodies. Specific absorption and subsequent purification of the antibody from normal sera using modified erythrocytes as the immunoadsorbent was also unsuccessful. Attempts to elute the antibody from the antigen, or the antibody-antigen complex from the red cells, by an increase in temperature or a decrease in pH or ionic strength, caused extensive hemolysis of the red cells. The resulting hemoglobin was so concentrated that it could not be removed by ultrafiltration or column chromatography.

The amount of anti-teichoic acid antibody in FII of normal guinea pig serum was calculated by determining the total nitrogen (N) in a 0.5ml aliquot of the fraction, and subtracting from this figure the total N remaining after absorption using PCE-modified guinea pig erythrocytes as the immunoadsorbent. Mixtures of aliquots of FII and equal volumes of a 20% suspension of modified red cells were incubated at 4°C for 3/4 hour with frequent agitation. The mixtures were centrifuged, the supernates removed and concentrated by ultrafiltration to the original volume of the serum fraction. As a control for nonspecific absorption, an equal volume of the fraction was incubated with a 20% suspension of unmodified guinea pig erythrocytes. Total N in
the untreated and absorbed fraction samples was determined by the Kjeldahl nitrogen assay (Kabat and Mayer, 1964). Two-tenths milliliter aliquots were placed in separate Kjeldahl boiling flasks. Two milliliters of a concentrated sulphuric acid solution saturated with CuSO₄, one gram of K₂SO₄ and a glass bead were added to each flask. The contents of each flask were boiled until the solutions were clear. The flasks were cooled to room temperature, placed in ice and the contents diluted with 10ml DDD water. The samples were mixed with 9ml saturated NaOH and the resulting NH₃ steam distilled in a Kjeldahl distillation apparatus constructed from common laboratory glassware following the description of Kabat and Mayer (1964). The distillates were titrated with exactly N/70 N HCl using a boric acid-methyl red indicator. The end point was reached when the sample had the same color as a blank. Total N was calculated to be:

\[ N = ml \text{ of } N/70 \times 0.2 \]

To determine the amount of free and bound antigen, four 0.3ml aliquots of varying concentrations of PGP-1-¹⁴C-glycine were placed separately in one compartment of each of 4 dialysis chambers. Three-tenths milliliter aliquots of the appropriate serum fraction were placed in the other compartment of each chamber, the compartments being separated by Visking Dialysis Tubing (Union Carbide Corp., Chicago, Ill.). The chambers were sealed and gently rocked for 24 hours in the cold. The solutions from each compartment were quantitatively transferred to liquid scintillation vials containing 9.7ml Scintisol-Complete and counted for radioactivity. Moles of bound PGP-1-¹⁴C-glycine were calculated by subtracting the average counts per minute (cpm) in the
compartment not containing the serum fraction (free PGP-1-\textsuperscript{14}C-glycine) from the average cpm in the compartment containing the serum fraction, and converting cpm to moles of PGP-1-\textsuperscript{14}C-glycine. To ensure that the polymer reached equilibrium in 24 hours, a chamber was run in which PBS, pH 7.3 was substituted for the serum fraction. Non-specific binding of the polymer to globular proteins was tested for by substitution of an absorbed guinea pig serum fraction for the test fraction.

**Macrophage Cytophilic Antibodies** Normal guinea pig \( \gamma_G \) and \( \gamma_2G \) and Sephadex FI from normal guinea pig serum were tested for the presence of macrophage cytophilic antibodies with teichoic acid specificity by the macrophage monolayer technique of Boyden (1964). Normal guinea pig PE cells were collected as described above, washed three times in HBSS and placed on sterile coverslips. The coverslips were incubated for 3 hours at 37 C in 5% CO\textsubscript{2}. The medium was drained and the adherent cells (macrophages) washed once with HBSS. The coverslips were flooded with the appropriate serum fraction in PBS, pH 7.3 and incubated for one hour at 37 C in 5% CO\textsubscript{2}. As a control, one coverslip was covered with a serum fraction absorbed with guinea pig erythrocytes modified with purified teichoic acid. The sensitized macrophages were washed twice with PBS and covered with a 1% suspension of modified guinea pig erythrocytes. The macrophage-RBC coverslips were incubated for 30 minutes at 4 C, rinsed twice with cold PBS, pH 7.3 and flooded with cold PBS. The cells were then examined by phase-contrast microscopy for red cell coated macrophages.

**Bactericidal Activity of Normal Guinea Pig Anti-teichoic Acid Antibodies** Sepahdex G-200 FI and FII of normal guinea pig serum were tested for
bactericidal activity against _Bacillus_ sp. O.S.U. 372. One-tenth milliliter of a bacterial suspension (1.5 x 10^5 bacilli/ml) was mixed with 0.5ml of the appropriate sterile serum fraction and 0.3ml of complement. Duplicate mixtures were made using serum fractions which had been absorbed with modified guinea pig erythrocytes, rendering them PHA negative. Three control mixtures which substituted saline for the bacillus, the serum fraction and the complement were included. All mixtures were incubated at 37 C for 1 hour. Five-tenths milliliter of each mixture was placed in separate tubes containing 10ml of melted and cooled Trypticase Soy Agar (Baltimore Biological Laboratory, Baltimore, Md.). The tubes were thoroughly mixed and plated out. All tests were performed in triplicate. The plates were incubated at 37 C for 24 hours and the number of resultant colonies were counted.
RESULTS

Characterization and Specificity of Immunochemical Reagents  Stepwise purification of IgM by column chromatography and agar-gel electrophoresis resulted in a preparation which gave only one precipitating arc when tested by immuneelectrophoresis (IE) versus rabbit anti-guinea pig whole serum (Plate Ia), and had anti-teichoic acid antibody activity when tested by passive hemagglutination. Immunization of rabbits with this preparation produced strongly precipitating antiserum within one week post-injection. Cross-reactions with serum proteins in Sephadex G-200 FII were noted (Plate Ib), probably due to anti-light chain antibodies. Absorption of the multispecific "anti-IgM" with FII removed these specificities. A cross-reaction was noted with an alpha globulin, present in FI (Plate Ic), due either to contamination with this component in the original IgM preparation or to a true cross-reaction with an alpha_2-macroglobulin. This specificity was completely absorbed with the anodic portion of Sephadex FI separated by agar-gel electrophoresis. Mixing of the resulting monospecific anti-IgM with Sephadex FI removed, by precipitation, all of the anti-teichoic acid antibody activity from that fraction, confirming the specificity of the reagent. Tests by IE against guinea pig whole serum resulted in a single arc identical to the original IgM preparation (Plate Id).

Purification of 
\[ \gamma_1 G \] and 
\[ \gamma_2 G \] by column chromatography on Sephadex G-200 followed by ion-exchange chromatography resulted in pure
Plate I. Immuneelectrophoresis of purified guinea pig IgM and various rabbit anti-guinea pig IgM antisera.
(a) purified guinea pig IgM vs. rabbit anti-guinea pig whole serum (arrow indicates IgM arc)
(b) Sephadex FII of guinea pig serum vs. unabsorbed rabbit anti-guinea pig IgM
(c) Sephadex FI of guinea pig serum vs. rabbit anti-guinea pig IgM absorbed with Sephadex FII of guinea pig serum (arrow indicates IgM arc)
(d) guinea pig whole serum vs. monospecific rabbit anti-guinea pig IgM (arrow indicates IgM arc)
immunoglobulin preparations as shown by IE versus rabbit anti-guinea pig whole serum (Plate II). Immunization of rabbits with \( \gamma_1 \)G produced antiserum which reacted with both \( \gamma_1 \)G and \( \gamma_2 \)G as shown by immunodiffusion (Plate IIa). Similar results were obtained with \( \gamma_2 \)G. No reactions with IgM or IgA could be detected by IE. Absorption of "anti-\( \gamma_1 \)G" with \( \gamma_2 \)G removed the cross-reacting antibodies (Plate IIa). However, absorption of "anti-\( \gamma_2 \)G" with \( \gamma_1 \)G removed all precipitating antibodies (Plate IIa).

Immunization of one rabbit with both \( \gamma_1 \)G and \( \gamma_2 \)G produced antiserum reacting with both serum proteins. This reagent gave IE patterns (Plate IIe) identical with commercial rabbit anti-guinea pig IgG.

Immunization of rabbits with fresh guinea pig whole serum produced anti-whole serum which reacted with all known guinea pig serum proteins detectable by IE. IE patterns with this reagent (Figure 1) were identical to those obtained with commercial rabbit anti-guinea pig whole serum.

**Distribution of Normal Anti-teichoic Acid Antibodies**

One-hundred percent of normal adult human, guinea pig and rabbit sera tested contained passive hemagglutinating antibodies specific for glycerol-teichoic acid (Table 1). Fifty-nine percent of all rat sera tested were positive and the percentage appeared to vary with strain (Table 1). No specific antibodies could be found in any of the germfree or conventional mouse sera tested (Table 1).

**Characterization of Anti-teichoic Acid Antibodies**

All adult normal human sera, adult normal guinea pig sera and immune guinea pig sera
Plate II. Immunodiffusion and immunoelectrophoresis of guinea pig $\gamma^1G$ and $\gamma^2G$, unabsorbed rabbit anti-guinea pig $\gamma^1G$ and absorbed rabbit anti-guinea pig $\gamma^1G$ and anti-$\gamma^2G$.

(a) unabsorbed rabbit anti-guinea pig $\gamma^1G$ (A1), absorbed rabbit anti-guinea pig $\gamma^1G$ (As1) and absorbed rabbit anti-guinea pig $\gamma^2G$ (As2) vs. purified guinea pig $\gamma^1G$ (1) and $\gamma^2G$ (2) (arrow indicates arc of reaction between $\gamma^2G$ and unabsorbed anti-$\gamma^1G$ which was later removed by absorption of anti-$\gamma^1G$ with $\gamma^2G$)

(b) guinea pig $\gamma^1G$ and $\gamma^2G$ (1-2) vs. unabsorbed rabbit anti-guinea pig $\gamma^1G$

(c) purified guinea pig $\gamma^2G$ vs. rabbit anti-guinea pig whole serum

(d) purified guinea pig $\gamma^1G$ vs. rabbit anti-guinea pig whole serum

(e) guinea pig whole serum (ws) vs. rabbit anti-guinea pig IgG
Figure 1. Immunoelectrophoresis of two guinea pig whole sera vs. rabbit anti-guinea pig whole serum prepared by the author (top) and commercial rabbit anti-guinea pig whole serum (bottom).
### TABLE 1

Distribution of normal anti-teichoic acid antibodies in adult sera of selected mammalian species

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of individuals tested</th>
<th>Percent positive for anti-teichoic acid antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>112</td>
<td>100</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>127</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Rat (total)</td>
<td>111</td>
<td>59</td>
</tr>
<tr>
<td>aWistar</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>Lewis</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
<td>Charles River</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Mouse</td>
<td>108</td>
<td>0</td>
</tr>
</tbody>
</table>

aWistar, Lewis and Charles River inbred rat strains tested

from animals 14-16 weeks old and from adults, when tested by PHA had anti-teichoic acid antibodies in both the 19S (IgM-containing), 2-mercaptoethanol sensitive and the 7S (IgG-containing), 2-mercaptoethanol resistant Sephadex G-200 fractions (Table 2). The PHA activity of the guinea pig 7S fraction was restricted to the $\gamma_1G$ subclass. No PHA activity could be detected in the guinea pig IgA-containing serum fractions obtained by ion-exchange chromatography. PHA activity was found in both the 19S and 7S fractions of some normal adult rat sera (Table 2). Other rat sera had PHA antibodies in the 19S fraction only. All PHA antibodies of normal adult rabbit sera were restricted to the 19S fraction (Table 2). All immune rabbit sera had anti-teichoic acid antibodies in both fractions. The identity of the immunoglobulin class
TABLE 2

Characterization of anti-teichoic acid antibodies in adult sera of selected mammalian species

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of individuals tested</th>
<th>Anti-teichoic acid antibodies present in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>Human</td>
<td>12</td>
<td>+(12)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>17</td>
<td>+(17)</td>
</tr>
<tr>
<td>immune</td>
<td>8</td>
<td>+(8)</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>12</td>
<td>+(12)</td>
</tr>
<tr>
<td>immune</td>
<td>6</td>
<td>+(6)</td>
</tr>
<tr>
<td>Rat</td>
<td>11</td>
<td>+(11)</td>
</tr>
</tbody>
</table>

\( ^a \)Number in parentheses indicates number positive
\( ^b \)NT—not tested
\( ^c \)neg—all animals tested were negative for antibodies in the fraction indicated

Of antibody present in human, guinea pig and rabbit serum fractions was confirmed by precipitation of all anti-teichoic acid antibody activity from these fractions with the appropriate antiserum.

Ontogeny of Natural Anti-teichoic Acid Antibodies in Germfree and Conventional Guinea Pigs. Forty-two conventional and 48 germfree guinea pigs were studied from birth. Individual sera from consecutive weekly bleedings, tested by radial immunodiffusion using monospecific antisera (Plate III), had levels of IgM and IgG classes of immunoglobulins as illustrated in Figure 2. Throughout the early life of both groups, comparable levels of IgM were present. The levels of IgG during the early
Plate III. Radial immunodiffusion patterns of guinea pig whole serum vs. monospecific rabbit anti-guinea pig IgM and IgG.

(a) whole serum from a conventional guinea pig at 8 weeks of age vs. monospecific rabbit anti-guinea pig IgG

(b) whole serum from a germfree guinea pig at 8 weeks of age vs. monospecific rabbit anti-guinea pig IgG

(c) whole serum from a conventional guinea pig at 8 weeks of age vs. monospecific rabbit anti-guinea pig IgM

(d) whole serum from a germfree guinea pig at 8 weeks of age vs. monospecific rabbit anti-guinea pig IgM
Figure 2. Relative levels of total IgG and IgM in germfree (□——□) and conventional (●——●) whole sera. Average diameters of radial immunodiffusion patterns of whole sera tested with monospecific rabbit anti-guinea pig IgG and anti-IgM.
life of the germfree animals dropped considerably but began to rise slowly at 8 weeks of age. No tests were carried out after 13 weeks of age in either group.

Consecutive sera from both groups were fractionated and tested for anti-teichoic acid antibodies by PHA. The resultant titers were averaged and plotted as shown in Figure 3. Both IgM and IgG antibodies of this specificity were present in low levels at birth and the average titers of both classes dropped gradually for the first four weeks. The IgM antibody titer then rose in both groups, the average rise in the germfree animals was more rapid, reached a higher titer, and remained surprisingly constant thereafter. The average rise in titer of the conventional group occurred more slowly, but eventually reached the same level. No IgG anti-teichoic acid antibodies could be detected in the sera of any of the young animals of either group after 6 weeks of age. In conventional animals, natural IgG anti-teichoic acid antibodies appeared at 20-21 weeks of age. Since the normal germfree animals could not be maintained longer than 16 weeks, no data was available on natural IgG antibodies in this group.

Ontogeny of Natural Cell-mediated Immunity to Glycerol-teichoic Acid in Guinea Pigs Although not enough animals were tested to provide a continuum, it appeared that those animals 19 weeks of age or younger did not display a natural cell-mediated immunity to glycerol-teichoic acid and all animals 26 weeks of age or older reacted positively, either by a delayed skin test or by the inhibition of macrophage migration (Table 3). Of the cells which infiltrated the positive skin test sites, 78% were monocytes and macrophages, 14% were small lymphocytes and 8% were
Figure 3. Average titers of specific anti-teichoic acid antibodies in IgM (Germfree■——■, Conventional○——○) and IgG (Germfree□——□, Conventional●——●) classes of immunoglobulins as measured by passive hemagglutination.
TABLE 3

Cell-mediated immunity to glycerol-teichoic acid in normal guinea pigs as demonstrated by delayed skin reactions or by the inhibition of macrophage migration

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Age (weeks)</th>
<th>Skin Test (mm induration)</th>
<th>Migration Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1449</td>
<td>6</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>7</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1427</td>
<td>7</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>5b</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1428</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1676</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1679</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1682</td>
<td>8</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1426</td>
<td>9</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1437</td>
<td>9</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1550</td>
<td>9</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1558</td>
<td>9</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1570</td>
<td>9</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1658</td>
<td>10</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1681</td>
<td>10</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1693</td>
<td>10</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1689</td>
<td>15</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1663</td>
<td>16</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>18</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1660</td>
<td>18</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>19</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>19</td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>1c</td>
<td>26</td>
<td>pos(6)</td>
<td>pos(29)</td>
</tr>
<tr>
<td>1696</td>
<td>26</td>
<td>pos(6)</td>
<td></td>
</tr>
<tr>
<td>1284</td>
<td>29</td>
<td>pos(8)</td>
<td></td>
</tr>
<tr>
<td>1695</td>
<td>29</td>
<td>pos(10)</td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>&gt;40</td>
<td>pos(58)</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>&gt;40</td>
<td>pos(33)</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>&gt;40</td>
<td>pos(16)</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>&gt;40</td>
<td>pos(14)</td>
<td></td>
</tr>
<tr>
<td>562</td>
<td>&gt;40</td>
<td>pos(29)</td>
<td></td>
</tr>
<tr>
<td>1023</td>
<td>&gt;40</td>
<td>pos(39)</td>
<td></td>
</tr>
<tr>
<td>1251</td>
<td>&gt;40</td>
<td>pos(49)</td>
<td></td>
</tr>
<tr>
<td>1263</td>
<td>&gt;40</td>
<td>pos(49)</td>
<td></td>
</tr>
<tr>
<td>1271</td>
<td>&gt;40</td>
<td>pos(49)</td>
<td></td>
</tr>
<tr>
<td>1275</td>
<td>&gt;40</td>
<td>pos(49)</td>
<td></td>
</tr>
<tr>
<td>1568</td>
<td>&gt;40</td>
<td>pos(42)</td>
<td></td>
</tr>
<tr>
<td>1887</td>
<td>&gt;40</td>
<td>pos(42)</td>
<td></td>
</tr>
</tbody>
</table>
cells of other types. No signs of any immediate-type reactions could be detected during the first 4 hours, nor at 8 or 12 hours after the test injection. The diameters of induration at the skin test sites of the animals in the 26-29 week old age group were less than the diameters at the test sites of the animals older than 40 weeks of age. Percent inhibition of migration of peritoneal exudate cells from positive donors ranged from 29% to 58%. No correlation of percent inhibition with the age of the animals could be determined.

Effects of Artificial Immunization on the Humoral and Cell-mediated Responses to Teichoic Acid  The average \( \log_2 \) of the whole serum titers, determined by arithmetic dilution, of the four groups of guinea pigs on day 0 and on days 4, 7 and 14 post-injection are presented in Figure 4. Synthetic PGP did not stimulate an increase in anti-teichoic acid antibody titer. Immunization with AP and PCE produced an apparent slight increase in antibody titer from 4 to 11 and 6 to 15 respectively. These increases in titers were transient, however, the level of anti-teichoic acid antibody dropping by day 7, with no significant change in titer by day 14 post-injection. The average increase in titer of normal, uninjected animals during the same period (11-13 weeks) rose from 2 to 8 (Figure 3). Injection of whole bacillus resulted in only a slight increase in titer (6 to 10) by day 4 post-injection. This increase continued, however, to 16.5 by day 7 and 32 by day 14 post-injection. The slope of the increase in the Bac group from day 4 to 14 (1.1) was not significantly different from the slopes of the lines representing the brief increases produced by injection with AP (0.88) and PCE (1.1). These slopes produced by injection were significantly
Figure 1. Average whole serum titers of four groups of guinea pigs on days 0, 4, 7 and 14 after intraperitoneal injection with $2.2 \times 10^{10}$ whole bacilli (□——□), 25 mg acid-precipitated teichoic acid (●——●), 180 µg purified teichoic acid (●——●) and 180 µg synthetic polyglycerophosphate (□——□).
greater than the slope of the increase in the average antibody titer of the normal, uninjected animals (0.21), but were still approximately one-half the slope of the increase in the germfree animals during the 7th week (2.3). Like both of the uninjected groups (germfree and conventional), all of the anti-teichoic acid antibodies in the sera of the injected guinea pigs, 11-13 weeks of age, were present only in the Sephadex G-200 F1 and were completely absorbed by anti-IgM.

The serum antibody responses of the four groups of rats (Figure 5) were quite different from those of the guinea pigs. Bac and PCE produced very little, if any, increases in antibody titers. AP and POP, on the other hand, produced significant increases in titers. These data are not as reliable as the data obtained from the guinea pig experiment since the preimmunization titers of the four groups of rats varied significantly when tested by the one-way analysis of variance, while the preimmunization titers of the groups of guinea pigs did not. Interspecies comparisons of response to injection may not be valid since, while other parameters (i.e., route, dosage) were identical, the guinea pig and rat populations were not comparable in age.

Skin tests of each guinea pig on day 17 post-injection were negative for the AP, PCE and POP groups and weakly positive (5.2-6.7 mm diameter of induration) for 3 of the 4 animals in the Bac group (Table 4). The 4th member of the Bac group was skin test negative. All 16 animals were negative when tested for the inhibition of macrophage migration on day 16 post-injection. No data was collected on any of these animals after 17 days post-injection.

**Chemistry and Labeling of Synthetic Polyglycerophosphate** When first
Figure 5. Average whole serum titers of four groups of rats on days 0, 4, 7 and 14 after intraperitoneal injection with $2.2 \times 10^{10}$ whole bacillus (□——□), 25mg acid-precipitated teichoic acid (●——●), 180µg purified teichoic acid (●——●) and 180µg synthetic polyglycerophosphate (□——□).
TABLE 4

Skin tests of guinea pigs artificially immunized with various forms of glycerol-teichoic acid

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Age (weeks)</th>
<th>Injected with</th>
<th>Skin Test (mm induration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1449</td>
<td>11</td>
<td>$2.23 \times 10^{10}$ Bac&lt;sup&gt;a&lt;/sup&gt;</td>
<td>neg</td>
</tr>
<tr>
<td>1682</td>
<td>12</td>
<td>$2.23 \times 10^{10}$ Bac</td>
<td>pos(5.2)</td>
</tr>
<tr>
<td>1768</td>
<td>13</td>
<td>$2.23 \times 10^{10}$ Bac</td>
<td>pos(5.3)</td>
</tr>
<tr>
<td>1679</td>
<td>13</td>
<td>$2.23 \times 10^{10}$ Bac</td>
<td>pos(6.7)</td>
</tr>
<tr>
<td>1453</td>
<td>11</td>
<td>25mg AP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>neg</td>
</tr>
<tr>
<td>1934</td>
<td>12</td>
<td>25mg AP</td>
<td>neg</td>
</tr>
<tr>
<td>1475</td>
<td>12</td>
<td>25mg AP</td>
<td>neg</td>
</tr>
<tr>
<td>1462</td>
<td>13</td>
<td>25mg AP</td>
<td>neg</td>
</tr>
<tr>
<td>1461</td>
<td>11</td>
<td>180µg PGP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>neg</td>
</tr>
<tr>
<td>1721</td>
<td>11</td>
<td>180µg PGP</td>
<td>neg</td>
</tr>
<tr>
<td>1703</td>
<td>13</td>
<td>180µg PGP</td>
<td>neg</td>
</tr>
<tr>
<td>1704</td>
<td>13</td>
<td>180µg PGP</td>
<td>neg</td>
</tr>
<tr>
<td>1452</td>
<td>12</td>
<td>180µg PCE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>neg</td>
</tr>
<tr>
<td>1707</td>
<td>12</td>
<td>180µg PCE</td>
<td>neg</td>
</tr>
<tr>
<td>1437</td>
<td>12</td>
<td>180µg PCE</td>
<td>neg</td>
</tr>
<tr>
<td>1459</td>
<td>13</td>
<td>180µg PCE</td>
<td>neg</td>
</tr>
</tbody>
</table>

<sup>a</sup>Bac—whole bacillus
<sup>b</sup>AP—acid-precipitated glycerol-teichoic acid
<sup>c</sup>PGP—synthetic polyglycerophosphate
<sup>d</sup>PCE—purified glycerol-teichoic acid

passed over Dowex 50Wx12 (H form), PGP was highly charged ($R_f = 1$ in organic solvents on thin-layer chromatography) and acidic (pH = 1).

This preparation required neutralization with NaOH or dilution in PBS, pH 7.3 prior to use in serologic reactions. The average chain length of various lots of PGP ranged from 1.8 to 2.56 glycerophosphoric acid units, which represented a molecular weight range from 309 to 440.

When fractionated by column chromatography on Sephadex LH-20 the longest polymeric chain detectable was estimated to be 3.6 glycerophosphoric units. These longer chains were in very small numbers as evidenced by
the small amount of the total phosphorus present in the fractions containing these longer polymers (Figure 6).

When the PGP was coupled to 1-\(^{14}\)C-glycine and chromatographed on LH-20, the effluent peaks of organic phosphorus, radioactivity and PHA-inhibiting activity corresponded (Figure 7). Thin-layer chromatography of the tagged polymer, followed by \(\text{H}_2\text{SO}_4-\text{KMnO}_4\) spray and radio-scanning, indicated that the radioactivity travelled with the PGP.

**Affinity of Natural \(\text{IgG}\) Anti-teichoic Acid Antibodies in Conventional Guinea Pigs**

The average affinity \((K_0)\) of natural \(\text{IgG}\) anti-teichoic acid antibody was determined for 4 different normal adult guinea pig sera. The concentration of \(\text{IgG}\) antibody and the average affinity (determined from plots of \(r/c\) vs. \(r\) (Plate IV)) for each serum is shown in Table 5. There appeared to be no direct correlation between antibody titer as measured by PHA, antibody affinity as determined by equilibrium dialysis and concentration of anti-teichoic acid antibody as measured by the Kjeldahl nitrogen assay. The average affinity for natural \(\text{IgM}\) anti-teichoic acid antibody had not been determined at the time of the writing of this dissertation because of the difficulty encountered during Kjeldahl assay of aliquots of absorbed and unabsorbed Sephadex F1.

**Macrophage Cytphillic Antibodies**

No rosette formation could be detected by microscopic examination of macrophage-modified RBC coverslips using any of the serum fractions tested (\(\text{IgM}, \text{IgG}, \text{IgA}\)).

**Bactericidal Properties of Normal Guinea Pig Anti-teichoic Acid Antibodies**

Of the five normal sera tested, only one demonstrated a significant reduction in the number of living bacteria remaining after
incubation (Table 6). This activity was found in both Sephadex G-200 FI and FII. It was also observed that a similar reduction in the number of viable bacteria occurred when saline was substituted for the complement. The PHA titers of the positive serum fractions were similar to the titers of the negative serum fractions. The age of the positive donor was over two years, while the ages of the negative donors were under one year.
Figure 6. Fractionation of synthetic polyglycerophosphate on Sephadex LH-20. Dots represent the amount of phosphorus in milligrams present in a 0.2ml aliquot of the tube represented. Bars indicate the average chain length of the PGP in that tube.
Figure 7. Fractionation of PGP-1-\textsuperscript{14}C-glycine on Sephadex LH-20.

O—O: Counts per minute; •—•: Milligrams organic phosphorus; bars indicate relative inhibition by an aliquot (4% of volume) of the tagged polymer in the tubes indicated.
Plate IV. Plots of moles of PGP-1-\textsuperscript{14}C-glycine bound per mole of anti-teichoic acid antibody/free concentration of PGP-1-\textsuperscript{14}C-glycine (r/c) vs. moles of PGP-1-\textsuperscript{14}C-glycine bound per mole of anti-teichoic acid antibody (r).

(a) serum no. 1606
(b) serum no. 1622
(c) serum no. 1712
(d) serum no. 1720
### TABLE 5
Concentration and average affinity of $\gamma^G$ anti-teichoic acid antibodies in normal adult guinea pig sera

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Concentration of $\gamma^G$ Anti-teichoic Acid Antibody (M)</th>
<th>Average Affinity ($K_0$, M$^{-1}$)</th>
<th>PHA Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1606</td>
<td>$1.75 \times 10^{-5}$</td>
<td>$1.0 \times 10^5$</td>
<td>2048</td>
</tr>
<tr>
<td>1622</td>
<td>$6.89 \times 10^{-6}$</td>
<td>$7.0 \times 10^5$</td>
<td>64</td>
</tr>
<tr>
<td>1712</td>
<td>$2.42 \times 10^{-6}$</td>
<td>$2.4 \times 10^5$</td>
<td>64</td>
</tr>
<tr>
<td>1720</td>
<td>$8.33 \times 10^{-6}$</td>
<td>$1.3 \times 10^6$</td>
<td>4</td>
</tr>
</tbody>
</table>

### TABLE 6
Bactericidal properties of normal adult guinea pig serum

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Number of living bacteria/mL in suspensions incubated with</th>
<th>Percent reduction of living bacteria by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FT (abs)$^a$</td>
<td>FI</td>
</tr>
<tr>
<td>467</td>
<td>1320</td>
<td>7160</td>
</tr>
<tr>
<td>1712</td>
<td>7310</td>
<td>7420</td>
</tr>
<tr>
<td>1606</td>
<td>7480</td>
<td>7720</td>
</tr>
<tr>
<td>1622</td>
<td>7430</td>
<td>7510</td>
</tr>
<tr>
<td>1720</td>
<td>7230</td>
<td>7420</td>
</tr>
</tbody>
</table>

$^a$abs—absorbed with 20% suspension of modified guinea pig erythrocytes
DISCUSSION

The procedures employed in this work for the purification of guinea pig IgM, $\gamma_1G$ and $\gamma_2G$ were quite adequate. These procedures, Sephadex column chromatography, ion-exchange chromatography and agar-gel electrophoresis, are very simple to perform, require no special equipment other than that found in a well-equipped immunology laboratory, and can be used to purify relatively large batches of material. The purity of the resultant immunoglobulins can be checked by immunoelectrophoresis and immunodiffusion, but should be confirmed by analytical ultracentrifugation. Foot pad injections of these serum proteins, mixed with an equal volume of Freund's Complete Adjuvant, produced strongly precipitating antisera within 1-2 weeks. This response is remarkably quick when compared with the times suggested for preparation of antisera to a variety of protein antigens (Williams and Chase, 1968).

Unabsorbed rabbit anti-guinea pig IgM cross-reacted with at least two serum proteins in Sephadex G-200 FII, most notably IgG. This cross-reaction was probably due to anti-light chain antibodies since the preparation of guinea pig IgM should have precluded the possibility of contamination with IgG. These cross-reacting antibodies were completely removed by absorbing the antiserum with Sephadex G-200 FII. A cross-reaction was also noted with a serum component in Sephadex G-200 FII. This component migrated as an $\alpha_2$-macroglobulin, similar to the $\alpha_2$-macroglobulin of human serum. Identity of this
guinea pig serum component has not been established in the literature. Whether the presence of antibodies in the anti-IgM which reacted with this component was due to a minor contaminant in the IgM preparation or to a true cross-reacting antibody could not be determined at this time. Absorption with the anodic portion of agar-gel electrophoresis of Sephadex F1 completely removed the antibody which reacted with this serum component, thus rendering the anti-IgM monospecific.

The production of antiserum which reacted with both $\gamma_1 G$ and $\gamma_2 G$ upon injection of purified $\gamma_1 G$ or $\gamma_2 G$ was expected, because of the close antigenic similarities of these two serum proteins (Thorbecke et al., 1963). Upon absorption of anti-$\gamma_2 G$ with $\gamma_1 G$ to remove cross-reacting antibodies, all of the precipitating antibody which reacted with $\gamma_2 G$ was removed as well. On the other hand, absorption of anti-$\gamma_1 G$ with $\gamma_2 G$ removed those antibodies which reacted with $\gamma_2 G$, but left some which reacted with $\gamma_1 G$. These results do not agree with the observations of Nussenweig and Benacerraf (1964) who have demonstrated unique antigenic determinants for each of these two subclasses. Perhaps the determinants of the $\gamma_2 G$ subclass were weakly immunogenic in this rabbit and thus did not produce specific anti-subclass antiserum during the short immunization schedule (7-14 days).

By employing these monospecific anti-IgM and anti-$\gamma_1 G$, as well as commercial antisera, the class or subclass of immunoglobulin(s) containing antibodies specific for glycerol-teichoic acid could be firmly established.

Prior to the characterization of the anti-teichoic acid antibodies, a survey of a number of mammalian species was carried out to
ascertain the distribution of these natural antibodies. Differences among species was well established. Adult humans, rabbits and guinea pigs were 100% positive for anti-teichoic acid antibodies, while rats were intermediate and mice entirely negative. The results in rats constitute new observations while the data for the other species is in agreement with previous reports from this laboratory (Chorpenning and Dodd, 1967; Chorpenning et al., 1969; Decker et al., 1972). Perhaps the carbohydrate nature of the antigen is a partial explanation for these differences although this is probably not the case since mice (0% positive for anti-teichoic acid antibodies) are excellent responders to pneumococcal polysaccharides (Baker et al., 1971a,b; Braley and Freeman, 1971). The level of antigenic stimulus required for antibody production in this species is not important since the injection of mice with large numbers of whole bacilli (10^{10} organisms) produced little, if any, antibody response in mice (Stamper, H.B., personal communications). It might be hypothesized that the handling of the antigen varies among species studied or that the antibody response to this antigen is under a true genetic control. Preliminary evidence for this latter suggestion would be the variation in response among the strains of rats studies. Much larger numbers of rats, as well as breeding studies, are needed to confirm or deny this hypothesis.

A difference with regard to the immunoglobulin class(es) of anti-teichoic acid antibodies also existed among the species studied. Adult human and guinea pig sera consistently (100%) had antibodies of this specificity in both the IgM and IgG3 classes, while normal rabbit
sera had anti-teichoic acid antibodies in the IgM class only. Rats were divided between those that had antibodies in both classes and those that had them in the IgM class only. Even though the rabbit has been noted to be a rather poor responder to certain carbohydrate antigens (Kabat, 1968), the rabbits injected here and elsewhere (Frederick, 1969) with whole bacillus, acid-precipitated teichoic acid or acid-precipitated teichoic acid-modified rabbit erythrocytes responded with significant increases in titers with some reaching as high as 4096. The injection of rabbits with teichoic acid in the forms mentioned also led, in every case, to the production of specific antibodies in the IgG class of immunoglobulins. An explanation for these results is suggested by the work of Kishimoto and Ishizaka (1972) who point out that the optimal concentration of antigen required may differ for IgG and IgM antibody formation. In the rabbit, the oral route of antigen presentation may provide a level of teichoic acid optimal for IgM serum antibody production but suboptimal for IgG serum antibody production. Intraperitoneal injection of antigen would increase the amount of antigen present in the host, reaching the optimum required for IgG anti-teichoic acid antibody synthesis. The presence of IgG anti-teichoic acid antibodies in humans and guinea pigs may reflect lower optimal levels of antigen required for IgM antibody production or better absorption of the teichoic acid antigen through the gut wall by these two species. A second explanation for the difference in immunoglobulin classes of natural versus immune rabbit anti-teichoic acid antibodies might be the presentation of the antigen to different immunocompetent cell populations due to the difference in route of
inoculation (i.p. for artificial immunization vs. orally for natural stimulus). Further investigation of the localization of radiolabeled teichoic acid within the host after introduction by various routes is required. In any case, the differences among species with respect to their antibody responses to teichoic acids provides a sound basis from which to begin studies of the relative contributions of genetic control and antigenic stimuli to the immune response to glycerol-teichoic acid.

The major portion of this work concerned itself, however, with the ontogeny of antibody and cell-mediated immune responses to glycerol-teichoic acid in guinea pigs. The natural antibodies of two groups of animals, germfree and conventional, were studied from birth. IgG antibody was present in all of the young (<2 weeks) animals tested (and in most up to 5 weeks) and the titer of this class of immunoglobulins dropped gradually during the first 4-6 weeks in both groups of animals. It is suggested that this early IgG was maternal in origin since Brambell (1958) has shown guinea pig IgG to be capable of crossing the placenta and Sell (1967) demonstrated that maternal IgG in guinea pigs is catabolized by the neonate within 4-6 weeks. Attempts were made to ascertain if there was any relationship between the decrease in IgG anti-teichoic acid antibodies and the level of total IgG in these young animals. Radial immunodiffusion with monospecific antisera revealed that the level of total IgG dropped in the germfree guinea pigs, while the level in conventional animals remained relatively constant. These lower levels of IgG in the germfree animals could have resulted from exposure to fewer antigens and to lower dosages
than in the conventional animals, if we assume that greater dosages are required to elicit secondary responses. The constant level of total IgG, a class of immunoglobulins with a relatively rapid turnover rate (Waldmann and Ghetie, 1971), in the conventional animal indicates this group is quite capable of producing immunoglobulins associated with secondary responses very early in life.

Another piece of data which might indicate the germfree guinea pigs were receiving less stimuli than their conventional counterparts is the rapid rise in antibody titer, exclusively IgM, between 6-7 weeks of age in the germfree group versus a slower, more prolonged rise in the conventional group. This early rapid rise of natural IgM anti-teichoic acid antibody suggests that the germfree animal may actually be responding to teichoic acid antigen under less antigenic competition than its conventional counterpart. This is in agreement with the observations of Kim and Watson (1968), who have demonstrated a more rapid rate of antibody production during the "true" primary response in germfree piglets. The fact that the average level of IgM anti-teichoic acid antibody remains quite constant in the germfree and conventional animals suggests the exhaustion of some limiting factor required for increase in natural antibody production. This factor could be antibody-producing cells, antigen-reactive cells or a humoral factor, all of which have been hypothesized to play a role in antigenic competition (Möller and Sjorberg, 1970; Albright et al., 1970; Adler and Möller, 1971). Since both germfree and conventional guinea pigs are equally immunocompetent (Pollard and Nordin, 1971; Gordon and Pesti, 1971) and their diets were nutritionally identical, the only immunologic
difference between the two groups is the amount and variety of antigenic stimuli, which also suggests the difference in the rates of IgM antibody production is influenced by antigen.

Just how much of a role antigen might play in the development of the immune capabilities during maturation of an animal was investigated by injecting groups of conventional guinea pigs, 11-13 weeks of age, intraperitoneally with various forms of teichoic acid. As was expected, the best response was produced by the whole organism. Transient responses were produced by acid-precipitated teichoic acid (AP) and purified teichoic acid (PCE). Because of the method of arithmetic dilution employed, these differences in antibody levels before and after injection were significant and were taken to mean that both the AP and PCE were weakly immunogenic. Since the slopes of the rates of increase in antibody titer produced by injection with these two antigens were not significantly different, this meant that the protein moiety of the AP contributed in no way to the antibody response to the carbohydrate portion of the molecule. The fact that the antibody titers produced by these two antigens fell back to the level of the uninjected, normal animals, while the level of antibody in the bacillus-injected animals continued to rise, may reflect differences in antigen processing or clearing. The more rapid rate of increase in antibody titers in the AP, PCE and whole bacillus injected animals, compared to the rate of increase in the uninjected, conventional animals may be due to an increase in the level of antigenic stimulus which might overcome antigenic competition, or the difference in route of antigen presentation. Studies by Bohl et al. (1972), Ogra and Karzon (1969),
Ogra et al. (1968), Tomasi and Bienenstock (1968) and others, suggest that the oral route of immunization leads, in many cases, to much less of an increase in serum antibody titers than other routes of injection. The fact that the synthetic PGP produced no increase in antibody titer was expected because of the relatively small size of the molecule (<8000w).

Although the whole bacillus-produced a significant increase in antibody titer in the young guinea pigs, all of the antibody produced was IgM even though 14 days (time after injection of last bleeding) was sufficient time to allow a secondary response to be produced. Earlier work by this author (Frederick and Chorpenning, 1972) demonstrated that normal guinea pigs, when injected at 16 weeks with AP in an amount (500μg) much less than that used in these experiments (25mg), responded with a very rapid increase in antibody titer and the appearance of IgG anti-teichoic acid antibodies. These data suggest that the maturation of the natural antibody response, i.e., the appearance of IgG anti-teichoic acid antibody, is not dependent upon the level of antigen since the injections of large amounts of antigen in the younger animals (11-13 weeks) did not lead to the production of IgG antibody.

The quite unexpected results in rats injected with the l forms of teichoic acid cannot be explained without a more precise determination of antibody titers by arithmetic dilution and the use of a homogeneous population with respect to pre-injection titers.

Because of the recent interest in T-cell responses to antigenic stimuli, a brief study of the ontogeny of cell-mediated immune responses and the effect of artificial immunization on this phase of
immunity was also undertaken. Indeed, it appeared that cell-mediated immunity (CMI) to teichoic acid developed naturally, much along the same lines as the humoral response. CMI, as measured by delayed skin reactions and the inhibition of macrophage migration, was absent in normal guinea pigs under 19 weeks of age. These data, along with the demonstration that incubation of PE cells with teichoic acid did not reduce cell viability, are proof that the skin reactions and inhibition of migration reactions observed were not due to antigen toxicity. All of the normal animals over 26 weeks of age were positive when tested by one of the two methods mentioned (no animals were tested by both methods). No animals between 19 and 26 weeks of age were tested. It is during this period (20-21 weeks) that IgG natural anti-teichoic acid antibody appeared in the normal, uninjected animal and it is quite tempting to hypothesize that the production of IgG antibody, in this case, is related to the appearance of CMI to the same antigen. A great deal of evidence in the literature supports the hypothesis that IgM antibody is independent of T-cell function while IgG antibody production involves T-cells in some way (see A Review of the Literature). The production of anti-IgM antibody in the absence of demonstrable CMI in young, uninjected guinea pigs, supports the former hypothesis. However, the production of IgG anti-teichoic acid antibody in response to injection of AP in the 16 week old guinea pigs (Frederick and Chorpenning, 1972) tends to refute the latter, since natural CMI could not be demonstrated at this age (16 weeks). But it may be that CMI is developing very slowly in response to the constant antigenic stimulus of natural oral immunization and that enough memory (T2) cells were
present at 16 weeks of age to cooperate in producing an anamnestic response, but not enough cells were present to produce a positive skin test or inhibition of macrophage migration. Since the 16-week-old injected animals, as well as all of the normal skin test negative animals were not retested to see if artificial immunization had stimulated enough T cells to give a positive response, this point awaits further investigation. A brief study in this direction was attempted by checking the 4 groups of guinea pigs artificially immunized at 11-13 weeks of age to see if any CMI could be detected post-injection. Indeed, weak positive skin tests were displayed by 3 of the 4 Bac group. None of the other animals were positive. This evidence of a cell-mediated immune response to injection of the whole bacillus at a time when no CMI could be detected in un.injected animals, suggests that the development of CMI to teichoic acid antigen may be antigen dependent, i.e., may reflect T1 to T2 maturation of the T-cell system. The fact that only the whole bacillus stimulated a cell-mediated immunity might be related to differences in antigen processing by macrophages if we assume that this step is necessary for the production of CMI to teichoic acid antigen. The whole bacterial cell may contain non-specific substances able to cause lymphocyte stimulation, resulting in an enhancement of the T-cell response to the cell-wall associated teichoic acid. (Further investigations into the area of the relationship of antigen complexity and processing to cell-mediated immunity, employing the 4 forms of teichoic acid used in these studies, seems warranted). If it could be shown that only the teichoic acid in the form of the whole bacillus, and not in the form of AP or PCE, interacted
with T cells, this might also explain the transient antibody response produced by injection of the AP and the PGE, since these antigens could be interacting directly with B cells responsible for IgM memory (Jacobson et al., 1970), and the proliferation of IgM B cells without the cooperation of T cells might be quite low. It seems plausible that all natural responses would be kept low so as not to overtax the immune system which constantly contacts large numbers of natural antigens. A state of partial tolerance could exist, whereby certain groups of cells able to respond to teichoic acid may never be turned on until a large dose of antigen is encountered by injection, or the level of antigen is built up naturally until these cells are triggered. This "trigger" would be the stimulation of large numbers of T2 cells resulting in the production of CMI to the antigen and the appearance of IgG antibody. Borel and David (1971) suggest a partial tolerance as the explanation for their results in guinea pigs where cell-mediated immunity to DNP-BSA is lacking and the IgG antibody response is restricted to the \( \gamma \)G subclass. Coincidentally, the IgG anti-teichoic acid antibody response in guinea pigs is also restricted to the \( \gamma \)G subclass, whether the antibodies are natural or are produced in response to injection, while guinea pigs artificially immunized with other antigens produce IgG of both subclasses (Benacerraf et al., 1963; Nussenweig et al., 1968; Sandberg et al., 1971). These data suggest that there may actually be an intrinsic difference between the naturally-occurring responses and responses to injection of an antigen encountered by an animal for the first time. It cannot be ruled out, however, that some aspect of the natural stimulus, such as the type of
antigen, the constant antigenic stimulus, or the oral route, could be responsible for this restriction to the \( \gamma_1 \) subclass.

Throughout this discussion, the specificity of the reactions under study has been called "glycerol-teichoic acid". Earlier work in this laboratory (Decker et al., 1972) has shown the specificity of this teichoic acid to reside completely in the polyglycerophosphate backbone. Complete inhibition of the reaction of normal and immune sera with teichoic acid (AP and PCE) modified erythrocytes could be accomplished with a synthetic PGP. This polymer was studied briefly in this work and was used as the hapten in equilibrium dialysis.

Chain-length analysis indicated the polymer to have an average length of 1.8 to 2.56 glycerophosphoric units, corresponding to a molecular weight of 309-440. Recycling of the polymer through Dowex 50WX12 did not increase its average chain length. The longest chain detected in any preparation was 3.57 units long. The shortness of the chain probably explains the high levels of PGP (850\(\mu\)g) required to inhibit PHA reactions when compared to the inhibition obtained with purified teichoic acid (<1\(\mu\)g) (Decker et al., 1972), with a molecular weight of at least 30,000 (non-dialyzable).

PGP could be covalently coupled to \( ^{14}C \)-glycine without increasing the average chain length, thus making it ideal for equilibrium dialysis studies. This preparation was stable at -20 \(^\circ\)C for at least 8 months (at the time of this writing) and it is anticipated that it could be employed for very sensitive methods of antibody detection and that PGP-\(^3H\)-glycine, prepared in the same manner as the carbon-14 tagged polymer, could be used for autoradiographic investigation of
the localization of antigen in the host.

Equilibrium dialysis of tagged PGP versus normal guinea pig Sephadex G-200 FII showed the average affinity of the anti-teichoic acid antibodies in this fraction to range from $1.0 \times 10^5$LM$^{-1}$ to $1.3 \times 10^6$LM$^{-1}$ for four different sera. If these $K_0$ are representative of the binding of most natural IgG-containing sera, the serum proteins responsible for the reactions with teichoic acid are true antibodies because their $K_0$ are comparable to those reported for antibody molecules ($10^3 \leq K_0 \leq 10^9$) (Davis et al., 1969). Their low affinities are similar to those of antibodies specific for p-azobenzenearsionate, p-azophenyl-β-lactoside, mono-DNP-ribonuclease and ovalbumin. Using the techniques described in this work, it is now possible to study the maturation of antibody affinity during natural and artificial stimulation in germfree and conventional animals. The results of such a study should provide evidence as to the role, if any, of antigen in the development of antibody affinity. If antigen acts by selecting for higher affinity antibody, then increases in antigen concentration in the host should cause a slowing of maturation of affinity as more lower affinity antibody-producing cells would have a greater opportunity to interact with antigen.

The molar concentrations of IgG anti-teichoic acid antibodies ranged from $2.1 \times 10^{-6}$M to $1.75 \times 10^{-5}$M and there appeared to be no correlation among affinity, molar concentration and PHA titer. Of course PHA probably does not measure all of the anti-teichoic acid antibodies in normal serum, most likely missing some low affinity molecules. Antibody affinity could also not be expected to correlate with
antibody concentration since there is heterogeneity of affinity among antibody molecules of the same serum as indicated by the curved lines of r/c vs. r, and the average affinity constant (K₀) for each serum is thus affected by the relative amounts of high and low affinity molecules. Because of these results, conclusions with respect to levels of anti-teichoic acid antibodies should only be made from data obtained by a single method of antibody detection and should not be compared to data obtained by another method unless a strict correlation can be shown to exist between the two methods. Total specific antibody, measured by the Kjeldahl method, appears to be the most desirable.

Two other properties of guinea pig serum antibodies, cytophilic attachment to macrophages and bactericidal capability, were studied in this work. IgM- ^G- and ^G-containing serum fractions were tested for their ability to attach to macrophages, mediated by the F₉ portion of the molecule. No such property could be detected in any of the three classes. Since guinea pig cytophilic antibody has only been consistently demonstrated in the G subclass (Jonas et al., 1965; Nelson and Mildenhall, 1966; Berken and Benacerraf, 1966; Gowland, 1968), these results confirm the lack of any specific anti-teichoic acid antibodies in this subclass.

Bactericidal testing of normal guinea pig serum fractions I and II showed that only one of the four sera tested significantly reduced the number of living bacteria, this ability being found in both fractions. Preparation of these fractions by Sephadex G-200 column chromatography precluded the involvement of normal non-antibody serum bactericidal factors, not found in either of these two fractions.
Since the IgM antibodies of most mammalian species can participate in bactericidal reactions, albeit against gram-negative bacteria, the results with FI of this serum could be due to bactericidal anti-teichoic acid antibodies. Other data (Stamper, H.B., personal communications) have suggested that this same serum (#167) might provide passive protection in mice, although the mechanism has not been established. However, the results with FII of this serum raises doubts about this interpretation since, of the guinea pig IgG immunoglobulins tested, only \( \gamma_G \) has been shown to fix complement (Bloch et al., 1963). The results obtained with this one serum may be due to a bacteriostatic effect similar to the mechanism suggested by Ehrenkranz et al. (1971) for the effect of human serum on Staphylococcus aureus. They suggest that the effect is attributable to antibodies and not to non-specific serum components and that the components of the cell wall of the bacteria which react with these antibodies are carbohydrate in nature.

The mechanism of this stasis is, as of yet, unknown, but it could be due to an alteration in the cell surface structure or charge resulting in an inhibition of exchange between the bacterium and its surrounding environmental nutrients. It is apparent, however, that anti-teichoic acid antibodies do not contribute significantly to protection in guinea pigs since this property was not demonstrable in the other three sera tested.

The data presented and discussed in this dissertation raises a number of new questions. What is the role, if any, of natural anti-teichoic acid antibodies? Is the development of T cells sensitive to teichoic acid dependent upon antigen? Does the form of immunogen, and
route of injection, favor one of these types of immunity over the other, or is the development of natural T and B cell immunity completely antigen independent? Also, is there a direct relationship between the manifestation of cell-mediated immunity and the appearance of IgG antibody? Why is the IgG anti-teichoic acid antibody in guinea pigs all of the \( \gamma \) subclass? Why does the antibody response to teichoic acid vary among species with respect to frequency and class(es) of immunoglobulins?

These questions have been asked throughout this dissertation and some have been partially answered. By using the techniques and reagents described here, many of them can be completely answered. These answers may, in turn, shed some light on very basic immunologic questions.
SUMMARY AND CONCLUSIONS

1. Sephadex G-200 column chromatography, ion-exchange chromatography and agar-gel electrophoresis were employed to prepare purified guinea pig IgM, $\gamma_1 G$ and $\gamma_2 G$ immunoglobulins.

2. Immunization of rabbits with these preparations produced cross-reacting antisera which could be rendered monospecific by absorption with the appropriate Sephadex or agar-gel serum fraction(s). Monospecific anti-$\gamma_2 G$ could not be prepared since it appeared that $\gamma_2 G$ did not produce specific anti-subclass antibody within the immunization period.

3. By employing these prepared antisera and commercial antisera, the class(es) of anti-teichoic acid antibodies present in a number of adult mammalian species were firmly established. One-hundred percent of normal human and guinea pig sera tested had anti-teichoic acid antibodies in the IgM and IgG classes of immunoglobulins, the guinea pig IgG antibody being exclusively of the $\gamma_1 G$ subclass. One-hundred percent of normal rabbits tested had antibodies only in the IgM class. Immunization of normal rabbits with acid-precipitated teichoic acid (AP) stimulated the production of IgG antibodies in every case. Fifty-nine percent of normal rats tested had antibodies in their sera, with some having antibodies in both the IgM and IgG classes, while others had them only in the IgM class. The frequency of positive sera appeared to vary with the species of rat tested. None of the normal mice
tested had anti-teichoic acid antibodies in their sera. These results were thought to be due to genetic control of serum antibody responses, differences in routes of antigen presentation in the case of normal versus immune rabbit antisera, and in the case of the restriction of guinea pig IgG antibody to the $\gamma_1$G subclass, a state of partial tolerance.

4. The levels of total IgM were comparable in germfree and conventional guinea pigs. The level of IgG in young germfree animals was lower than in conventionals. This was thought to be due to fewer antigenic stimuli in the germfree animals. Sustained levels of IgG in the conventional animals suggests these animals were capable of producing immunoglobulins associated with secondary responses at this age.

5. The ontogeny of the natural humoral antibody and cell-mediated immune responses to teichoic acid in guinea pigs was investigated. Early antibody was thought to be maternal. The natural antibodies produced by animals under 20 weeks of age were exclusively of the IgM class. No natural cell-mediated immunity could be detected in these same animals. These results were interpreted to mean that the development of IgM antibody was T cell independent. The early rise in IgM antibodies was more rapid in germfree than in conventional animals, probably due to less antigenic competition in the germfree animals.

6. Immunization of 11-13 week old conventional animals with acid-precipitated teichoic acid (AP), purified teichoic acid (PCE) or whole bacillus (Bac) caused a more rapid increase in antibody titers than in the uninjected animals. Since all of the anti-teichoic acid antibody was IgM, this suggests that the increase in antigenic stimulus or the
different route of antigen presentation simply increased the number of responding B cells or caused a more rapid proliferation of committed plasma cells. Injections with Bac increased antibody titer to 32, while injections of AP and FCE produced transient antibody responses. This difference is probably the result of different antigen processing steps in the handling of the three antigens. Bac injections also produced a weak cell-mediated immune response to teichoic acid suggesting that the development of cell-mediated immunity is antigen dependent.

7. Injections of 16-week old guinea pigs with AP, in an amount much less than that given the 11-13 week old animals, stimulated a much greater antibody response and caused the appearance of IgG antibody (Frederick and Chorpenning, 1972). Natural IgG appeared at about 20-21 weeks and natural cell-mediated immunity was detected at 26 weeks. No animals were tested for cell-mediated immunity between 19 and 26 weeks of age. The natural development of IgG (G) anti-teichoic acid antibody is not directly dependent on antigen, but may be dependent upon the manifestation of cell-mediated immunity to teichoic acid.

8. The reaction of anti-teichoic acid antibody was completely inhibited by synthetic polyglycerophosphate (PGP), a polymer of molecular weight 309-140. Covalent coupling of PGP to 1-1G-glycine produced a stable reagent used for equilibrium dialysis. The average affinity constants (K0) for 1 guinea pig G-containing serum fractions indicated the serum proteins responsible for binding were antibodies with relatively low intrinsic association constants.

10. One of four normal guinea pig sera tested demonstrated specific anti-teichoic acid antibody mediated bacteriostasis.
REFERENCES

Abdoosh, Y.B., 1936. Natural and immune bactericidins for the gonococcus. J. Hyg. 26:355


Dienes, L. and Mallory, T.B., 1932. Histological studies of hypersensitive reactions. Am. J. Path. 8:689


Kerman, R., Segre, D. and Myers, W.L., 1970. The role of immune and natural specific antibodies in immunologic paralysis and immunity of mice to pneumococcal polysaccharide type III. J. Immunol. 104:656


Marres, M., 1932. Inheritance to resistance and susceptibility to infectious abortion. J. Inf. Dis. 51:30


Nussenweig, V. and Benacerraf, B., 1961. Studies on the properties of fragments of guinea pig $\gamma_1$G and $\gamma_2$G antibodies obtained by papain digestion and mild reduction. J. Immunol. 92:1008

Nussenweig, V., Green, I., Bassalli, P. and Benacerraf, B., 1968. Changes in proportion of guinea pig $\gamma_1$G and $\gamma_2$G antibodies during immunization and the cellular localization of these immunoglobulins. Immunology 14:601


Sterzl, J. and Hrubesova, M., 1959. Attempts to transfer tuberculin hypersensitivity to young rabbits. Folia Microbiol. (Prague) 4:60


Strauss, H.W., 1931. Artificial sensitization of infants to poison ivy. J. Allergy 2:137


