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The Ohio State University, Ph.D., 1976
Health Sciences, Immunology
RESPONSES TO ENVIRONMENTAL TEICHOIC ACID
IN CONVENTIONAL AND GERMFREE RATS

DISSERTATION

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

By
Ronald Wayne Bolton, B.S., M.S.

* * * * *

The Ohio State University
1976

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ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. Frank Chorpenning for his guidance during this investigation, to Harry Rozmiarek, D.V.M. for his assistance in maintaining the germfree animal colony and for developing a liquid diet which was nutritionally adequate for rats, to Harold Cooper and Thomas Alexander for their chemical analysis of the teichoic acid antigen, and to Rollie Cruze for his technical support during this investigation.
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INTRODUCTION

Naturally-occurring responses to glycerol-teichoic acid have been studied extensively by a number of investigators. Decker et al. (1972) found natural antibodies to teichoic acid in humans, guinea pigs, and rabbits, and showed them to be of polyglycerophosphate (PGP) specificity. Frederick (1972) and Bolton and Chorpenning (1974) reported the occurrence of natural anti-PGP antibodies in rats. Naturally-occurring cell-mediated responses of this specificity were reported by Frederick et al. (1972) in guinea pigs and by Bolton and Chorpenning (1974) in rats. Both investigators found a temporal relation in the onset of natural cell-mediated and IgG responses to teichoic acid. Frederick and Chorpenning (1972) reported the occurrence of natural anti-PGP antibodies in germfree guinea pigs and suggested that such responses were the result of ingesting killed Gram-positive bacteria, however no direct proof was established prior to the present investigation. Previous investigations suggested that environmental antigens were a source of stimulation for natural antibodies (Springer et al., 1959), (Wagner, 1959), (Sterzl et al., 1962), (Ikari, 1964).

The previous reports of natural responses to the well-defined PGP specificity prompted an investigation of the ontogeny of such responses as well as an attempt to clarify their sources of
stimulation. Such studies provide an important addition to our understanding of basic immune functions since most previous work utilized poorly defined antigens in artificially immunized animals. The experiments employed in the present investigation permitted observation of the immune response as it matured in the normal animal, a situation quite unique when compared with previous ontogeny studies (Solomon, 1971).

The rat was chosen for this investigation since some preliminary information on responses to teichoic acid in this species was available (Bolton and Chorpenning, 1974), large number of germfree and conventional animals could be obtained, and previous studies in the rat involved use of poorly defined antigens which were administered parenterally (Solomon, 1971). A large population of rats was serially bled for a one-year period and sera examined for naturally-occurring antibodies to teichoic acid using the passive hemolysis test. The natural sheep erythrocyte hemolysin response was used as a control specificity for all experiments since such responses were previously reported in the rat (Solomon et al., 1972) and thought to occur as the result of stimulation from gut flora (Cheng and Trentin, 1967). A large number of germfree and conventional rats were also examined in group studies with ages selected to correspond with the times of serial bleeding. These animals were examined for naturally-occurring cell-mediated responses to teichoic acid and sera were fractionated and characterized with respect to antibody class. Whole serum and fractions were examined for presence of natural hemolysins of sheep erythrocyte and teichoic acid specificities. This
work clarified the temporal relation in the onset of natural cell-mediated and IgG responses to teichoic acid first observed in the rat by Bolton and Chorpenning (1974). The failure of germfree rats to produce naturally-occurring cell-mediated and IgG antibody responses to teichoic acid suggested that antigen dosage may affect the class of natural antibody produced and whether or not naturally-occurring cell-mediated responses occur. As a test of this theory, a number of weanling rats were fed Gram-positive bacteria in controlled amounts. After several months the animals were examined for the presence of cellular and humoral responses to teichoic acid. Feeding bacteria to these rats did stimulate cell-mediated responses to teichoic acid along with a shift to IgG antibody production, concurrent with the presence of IgM, while control animals produced only IgM and no cell-mediated responses of this specificity. The results show that antigen, administered orally, could have a significant impact upon responses to teichoic acid as previously suggested by Frederick and Chorpenning (1973).

The results of the oral immunization studies along with the observation of natural antibodies to teichoic acid in germfree rats suggested that food and bedding may be the major source of stimulation for such responses. An attempt was made to create a teichoic acid-free environment in order to eliminate the source of stimulation, and therefore the response. Germfree rats were reared on a liquid diet which was shown to be free of detectable teichoic acid and contained very few bacterial bodies. Wire-bottomed cages were used instead of bedding. Bacteria were included in the water of some
of these animals and after several months both normal and orally immunized rats on the teichoic acid deficient diet were examined. Only those which were fed bacteria produced antibodies to teichoic acid. The results are discussed in relation to the environmental nature of teichoic acid.

Examination of natural antibodies to teichoic acid in individual rats included in the ontogeny serial studies revealed a cycling of antibody production, a phenomenon not previously described in normal animals with a defined antigen. A confirmatory experiment was then designed using additional normal rats and a group of immunized animals, which were examined at four-day intervals. Cyclical production of antibody to teichoic acid was again demonstrated in both serum and spleen cells. That such a phenomenon was demonstrated in normal rats suggested that it may play an important role in immunoregulation.

The large number of parameters being investigated here prompted a division of the work into several parts in order to simplify presentation. Part I includes preliminary work and development and standardization of hemolysin tests. The ontogeny of natural responses to teichoic acid and sheep erythrocytes in germfree and conventional rats are examined in Part II. The source of stimulation for such responses is clarified by the results in Parts III and IV, while Part V describes cyclical production of antibodies to teichoic acid. These results, when considered together, provide a complete overview of naturally-occurring responses to teichoic acid, including source of stimulation, relationship of dosage to cellular and humoral aspects of the response, and self regulation by antibody cycling.
REVIEW OF THE LITERATURE

The broad scope of this investigation warranted a review of previous work in several areas of immunology. A major area of significance involved a comparison of naturally-occurring responses in germfree and conventional rats as these animals mature. Previous investigations in gnotobiotics, natural antibodies, ontogeny, and development of the immune response after antigenic stimulation all become very pertinent when considering the results of this research. A brief discussion of immunity and aging is included since serial studies in this work entailed examination of the status of natural antibodies in rats over a one-year span. The relationship of the occurrence of delayed hypersensitivity and circulating antibody is an important problem when one considers the natural responses to glycerol-teichoic acid in rats as evidenced by previous work (Bolton and Chorpenning, 1974) and therefore a discussion of the literature on this subject is necessary in any attempt to explain these findings. Finally, preliminary observations of natural antibody cycling prompted a review of previous work in this area to determine the significance of the observation and to lay a foundation for a more elaborate investigation of the antibody cycling phenomenon using the teichoic acid specificity.
ONTGENY OF THE IMMUNE RESPONSE

Cells involved in the immune response. Immunology evolved from bacteriological studies of resistance to infection. Almost from the beginning investigators were aware of the cellular and humoral facets of immunity. Much debate arose over the roles of antibodies and phagocytic cells (Metchnikoff, 1905). The terms "cellular" and "humoral" immunity now take on a meaning somewhat distant from their original bacteriological connotations. Cell-mediated immunological phenomena are those transferrable by cells and not by serum, such as delayed hypersensitivity, graft rejection, and immunity to certain bacterial infections. The term is also applied to in vitro tests which are thought to be analogous to the above in vivo phenomena. Humoral immunity encompasses all immunological responses transferrable by serum (MacKaness and Blanden, 1967).

Lymphocyte involvement in immune responses has long been suspected (Murphy, 1926). However, only since the late 1950's has a definite role for these cells been established. Other functions for lymphocytes were postulated by Thomas (1959), who proposed that they eliminated aberrant autologous cells, and by Burch and Burwell (1965), who suggested they regulated tissue growth. Convincing evidence for the role of the lymphocyte in immune responses was obtained by cell depletion and reconstitution experiments which impart or impair immunological competence (reviewed by Gowans and McGregor, 1965). Purified populations of lymphocytes could transfer delayed hypersensitivity (Wesslen, 1952), skin allograft rejection
(Billingham and Silvers, 1963), and tumor immunity (Mitchison, 1966). The work of McGregor and Gowans (1963) suggested that lymphocytes were also responsible for primary antibody responses and that of Cochrane and Dixon (1962) placed the responsibility for immunological memory upon lymphocytes.

The precise role of lymphocytes in the immune response remained obscure until more precise tests were developed, such as the cytopathic effect of lymphocytes on target cells (Wilson, 1963), inhibition of macrophage migration (David et al., 1968), proliferation of antigen stimulated lymphocytes (Permain et al., 1963), and proliferation stimulated by allogeneic cells (Bain et al., 1964). Plasma cells, which were known to secrete antibody, were not believed at first to be of lymphoid origin (Marshall and White, 1950). Makela and Nossal (1961) demonstrated antibody production by lymphocytes and Braams (1961) found lymphoid cells could take on an intermediate appearance between lymphocytes and plasma cells. These investigations provided strong arguments for the role of lymphocytes in antibody formation.

A functional dichotomy of cellular and humoral responses was first established by the work of Glick et al. (1956), which showed the Bursa of Fabricius was the primary lymphoid organ responsible for the development of humoral immune responsiveness in avians and by the work of Cooper et al. (1966b) which showed differential effects of bursectomy and thymectomy on the two arms of the response in the chicken. Roitt et al. (1969) categorized the two lines of immunological responses and suggested the term T-cell for those lymphocytes
mediating thymus-dependent cell-mediated reactions and the term B-cell for those mediating bursal cell dependent (bursal equivalent cell in mammals) reactions.

Ontogeny of the immune response in the chicken. The first small lymphocytes found in the avian embryo are formed in the thymus (Greaves et al., 1973). Early in these investigations a controversy arose over the origin of the precursors of these cells. Some advocated a direct transformation of thymic epithelium while others advocated the migration of stem cells into the thymus (quoted from Cooper and Lawton, 1972). The work of Moore and Owen (1967) clarified the controversy. Experiments using parabiosed chick embryos, whose lymphocytes were traced by sex chromosome markers, indicated that lymphoid stem cells migrated to the thymus via the blood. Owen and Ritter (1969) found stem cell migration into the thymus in 6-9 day-old chicken embryos. Dwyer and Warner (1971) reported specific antigen binding by thymus cells by day 14 gestation, thus suggesting that antigen receptors are present on T-cells at this early stage of embryogenesis. The avian thymus also harbors B-cells, which have been shown to migrate from the Bursa of Fabricius (Linna et al., 1971).

The Bursa of Fabricius has been extensively studied and shown to be the primary lymphoid organ responsible for the development of humoral immune responsiveness in avians (Glick et al., 1956), (Cooper et al., 1966b). Removal of the bursa was shown to impair antibody formation but not cellular immunity. Stem cells, which migrate from the yolk sac, were detected in the bursa by day 13 of
embryonic growth (Moore and Owen, 1966). IgM containing cells were found within the bursa by day 14 (Kincade and Cooper, 1971). The number of these cells was found to increase over the next several days with the average generation time being 7-9 hours.

A number of investigators using thymectomized and bursectomized chickens have immensely clarified the initial investigations of the role of the bursa and have shown the functional dichotomy of cellular and humoral responses which exists in this species (Cooper et al., 1965), although it is now common knowledge that such a division is not absolute (Alm, 1971). Van Alten et al. (1968) found neonatally bursectomized birds manifested a poor primary response, but a normal secondary response at four weeks age. Birds bursectomized at 19 days gestation produced feeble primary and secondary responses. Restoration of responsiveness was achieved by injection of syngeneic bursal cells. Thorbecke et al. (1968) reported the bursa to be the first lymphoid tissue capable of synthesizing IgG and that this ability developed later than IgM synthesis. Van Meter et al. (1969) found that serum IgM levels in the chicken rose to peak levels before IgG did so and that neonatal bursectomy delayed the rise in serum IgG concentration but did not affect IgM levels. Kincade and Cooper (1971) found no IgG containing cells in the Bursa of Fabricius until hatching, as compared with the presence of IgM producing cells on day 14 of gestation. This developmental sequence was later found to be repeated in the spleen, tonsil, and thymus after birth. No IgG was found in extrabursal sites until day 4 after birth while IgM was found by day 17 gestation. The switch from IgM to IgG was thought to
occur in the bursa since anti-IgM given to day 13 embryos which were then bursectomized at hatching prevented both IgM and IgG synthesis (Kincade et al., 1970). In a mature chicken 50% of the bursal cells were shown by immunofluorescence to contain both IgM and IgG, again suggesting a switch occurring within the bursa since these cells were rarely found in the spleen (Kincade and Cooper, 1971). This switch apparently can not occur outside the bursa since bursectomy before hatching yields large amounts of IgM, but an irreparable deficit of IgG (Kincade et al., 1970).

The cells in the bursa have been shown to migrate to peripheral lymphoid tissue where they develop into antibody forming cells following antigenic stimulation. Peripheral development of the plasma cell line in the chicken may be aborted by bursectomy no later than the 17th day of gestation, chemical bursectomy by testosterone, or by bursectomy and x-irradiation at hatching. Such animals lack bursal cells, plasma cells, germinal centers, and are agammaglobulinemic (Cooper et al., 1969, 1972a).

Lymphoid development within the bursa appears to be antigen independent since it occurs during gestation at a time when no circulating immunoglobulins are detectable (Cooper et al., 1972a). Bursal development was found normal in germfree chicks whereas development of lymphoid tissue in extra-bursal sites was retarded (Kincade and Cooper, 1971). The extensive research on the ontogeny of the immune response in the chicken, of which only the major points were discussed above, prompted Cooper et al. (1972b) to propose a two stage developmental sequence for antibody producing cells. Stage
one occurs in microenvironments within primary lymphoid tissue and is independent of foreign antigens. Multiple clones of antibody-producing cells are generated from stem cells of hematogenous origin. Immunoglobulins synthesized in the first stage are incorporated as receptors on cell surfaces. An orderly intraclonal switch from IgM to IgG to IgA at this level generates diversity of immunoglobulin classes. The second stage, as described by Cooper et al. (1972b), occurs in the circulation and peripheral lymphoid tissue. Clonal selection of B-cells by antigen and differentiation to plasma cells occur here. It has since been demonstrated that IgG and IgA forming cells do arise from IgM antibody-forming cells (Martin and Leslie, 1974).

Ontogeny of the immune response in mammals. There are three lines of evidence supporting a functional dichotomy of the immune response in mammals which is similar to that described above in avians. (1) Cellular and humoral immunity are found in early vertebrates long before the emergence of birds and mammals (Papermaster et al., 1964). (2) The roles of the avian and mammalian thymus have been proven to be parallel (Good et al., 1962a), (Good and Gabrielson, 1964), (Cooper et al., 1966b), (Mitchell and Miller, 1968), although there were some early attempts to attribute the development of B-cell function in mammals to the thymus (Cooper and Lawton, 1972). The discovery of natural immune deficiencies of T-cell or B-cell lineage further supported the hypothesis of the parallel role advocated for the avian and mammalian thymus (Good et al., 1962b). (3) Neither avian nor mammalian thymus appears to affect plasma cell development
directly (Cooper et al., 1966a).

Much of the information on lymphopoiesis in mammals has been obtained from mice. Owen and Ritter (1969) found a stem cell influx into the murine thymus at 11-12 days gestation, which were characterized as large basophilic cells containing dense cytoplasm (Mandel and Russel, 1971). Some investigators have claimed thymus cells arise from epithelium within the organ itself (Turpen et al., 1973), but it is now generally accepted that the stem cells migrate into the thymus from an outside source (Leuchars et al., 1973) and move from the thymic cortex to the medulla as they mature (Borum, 1968).

Stem cells, which have been found as early as day 11 of gestation in the murine thymus (Tyan, 1968) begin differentiation into thymus cells by day 14 of gestation (Owen and Raff, 1970). Stites et al. (1970) reported responsive cells within the human thymus by 10 weeks gestation. Owen and Raff (1970) cultured murine 14 day embryonic thymus cells which initially did not possess the theta or TL antigens and after 4 days the cells gained both of these which is indicative of T-cell maturation. Ritter (1972) observed an increased sensitivity to radiation over this same period. Murine thymus cells were shown to migrate to peripheral lymphoid tissue at day 18 of gestation (Owen and Ritter, 1969), while Dwyer and McKay (1972) found competent lymphocytes in peripheral human lymphoid tissue by 12 weeks gestation. Aoke et al. (1969) found peripheral lymphocytes contained less theta antigen on their surfaces. This observation later prompted hypotheses that a second step of differentiation from thymus lymphocyte to T-cell must occur. Supportive of such ideas was the discovery of
subpopulations of cells in the murine thymus (Greaves et al., 1973). One subpopulation (3-5%) had the properties of mature peripheral blood lymphocytes in that they were cortisone resistant, had little theta antigen, and homed to the lymph nodes, whereas the majority of thymus cells were cortisone sensitive, had dense theta antigen on their surfaces, and homed to the spleen. Raff and Cantor (1971) have called the more mature cells T2 as opposed to the major population of immature T1 cells. Several other investigators have reported or suggested that subpopulations of T-cells exist and may be immunoregulatory as well as participate in cell-mediated immunity (Baker et al., 1973), (Folch and Waksman, 1974), (Wu and Lance, 1974).

The presence of the subpopulation of mature T2 cells in the thymus has been attributed to environmental antigenic stimulation, although this has not been proven (Greaves et al., 1973).

Differentiation of thymus-derived cells is thought to be hormonally regulated (reviewed by Miller and Osaba, 1967). Most of this work was performed by thymus implantation into mice using cell impermeable diffusion chambers. Bach and Dardenne (1972) found a substance in normal mouse serum which conferred T-cell characteristics on bone marrow cells. This substance was not found in thymectomized mice, however Kruger et al. (1970) found no effect when thymic extract was injected into thymectomized rats.

There is no direct evidence for the existence of a bursal equivalent organ in mammals, however the mammalian fetal liver (Tyan and Herzenberg, 1968) and bone marrow (Mitchell and Miller, 1968) are sources of stem cells which mature into competent B-cells. The gut-
associated lymphoid tissue (GALT) hypothesis has been offered by some as an answer to the mammalian bursal equivalent (Cooper and Lawton, 1972). The GALT Theory suggests that stem cells of hematopoietic tissue origin migrate to special microenvironments in close association with intestinal epithelium where they are influenced to begin lymphoid differentiation. These follicles supply the rest of the animal with competent cells. A number of bursal equivalent organs have been suggested as the site of stem cell differentiation: appendix (Archer et al., 1963), tonsil (Peterson et al., 1965), and Peyer's patches (Cooper et al., 1966a) (Chapman et al., 1974). One confirmation of these suggestions was the work of Perey et al. (1968) who removed appendix, Peyer's patches, and sacculus rotundus from irradiated rabbits which were later reconstituted with fetal liver. They were unable to detect antibody responses in these animals. Several investigators have cast doubt on the GALT Theory and it is no longer readily accepted. Bauer (1968) found the gut-associated lymphoid tissue of germfree mice to be poorly developed suggesting an antigen dependency, unlike the antigen independent bursal development. The observations of Shultz et al. (1973) suggested that the GALT in the bovine fetus develops more rapidly following antigen exposure. Friedberg and Weissman (1974) further discredited the GALT Theory by showing that cell proliferation in the Peyer's patches of neonatal mice was insufficient to account for the rapid increase in splenic immunoglobulin bearing cells during the first four days after birth. Cooper and Lawton (1974) now advocate the fetal liver as a good candidate for the mammalian bursal equivalent. They
observed the appearance of IgM and IgG bearing cells in 12-15 day murine fetal liver cell cultures after one week incubation in synthetic medium without antigen. They also reported de novo appearance of B-cells in 16 day murine fetal spleen cultures, however they argued that the fetal liver was the better candidate for the bursal equivalent since immunoglobulin-bearing cells appeared earlier in gestation and it was epithelial in origin as is the bursa, while the spleen is derived from mesenchyme.

A number of investigators have studied the ontogeny of the immune response in mammals with an emphasis on the age of development. Silverstein et al. (1967) found antibody responsiveness in the fetal lamb to viral antigen at 40 days gestation, but only of the IgM class. As the fetus matured it became responsive to other antigens and on day 85 could reject grafts as well as an adult. This group also observed the same sequence of development in monkeys. Sherwin and Rowlands (1974) also observed a sequential maturation of responsiveness in irradiated mice reconstituted with fetal liver suggesting ordered maturation of responsive cells. Cells containing mu and light chains were found at 55 days of gestation in the Peyer's patch of fetal pigs, followed by gamma chain-containing cells at 70 days gestation (Chapman et al., 1974). Lymphoid follicles did not develop fully until after birth in these animals suggesting that the Peyer's patches were stimulated by antigens in the gut. Little circulating antibody was detected before birth in the pig.

Thorbecke and Van Furth (1967) claimed that rabbits, mice, and rats produce no circulating antibodies until birth while humans, sheep
and guinea pigs all produce serum IgM during gestation. Stecher and Thorbecke (1967) first detected IgM production in spleen cell cultures of 9 day old rats, however IgG synthesis was not found before rats were 15 days old. Germfree conditions did not affect the appearance of IgM, but IgG levels were much less than in conventional animals. Fahoy and Barth (1965) found autogenous IgM at 1-3 weeks age in mice and IgA at 4-6 weeks. Herzenberg et al. (1967) found IgG 3 weeks after birth in mice using anti-immunoglobulin of paternal allotype. Serum immunoglobulins are barely detectable in neonatal rabbits and cell cultures revealed IgM, but not IgG production up to one week after birth (Stecher and Thorbecke, 1967). Shifrine et al. (1971) observed immunocompetence in dogs only at or near the end of gestation. One may generalize by stating that those animals with long gestation periods develop circulating antibodies before birth (Thorbecke and Van Furth, 1967), with the earliest physiologically equivalent age at which antibodies are detectable being in the bursa of the chicken (Solomon, 1971).
When considering the development of immunity it becomes necessary to be concerned with the involution of immunity that occurs with aging. The thymus of older animals tends to be small and fibrous rather than lymphoid as it appears in younger animals (Good, 1971). The organ reaches maximal development at sexual maturity, followed by involution and impairment of T-cell function much later in the life of the animal. The bursa also involutes at sexual maturity, but the ability to maintain immunoglobulin levels persists more adequately than the thymus dependent immune system (Good, 1971).

The ability to produce antibodies decreases as a function of age in several species (Walford, 1969), an idea first suggested by Thomsen and Kettel (1929), who measured the levels of natural iso-agglutinins to blood group antigens in humans. Makinodan and Peterson (1962) reported an age-associated decrease in antibody formation to foreign erythrocytes in mice. This decrease has been associated with a decline in the number of precursor cells (Price and Makinodan, 1972). The loss is more rapid for IgG than IgM antibody in mice (Makinodan and Peterson, 1966). This decline in responsiveness apparently is not caused by poor antigen processing in the macrophage population (Heidrich and Makinodan, 1973), but may be prompted by a decline in T-B cell interaction (Price and Makinodan, 1972). Regulatory factors within aged animals which influence cell proliferation may also affect the decline in responsiveness (Price and Makinodan, 1972b).
Observation of the immune response at the cellular level also reveals a decline in responsiveness with aging. Konen et al. (1973) observed a decline in mixed lymphocyte reactivity in spleens from aged mice, however Walters and Claman (1975) reported no drop in contact sensitivity or mixed lymphocyte culture responsiveness but a drop in mitogen responses. Hallgren et al. (1973) reported a decline in phytohemagglutinin responsiveness of peripheral blood lymphocytes in aged humans. The drop coincided with an increase in antibodies to thyroglobulin and DNA as well as an increase in Rheumatoid factor. Nielson (1974) and Jaroslow et al. (1974) suggested a lag in proliferative ability as the responsible factor in the decline in cellular responsiveness observed with age. One might hypothesize that the lag in cellular proliferation and responsiveness observed may affect T-cell regulation of B-cell activity and may be indirectly responsible for the high incidence of autoantibodies reported in aged humans (Hallgren et al., 1973). Age-associated changes in the immune response have been associated with the high incidence of certain diseases in older animals as well as man (Walford, 1969).
The development of cell-mediated immunity which occurs following stimulation by certain antigens, i.e., sensitization, challenge, production of lymphokines by activated T-cells, has been studied in depth in many vertebrates and found to be similar in most species (reviewed in Davis et al., 1973). A major point which remains unclear is the relationship of cell-mediated and humoral immunity, a subject which is reviewed later in this thesis.

Examination of the development of antibody responses to antigenic stimulation presents a problem to the investigator since the animals used may have been previously exposed to environmental antigens of the same or of cross-reacting specificities, and therefore he can not be absolutely certain that a true primary response occurs following parenteral immunization. Many investigators have presented evidence that the first antibody synthesized is of the 19S class (Uhr and Finkelstein, 1963), (Fahey and Humphrey, 1965), however 19S antibodies are also produced in the secondary response (Bradley et al., 1963). These investigators may not have considered the immunological status of the animals they were using. It has been known for many years that the normal flora may act as an antigen stimulus (Ingalls, 1937), therefore the primary responses described by some investigators may actually have been the result of anamnesis. Solomon (1971) stated that a true primary response could not be attained unless experimental animals were reared in an antigen-free environment. Kim et al. (1966a,b) performed experiments using germfree colostrum-deprived
miniature piglets. They reported these animals were free of detectable serum immunoglobulins and that they were immunocompetent. The initial antibody response was a 19S gamma globulin followed later by a 7S globulin, both of which preceded IgG and IgA (Kim et al., 1968). Actinophagae was the antigen used in these experiments. Kim et al. (1974) concluded from their findings with the germfree piglets that antibodies do not develop without antigenic stimulation.

Solomon (1971) reviewed the development of the immune response following antigenic stimulation as it occurs in several species, however development in the rat warrants further comment since it is the species under investigation and does possess certain peculiarities. This species is reluctant to express Arthus reactions, produces an unusual anaphylactic response, exhibits poor delayed hypersensitivity reactions, and produces only weakly precipitating antibody (Winebright and Fitch, 1962), (Flax and Waksman, 1962), (Waksman, 1968). Rats show no immune function during fetal life (Solomon, 1971).

Neonatal rats of some strains reluctantly reject allografts and can be tolerated by injection of allogeneic cells. Rawles (1955) placed 20 day allogeneic fetal grafts on neonatal recipients and found a mean survival time of 2-3 months. Medawar and Woodruff (1958) found that adult skin was more readily rejected than fetal skin when grafted to neonatal rats. Peterson (1959) was able to tolerate one-day-old rats with spinal cord extract as evidenced by failure to develop autoimmune encephalomyelitis upon challenge as adults. The results suggested that only a T-cell tolerance was induced since anti-spinal cord antibody was produced in these animals. Steinmuller
(1961) found Lewis rats rejected BN allografts as rapidly as an adult when only 3 days old, but BN rats did not develop full immunocompetence to reject Lewis grafts until 10 days of age. Apparently the maturation of cellular immunity in the rat is dependent upon the strain and therefore any general conclusions about the species would be invalid.

The humoral limb of the immune response in the rat does not produce detectable circulating antibodies until birth. Bauer et al. (1956) injected sheep erythrocytes into 15 day fetal rats. Ten weeks after birth the rats were challenged and no difference was found in hemolysin titers of the prenatally injected rats and controls. No priming or tolerizing had occurred. Nossal (1958) tolerized one-day-old rats to foreign erythrocytes with a single injection, suggesting that the immune system was still in an under-developed stage. Rowley and Fitch (1965) studied the maturation of the plaque-forming cell response following antigenic stimulation with sheep erythrocytes. They found the peak number of plaque-forming cells occurred at day 4 post-injection. Newborns could not be stimulated above background. A significant increase in plaque-forming cells occurred during the third week of life with the response reaching adult levels at day 34. No further increase in plaques was observed up to 146 days. IgG plaque-forming cells could not be stimulated until rats were 21 days old. Halliday (1957) injected ten-day-old rats with Salmonella pullorum and obtained antibodies after eight days, however if eleven-day-old rats were used, serum antibody was detectable after only 3 days. Halliday (1964) found serum antibody to Brucella abortus in
ten-day-old rats which were injected neonatally, however the level of response did not reach adult levels in the time of onset until the rats were 16 days old before injection. The amount of antibody produced by injection of *Brucella* increased ten-fold during the first month of age and then remained constant as revealed by injection of rats in various age groups. Hervey (1966) reported agglutinating antibody to *Brucella* at 7 days after injection of neonatal rats. Malberg and Rierl (1969) immunized ten-day-old rats with sheep erythrocytes and found one plaque-forming cell/10⁶ spleen cells on day 4 post-injection, however in 15-day-old rats the number of plaque-forming cells had increased one hundred times. It should be emphasized here that detection of native antibody in early postnatal life would depend upon the level of maternal antibody of the same specificity, which could inhibit antibody production. The half-life of maternal antibodies is 5.5 days (Bangham and Terry, 1957), a point worth considering in evaluating the results of the above investigations. The delay in antibody production in young rats to some of the antigens described above may be dependent upon the T-cell dependency of the antigen in question. Veerman (1975) found that rats responded to thymus-independent antigens at a much younger age (9 days) than to a thymus-dependent antigen (15 days). Histologic examination of spleens revealed the thymus-dependent areas to be poorly developed until rats were 15 days old suggesting that the delay in antibody responses was caused by a helper T-cell deficiency. Nossal et al. (1964) reported a brisk IgM response to flagella or polymerized flagellin in adult rats, followed by a prolonged IgG response. Williams (1966) found
rats unresponsive to polymerized flagellin until they were 20 days old which casts doubt on the work of Veerman (1975) described above since polymerized flagellin is a thymus-independent antigen.

Few studies on the development of the natural immune response in rats have been reported. Nossal and Ada (1971) discussed natural antibodies and their influence on the immune response to flagellin, suggesting that a true primary response was not attainable without a germfree environment. Jankovic et al. (1962) reported natural antibody titers to heterologous erythrocytes were not affected by neonatal thymectomy. Solomon et al. (1972) investigated the normal plaque-forming cell response in the rat. Hemolytic plaques were first detectable at 15 days age and reached adult levels by 31 days. Bolton and Chorpenning (1974) reported a switch from IgM to IgG production and the onset of delayed hypersensitivity in the natural response to glycerol-teichoic acid antigen at 20 weeks of age in normal rats.

In summary one may conclude from the above observations that the rat is capable of an immune response at 7-10 days from birth, but may not reach its full immune capability for several weeks depending on the nature of the antigen and strain of rat. The IgG response in the rat lags several days behind the appearance of IgM as evidenced by investigations of both serum antibodies and plaque-forming cells. This lag may be the result of a helper T-cell deficiency since rats responded to a thymus-independent antigen at an earlier age than to thymus-dependent antigens. This delay in helper T-cell function has been confirmed in the mouse by Chiscon and Golub (1972).
The interaction of cells involved in the immune response has been established for some time (Katz and Benacerraf, 1972), however the relationship between the occurrence of cellular and humoral responses has not been clarified and several conflicting reports on the nature of such a relationship are in the literature.

Dienes and Mallory (1932) suggested that delayed hypersensitivity was the first stage of the immune response on the pathway to antibody formation. Jones and Mote (1934) reported delayed hypersensitivity responses preceded immediate responses when humans were given injections of guinea pig peritoneal fluid. Simon and Rakeman (1932) observed a similar sequence of events when humans were injected with guinea pig serum. Salvin (1958) injected guinea pigs intradermally with ovalbumen and diphtheria toxoid and observed an initial delayed hypersensitivity response which disappeared with the onset of Arthus reactivity and the appearance of circulating antibody. He found that an increase in the quantity of antigen administered shortened the transition time from the cellular to the humoral response. Sell and Weigle (1959) observed development of delayed hypersensitivity in guinea pigs following injection of antigen-antibody complexes. This reaction gave way to Arthus reactivity. They suggested that delayed hypersensitivity responses appear during immune elimination and when elimination is complete, free circulating antibody and Arthus reactivity appear with a decline in the delayed response.
Benacerraf and Gell (1959a,b) induced delayed hypersensitivity by injection of antigen-antibody complexes. They suggested that the specificity of the delayed response is broader and precedes the more specific antibody response. They also induced delayed hypersensitivity to protein carriers and antibody to conjugated haptens, demonstrating the phenomenon of carrier specificity. Gell and Benacerraf (1961a) later proposed carrier specificity to be broader than hapten specificity.

The preceding investigations suggested that delayed hypersensitivity developed as an early stage of the immune response preceding antibody production. Gell and Benacerraf (1960) cast doubt on this theory by demonstrating that delayed hypersensitivity responses can be produced without boosting the antibody response, suggesting the two are independent, although coincident in some instances. Bolton and Chorpenning (1974) presented evidence which further refutes the early theories discussed above. They found naturally-occurring antibodies in rats before any cell-mediated responses were detectable using the chemically-defined glycerol-teichoic acid antigen.

The likelihood of relationship between cellular and humoral responses does exist, however the mechanisms remain unclear at present. Several investigators have demonstrated selective depression of either delayed hypersensitivity or antibody production. Asherson and Stone (1965, 1966) reported that animals pretreated with antigen in saline failed to develop delayed hypersensitivity upon subsequent challenge of the same antigen incorporated in adjuvant. There was little effect on the antibody response. This phenomenon was referred to as immune
deviation (Asherson, 1966) or split tolerance (Crowle and Hu, 1966). Parish (1971a,b) produced a series of acetoacetylated flagellin derivatives which he used to immunize rats. He found that as the flagellin was more heavily acetoacetylated, it lost its antibody affinity and its ability to induce antibody formation, but was enhanced in its ability to produce delayed hypersensitivity and antibody tolerance. Parish (1971a,b) suggested an inverse relation between delayed hypersensitivity and antibody production. Antigen with a high affinity for immunoglobulin receptors on cell surfaces would stimulate antibody production, whereas low affinity antigenic determinants would induce delayed hypersensitivity. Benacerraf and Gell (1959a) had previously reported chemical modification of a protein enhanced delayed hypersensitivity without affecting antibody production.

Neta and Salvin (1973) suggested a different relation between cellular and humoral responses than those mentioned above. They found a decline in delayed responses one day preceding the appearance of circulating antibody when animals were sensitized with the use of incomplete Freund's adjuvant. They could adoptively transfer the suppression of delayed hypersensitivity with spleen and peritoneal exudate cells in previously sensitized recipients. This suppression was not dependent on the amount of circulating antibody or the antigen dose, but was later shown to possibly involve a suppressor B-cell (Neta and Salvin, 1974).

Several investigators have proposed that the antigenic determinants for cellular and humoral immunity are different (Gell and Benacerraf, 1961b), (Turk, 1967), (Schlossman and Levine, 1967).
A striking example of these differences was reported by Mauel et al. (1970) who demonstrated that lymphocytes of C3H mice which had been immunized with mastocytoma cells derived from DBA/2 mice destroy the mastocytoma cells in vitro. These two strains differ at the H-2 locus by 12 serologically distinct antigenic determinants. C57Bl/6 mice bear 6 determinants which DBA mice possess and C3H lack. Moreover, sera produced by C3H mice against DBA/2 cells will interact with determinants of C57Bl/6 mice. Cells from C3H mice immune to DBA cells will not produce any cytotoxic effect on C57Bl/6 cells even though serological determinants are shared with DBA mice. This work demonstrated the broader specificity of the determinants required for cell-mediated immunity as compared with the narrower serological specificity. Parish (1971b) also suggested a difference in specificity requirements for cellular and humoral responses which was suggested by his work with acetoacetylated flagellin. One explanation for the less rigorous specificity requirement for delayed hypersensitivity responses is the lower density of receptors on T-cells as compared to B-cells (Enger and Unanue, 1974).
THE GNOTOBIOTIC ANIMAL AS A TOOL FOR THE STUDY OF NATURAL ANTIBODIES

The idea of rearing germfree animals stems from the pure culture concept in bacteriology and was initially that of Pasteur (1885), who proposed the use of a chicken egg which could be cleaned and placed in an isolated environment before hatching. The term gnotobiotic which means "know life complete" is used in referring to such animals, however a better term may be gnotobiota as suggested by Trexlar (1959) since one can not define the complete biological isolation of an animal. Nencki (1886) replied to Pasteur's idea with a statement arguing life was impossible without bacteria because of their necessity in the digestion process, however he predicted that a germfree animal, if obtained, would be healthier and live longer. The work of Kijanazzen (1895) discredited the idea. Rabbits raised in a sterile environment lost weight and died; their death being attributed to loss of microorganisms.

Germfree animal research actually began with the experiments of Nuttall and Thierfelder (1895-1896) who tried rearing germfree guinea pigs. Their primary purpose was to determine if life without microorganisms could be sustained. They concluded that bacteria were needed to digest cellulose containing materials, but not for digestion of food of animal origin. Nuttal and Thierfelder faced all of the problems encountered today in germfree research. They learned to determine parturition time, developed anesthetic, sterilization, and filtering procedures, as well as an isolator. Several groups over a 60 year period tried rearing healthy germfree guinea pigs, but it
wasn't until 1960 that B.A. Teah successfully reared these animals through a full generation.

Germfree rats presented more of a problem since they must be nursed at birth whereas the guinea pig could live off solid food. Reyneirs and Trexlar (1943) reported difficulty in rearing these Caesarian-derived animals because of their small size at birth. Gustafson (1947) obtained better results by feeding the newborn rats a milk diet. The first successful germfree rat colony began in 1950 at the Lobund Institute. Reyneirs (1959) reported these rats had survived 12 generations. The problems in rearing these animals and some solutions were outlined by Pleasants (1959) in experiments where 83% of the newborn rats were successfully weaned.

Nutrition has played a leading role in the development of germ-free research. Serious failures can be attributed more to poor nutrition than to unresolved contamination problems. Nuttal and Thierfelder (1896) and Schottelius (1908) both reported nutritional deficiencies in their attempts to maintain germfree animals. Sterilization of diets, liquid or solid, constantly caused problems because of denaturation of nutrients, protein coagulation, and precipitation. This problem has been greatly alleviated by the use of enriched solid diets and water-soluble liquid diets for rearing newborns. More is known about the nutritional requirements of the rat than any other species because of this dietary problem and it prompting the development of chemically-defined water-soluble diets (Greenstein et al., 1957, 1960), (Pleasants et al., 1970).
The immunologist studying natural antibodies in germfree animals must be aware of the nutritional problems mentioned above. Initially one would expect the germfree animal to be an ideal model for investigating naturally-occurring responses, however there are some faults in such an animal model. Most diets fed to germfree animals are antigenic. Wagner (1959) reported Micrococcus sp. in autoclaved food still remained antigenic. Michael et al. (1962) and Landy et al. (1962) reported lower levels of natural antibodies in germfree animals, but not total absence as reported by Springer et al. (1959) in his study of natural heteroagglutinins in germfree chicks. Nordin (1968) reported the occurrence of plaque-forming cells in germfree mice and Westmann et al. (1971a) reported a very slow buildup of immunoglobulins in germfree rats even though they were reared on a chemically-defined water-soluble diet of low antigenicity. Germfree mice produced no detectable immunoglobulins until they matured when reared on the same type of diet, but did produce plaque-forming cells (Westmann et al., 1971b). Frederick and Chorpenning (1972) and Rozmiarek et al. (1969) found naturally-occurring antibodies to glycerol-teichoic acid in germfree guinea pigs. One can not be certain whether or not the animals in the above experiments were exposed to antigenic specificities being examined since no effort was made to specifically remove such determinants from their environment. Therefore, it remained difficult to utilize the germfree animal in the study of origins of natural antibodies. Kim et al. (1966a,b) solved part of this environmental stimulation problem by rearing colostrum-deprived germfree piglets on an artificial diet. They reported that
these animals were free of detectable immunoglobulins and were immuno-
competent. The group later concluded that natural antibodies do not
arise without antigenic stimulation (Kim et al., 1974). Sterzl et al.
(1965) reported colostrum-deprived germfree piglets fed a synthetic
diet produced no antibodies to bacterial, viral, or protein antigens.
A fault with these experiments was that they were continued for only
short periods of time and with young animals.

General immunologic status of the germfree animal. Wostmann
(1959) reported germfree rats produced reduced levels of serum im-
munoglobulins, but that contamination with bacterial flora resulted
in a slow increase of these proteins to conventional levels (Wostmann
and Gordon, 1960). Miyakawa (1959) found that peripheral lymphoid
tissue in adult germfree guinea pigs was similar to tissue found in
conventional neonates. Olson and Wostmann (1966) found 8-12 times
fewer blast cells and plasma cells in germfree mice, however both
germsfree and conventionals responded equally well to antigenic chal-
lenge. These findings indicated germfree animals possessed poorly
developed, but functionally competent immune systems. Several other
investigators have confirmed this. Gustaffson and Lowell (1958,1959)
reported the concentration of gamma globulin in germfree rats to be
10-15% of that in conventional animals, but upon contamination with
flora, the levels rapidly increased. Cooper et al. (1968) found germ-
free rats able to produce germinal centers and hemolytic antibody when
injected with sheep erythrocytes in the Peyer's patches, tissues which
are practically non-existent in the unstimulated germfree rat. Bosma
et al. (1968) presented evidence indicating the full competence of
lymphoid cells in the germfree rat. Germfree donor lymphocytes were capable of restoring normal responses to irradiated conventional recipients.

Some investigators have reported better responses to antigenic stimuli in germfree than in conventional animals. Ikari (1964) found that bactericidal antibody titers rose more sharply in germfree than in conventional mice. Stashak et al. (1970) found germfree and conventional mice equally responsive to bacteriophage OX-174 with respect to time of onset, but the germfree animals produced higher titers of neutralizing antibodies. The findings in immunized germfree animals were analogous to the natural antibody levels to glycerol-teichoic acid found in germfree guinea pigs by Rozmiarek et al. (1969), in which titers rose much sooner in the germfree when compared to conventionals. In contrast to the above, Wostmann et al. (1971) reported that germfree rats were poorly competent in fighting off infection with Salmonella typhimurium, which could possibly be the result of a defect in cell-mediated responses as demonstrated in germfree guinea pigs (Pollard and Nordin, 1971).

In summary, one may conclude that the germfree animal provides an acceptable model for studying host-environment relations provided one defines his systems carefully. The peripheral lymphoid system in these animals is small and relatively inactive, but functionally intact. Germfree and conventional animals produce quite similar responses to parenteral antigenic challenge in most instances, however their natural responses may be altered depending on the conditions under which the animals were reared. The isolated conditions under
which gnotobiotic animals are maintained provides an excellent model for studying the influence of environment on immune responses with a possibility of initiating true primary responses. Results of such experiments may have a significant impact on our understanding of the immune system since much of the previous work has been performed on conventional animals which may have been previously exposed to the antigenic specificities studied in any given experiment, as it is extremely difficult to prove or disprove whether previous exposure has occurred without having complete control of the animals' environment. In this investigation the germfree rat was utilized in the study of natural responses to glycerol-teichoic acid. An environment and diet free of detectable teichoic acid was created in a gnotobiotic isolator with hope of producing an animal which was immunologically virgin to this antigen specificity. Results of such investigations may clarify the role of teichoic acid as an environmental antigen in stimulation of natural responses.
A number of authors have reviewed the subject of natural antibodies (Wilson and Miles, 1964), (Boyden, 1966), (Michael, 1969). A complete review is beyond the scope of this monograph and therefore the discussion will be limited primarily to literature describing natural antibacterial antibodies since this investigation involved an examination of natural antibodies of the glycerol-teichoic acid specificity.

One of the earliest studies of natural antibacterial antibodies was by Burgi (1907), who found normal sera from a number of mammalian species would agglutinate bacteria, however much of the activity was considered to be non-specific. Gibson (1930) tested normal sera from several species including the rat and found by absorption that some specific factors were involved in the agglutination of several species of enteric bacteria. Mackie and Finkelstein (1930, 1931, 1932) found natural complement-fixing and bactericidal antibodies in a number of species to a wide range of bacteria, mostly Gram-negative. They also showed the bactericidal effect to be complement dependent. Neter et al. (1959) found a low incidence of natural antibodies to Shigella dysenteriae and Salmonella paratyphi A in pooled human serum from the United States and Japan, but a high incidence in sera from India where infection is prevalent. Michael et al. (1962) demonstrated in the sera of man, rabbits, guinea pigs, rats, and mice bactericidal antibodies to Salmonella typhi, Escherichia coli, Shigella dysenteriae, Pseudomonas aeruginosa, and Serratia marcescens. These
antibodies were heat stable, specific, and as avid as those obtained by immunization. Neonatal mice obtained these natural antibodies at one week of age, but rats did not acquire them until four weeks of age. Germfree rats had no antibodies to *E. coli* or *S. typhi* while germfree mice produced as many as conventional animals. Michael et al. (1962) proposed that the rapid acquisition of natural antibodies after birth indicated their elicitation by an external stimulus and that the wide variation in titer to seven different antigens ruled out spontaneous origin.

Bactericidal tests were employed by Michael et al. (1962) and Muschel (1961) to detect natural antibodies. Serum-sensitive strains were found in each Gram-negative species and their test was able to measure as little as 0.0005 ug antibody nitrogen. Landy and Weidanz (1964) found bactericidal antibodies in all normal mammalian species tested. Serial studies in rabbits and humans showed that levels remained constant for several years.

Many studies on natural antibodies to Gram-positive bacteria have utilized staphlococci. Cohen et al. (1961) found natural anti-staphlococcal antibody in the serum of normal pathogen-free rabbits and later (1963) in mice. Jensen (1958) identified a precipitating natural antibody against the A antigen of staphlococcus in all human sera tested. Stamp and Hobbs (1967) reported natural antibodies to *S. pyogenes* in rabbits. Chorpenning and Dodd (1966, 1967) found that normal human serum contained natural antibodies which reacted with heterogenetic bacillary antigens extracted by boiling from Gram-positive bacteria. Anderson et al. (1967) reported inhibition of these
reactions with bacillary antigens by glycerophosphate as well as a number of other substances. Chorpenning et al. (1969) found natural anti-bacillary antibodies in rabbits, sheep, swine, and guinea pigs. The bacillary antigen described above was characterized as a glycerol-teichoic acid of polyglycerophosphate specificity by Decker et al. (1972), who reported the occurrence of natural anti-PGP antibodies in man, guinea pigs, and rabbits with a 100% frequency. Bolton and Chorpenning (1974) found natural anti-PGP antibodies in rats with a frequency approaching 100%. Natural cell-mediated responses to teichoic acid were reported by Martin et al. (1966) in humans, in guinea pigs by Frederick et al. (1972), and in rats (Bolton and Chorpenning, 1974).

**Origin of natural antibodies.** Early investigators pondered over the origin of natural antibodies as to whether they were produced due to the genetic composition of an animal or whether these antibodies arose because of prior infection or environmental stimulation (reviewed by Wilson and Miles, 1964). The question arose because of many reports which showed a lack of correlation between the presence of natural antibacterial antibodies in an animal and its past history of disease along with composition of normal flora (Burgi, 1907), (Gibson, 1930). Other authors found good correlations between natural antibodies and normal flora (Von Bunza, 1937), (Silverthorne, 1936). It was not until recent times that more conclusive results were obtained.

The more recent investigations of natural antibodies used young animals which were mostly incapable of antibody production and had
little chance of contacting large amounts of environmental stimuli, however Michael et al. (1962) and Landy and Weidanz (1964) found sera of newborn rabbits, rats, guinea pigs, and mice to have low levels of maternal bactericidal antibody, thus making it difficult to draw conclusions as to the origin of the stimulus. The gradual appearance of antibacterial antibodies during development may be caused by increased exposure to environmental stimuli or by maturation of the immune system (Michael et al. 1962). The development of germfree animal models provided a controlled environment for studying origins of natural antibodies and helped clarify some of the controversy, however in many instances the food given these animals still contained bacterial antigens and the animals produced natural antibodies (Wagner, 1959), (Michael et al., 1962), (Landy et al., 1962). In contrast, Landy and Weidanz (1964) observed lower antibody levels in germfree animals when compared to conventional animals. In their initial studies solid food was used whereas in the later work autoclaved cow's milk was used as a diet, which probably accounted for the discrepancy in their findings. Springer et al. (1959) reported an absence of natural anti-human group B agglutinins in germfree chickens, however these animals could be stimulated to produce such antibodies by addition of E. coli to their environment. Ikari (1964) observed fewer bactericidal antibodies in germfree than in conventional mice. Sterzl et al. (1962) found no bactericidal activity in germfree colostrum-deprived piglets, however when the piglets were colonized with E. coli, their serum became bactericidal within a week. Kim et al. (1966a, 1966b) reported that germfree colostrum-deprived piglets
were free of immunoglobulins but were immunocompetent and later stated that antibodies do not develop without antigenic stimulation (Kim et al., 1974). In contrast, Wostmann et al. (1971a, 1971b) found a very slow immunoglobulin buildup in germfree rats reared on a low molecular weight diet. Considering this finding along with those of Michael et al. (1962) in which rats were shown not to develop bactericidal antibody to S. typhosa until they were eight weeks old, one must conclude that previous short-term experiments with germfree systems have not satisfactorily defined the origin of stimulation for natural antibodies and therefore experiments of long duration are needed since these antibodies apparently develop slowly as the animal matures and is exposed to greater amounts of antigen in his environment.

A number of investigators have used the immunosuppressive effect of irradiation to study the origin of natural antibodies. Michael et al. (1962) observed no reduction of natural antibody levels to enteric bacteria in irradiated mice. Steward et al. (1964) obtained similar results with irradiated guinea pigs, however Hook et al. (1963) reported that bactericidal antibodies to Shigella declined in irradiated guinea pigs. Muschel et al. (1968) suppressed the immune response in dogs to Salmonella vaccine by injection of anti-lymphocyte serum, however the production of natural antibody to Shigella was not affected. One may argue that these immunosuppressive agents act only on the inductive phase of antibody formation and therefore if the antigen and sensitized cells are already present upon administration, the response remains unaffected.
Nature and immunological significance of natural antibodies.

Natural anti-bacterial antibodies do not cross the placenta in most species (Michael, 1969), the reason being that high molecular weight antibodies do not penetrate the placenta (Gitlin et al., 1963). Michael and Rosen (1963) showed natural antibodies as measured by mouse protection, passive hemagglutination, bactericidal tests, and opsonic tests, were macroglobulins (IgM). Rowley and Jenkin (1962) found 19S opsonic antibodies in normal pig serum while Turk (1959) found natural antibacterial antibodies in the pig which were 7S gamma globulins. Cohen and Norins (1966) found natural 7S gamma globulin antibodies in human serum against Neisseria by indirect fluorescence. Decker et al. (1972) reported that natural antibodies to glycerol-teichoic acid were of both 19S and 7S classes in humans and guinea pigs as measured by passive hemagglutination. Bolton and Chorpenning (1974) found both 19S and 7S antibodies of the same specificity in rats. Frederick and Chorpenning (1974) characterized natural antibodies to teichoic acid and found them not unlike immune antibodies of the same specificity. It should be noted that most assays used above for detecting natural antibodies are most sensitive in detecting IgM which could account for discrepancies among different investigations.

Natural anti-bacterial antibodies may play an important role in host defense. Keyes (1916) reported bactericidal antibody in pigeons to pneumococcus, an organism to which pigeons are naturally resistant. Lovell (1951) showed a clear correlation between the incidence of anti-\( \text{E. coli} \) natural antibodies in cows and their resistance to
scours. Michael et al. (1961) found that injection of endotoxin increased natural antibody levels and improved anti-bacterial defenses of the animal. Landy et al. (1962) found no difference in the ability of germfree and conventional mice to resist Salmonella infection, whereas Wostmann et al. (1971) found germfree mice more susceptible. Several investigators have suggested that teichoic acid antibodies are involved in protection (Mudd et al., 1963), (Ekstedt, 1966), (Yoshida and Ekstedt, 1968), (Cameron, 1969), although Shayegani et al. (1970) reversed the earlier conclusions of Mudd et al. (1963). Stamper (1972) recently showed in preliminary experiments that naturally-occurring guinea pig antibodies which are specific for PGP protect mice against lethal doses of Bacillus species (OSU No. 372).

In summary, one can state that natural antibodies are present in most mammalian species and they are immunochemically similar to immune antibodies. There is much evidence pointing to environmental antigens as the source of stimulation for these naturally-occurring antibodies. Results of some investigations suggest a protective effect of natural antibody against bacterial infection, however the interaction of the animals' immune system and the environment produces so many variables that it becomes difficult to make generalizations from previous work performed under such a wide variety of conditions with so many different species.
Rantz et al. (1952) found that filtrates of certain Gram-positive bacteria (group A streptococci, Staphlococcus aureus, pneumococci, and enterococci) modified erythrocytes so that they were hemolyzed by human sera and complement. This activity was later found to be absent from all Gram-negative organisms (Rantz et al., 1956). The substance appeared to cross species lines and was therefore named non-species-specific substance (NSS). It was heat stable in acid, non-dialyzable, and thought to be a polysaccharide.

In 1958, Armstrong et al. found that polymers of ribitol and glycerol-phosphate were present in the cell walls of some bacteria. Baddiley et al. (1958) isolated these polymers from Lactobacillus and Bacillus species and later McCarty (1959) extracted a substance from streptococci and identified it as a polyglycerophosphate. Neter et al. (1959) demonstrated that Staphlococcus aureus and Bacillus subtilus possessed cross-reacting antigens. Antibody to these antigens was detectable in normal human serum by passive hemagglutination. They later suggested that this substance was the Rantz antigen and that it was polyglycerophosphate (Neter et al., 1960). Anzai et al. (1960) studied 18 strains of the genus Bacillus and found all but one species, Bacillus brevis, contained the Rantz antigen. The name teichoic acid was given to polymers of ribitol and glycerol-phosphate by Archibald and Baddiley (1966). Jackson and Moskowitz (1966) and McCarty (1964) found D-alanine to be a constituent of the teichoic acid molecule they were studying, however the serological specificity of the precipitin
reaction appeared to be directed to the PGP portion of the molecule (McCarty, 1959), since the reaction was inhibited with synthetic PGP. Alanine was probably not detectable in the earlier investigations because of its labile ester linkage to the PGP backbone of the teichoic acid antigen (Jackson and Moskowitz, 1966). Ofek et al. (1975) showed that a purified streptococcal lipoteichoic acid, lacking detectable alanine, retained erythrocyte-coating activity and provided evidence indicating lipid was involved in the cell coating. Wicken and Knox (1975) also suggested that the teichoic acid antigen could be immunogenic and could spontaneously coat erythrocytes only if it were a lipoteichoic acid. Teichoic acid, as it exists in Gram-positive bacteria, has been studied extensively in a number of laboratories, however only recently Gmeiner (1975) reported that ribitol phosphate is a constituent of lipopolysaccharide from Proteus mirabilis. The significance of Gmeiner's finding remains questionable since the ribitol-teichoic acid found in Gram-positive bacteria consists of polyribitolphosphate.

Chorpennning and Dodd (1965) found a Bacillus species in the donor bottle of a patient who had experienced a bacteriogenic transfusion reaction, and later reported that all Gram-positive organisms altered erythrocytes in a similar way except for Corynbacteria. They also demonstrated the activity in extracts from B. brevis. Altered cells reacted with specific bacterial antisera and with normal human sera (Chorpennning and Dodd, 1966). Anderson et al. (1967) showed passive hemagglutination inhibition of these altered erythrocytes by sugars, uronic acids, amino acids, and glycerol-phosphate. Schmidt (1968)
found that the major component of the antigen was PGP and that it could be precipitated at low pH from crude bacillary extracts. Decker et al. (1972) purified and characterized the glycerol-teichoic acid antigen extracted from a *Bacillus* species (OSU No. 372), a preparation which was capable of erythrocyte coating in concentrations as low as 0.005 μg/ml. Synthetic polyglycerophosphate inhibited the passive hemagglutination reaction when this purified antigen was used to coat erythrocytes. Chorpennning and Stamper (1973) found that optimal erythrocyte coating by the teichoic acid antigen was time, temperature, and pH dependent. It was also reversible.

A glycerol-teichoic acid was extracted from *Streptococcus mutans* BHT by Chorpennning et al. (1975). The antigen was determined to be a glycerol-phosphate by passive hemagglutination inhibition with synthetic polyglycerophosphate and also produced bands of identity in gel diffusion with extracts of *Staphlococcus aureus* and *Streptococcus pyogenes* against antisera to *Bacillus* species (OSU No. 372) and *Streptococcus mutans* BHT and AHT. Cooper et al. (1975) analyzed the cell wall components of *S. mutans* BHT and found strong evidence for the presence of cell-wall associated glycerol-teichoic acid.

Chorpennning et al. (1969) found naturally-occurring anti-bacillary antibodies in rabbits, sheep, swine, and guinea pigs. Decker et al. (1972) reported the occurrence of natural antibodies to teichoic acid in humans, guinea pigs, and rabbits with a 100% frequency. Frederick and Chorpennning (1974) characterized these natural antibodies to teichoic acid and found them not unlike immune antibodies of the same specificity. Frederick et al. (1972) reported natural
cell-mediated immunity to teichoic acid in guinea pigs and Bolton and Chorpenning (1974) found naturally-occurring cell-mediated and humoral responses to glycerol-teichoic acid in rats.
The idea that antibody plays a regulatory role in the immune response was first suggested by Uhr and Bauman (1961), who found passive administration of antibody suppressed antibody formation of the same specificity. It was later demonstrated that the suppression resulted from the covering of specific antigenic determinants, thus inhibiting stimulation (Dixon et al., 1967). Apparently only B-cell responses were inhibited in these experiments since anamnestic responses were not susceptible to inhibition, possibly because of failure to inhibit helper T-cell activity (Uhr and Bauman, 1961), (Weigle, 1975).

Further evidence for the role of antibody in regulation of the immune response arose from experiments in which antibody cycling was observed. Moller (1965) observed a fluctuation in antibody levels after a single injection of antigen. He observed two peaks of antibody-producing cells in mice after a single intravenous injection of lipopolysaccharide (LPS), a T-cell independent antigen. Britton and Moller (1968) observed four cycles of direct plaque-forming cells (PFC) over a 46 day period in mice injected once with E. coli. They interpreted their findings as evidence that IgM antibody produced during the immune response blocked antigenic stimulation of competent cells. After the antibody coating the antigen was catabolized, free antigen could again stimulate antibody-forming cells, thus giving a cycling effect.
Several investigators have reported antibody cycling following a single injection of sheep erythrocytes (SRBC), a T-cell dependent antigen. Wortis et al. (1966) found a biphasic appearance of both IgM and IgG PFC in mice following a single injection of antigen, with peaks occurring at days 4-6 and 9-11. Sell et al. (1970) repeated this experiment with a different strain of mice and found cycling of IgG PFC, but not of IgM. Recent investigations of antibody cycling have involved the use of aggregated gamma globulins. Rabbits were injected intravenously with aggregated human IgG (A Hu IgG) and produced cycling in both IgM and IgG PFC with peaks at 5 and 12 days (Romball and Weigle, 1973). They repeated the experiment with aggregated turkey IgG. A biphasic response was observed only in IgG PFC. The circulating precipitating antibody titers lagged several days behind the peak PFC responses. Plaque tests were performed on peripheral blood lymphocytes in these experiments allowing individual animals to be observed for long periods of time.

Nossal and Ada (1971) have elaborately described the effect of route of antigen administration on the organ of localization and persistence. The cyclic production of antibody in any given lymphoid organ may depend on the localization of antigen in that tissue. Britton et al. (1968) demonstrated the persistence of SRBC for 14 days and LPS for 45 days following intravenous injection. Romball and Weigle (1973) using a similar system of cell transfer experiments showed aggregated human IgG $^{125}$I to persist in the spleen of rabbits following intravenous injection. Autoradiography revealed a dense localization in splenic germinal centers 10 days post-injection,
but no localization in the mesenteric lymph nodes. Plaque-forming cells were also found in the spleen, but not in the mesenteric lymph nodes. Romball and Weigle (1973) also reported intraperitoneal injection of xenogeneic tumors resulted in cycling of cell-mediated immunity in the spleen, but not in the lymph nodes. Eldenger and Pross (1967) found that intradermal injection of SRBC into mice resulted in cycling of PFC in the draining lymph nodes, but not in the spleen whereas intravenous injection produced cycling in the spleen and not in the lymph nodes. One may conclude from the above investigations that the organ in which antibody cycling occurs depends upon both the nature of the antigen and route of administration.

Previous failures to observe cycling in secondary responses (Schlegel, 1974), (Romball and Weigle, 1973) may have been due to a lack of persisting antigen since it has been shown there is more rapid disappearance of antigen when administered to a previously exposed animal (Weigle, 1975). Antigen persistence appears to be correlated with antibody cycling, however there is insufficient data to warrant generalizations. Further experimentation with the more persistent polysaccharide antigens could yield valuable information on the matter and possibly reveal cycling in an anamnestic response. Chen et al. (1973) observed cyclic variations in rabbits when given several injections of pneumococcal polysaccharide, however the patterns were not the same as the single injection experiments and therefore different mechanisms may have been involved, making it difficult to draw any conclusions.
Several mechanisms have been proposed in explanation of antibody cycling. It is well documented that passive antibody can inhibit the immune response and, after it is catabolized, the exposed antigen can again stimulate competent cells (Uhr and Moller, 1968). Weigle (1975) proposed that the newly released antigen contacts memory cells thus causing the sharp multiple PFC peaks he observed in the aggregated human IgG-injected rabbits (Romball and Weigle, 1973). The basis for this hypothesis was that when the rabbits were injected with low doses of antigen, a small first peak of PFC was observed followed by a large second peak suggesting that most cells contacted by antigen became memory cells, whereas if large doses were injected, the second peak was much smaller than the first since most cells had differentiated to antibody-forming cells upon initial contact, leaving few memory cells. Other suggested mechanisms for explaining antibody cycling include release of stored antigen from macrophages (Britton and Moller, 1968), different subclasses of immunoglobulin-forming cells having different response times (Wortis et al., 1966), and that responses to different antigenic determinants may occur at different times (Wortis et al., 1966). Weigle (1975) also proposed an alternation of suppressor and helper T-cell influence on B-cells as a possible cause of cycling. None of these ideas have been definitely proven or disproven by any of our current knowledge of the subject. Additional work is needed to clarify the mechanism and significance of the cycling phenomenon.
**Part I PRELIMINARY EXPERIMENTS**

**MATERIALS AND METHODS**

**Animals.** Albino rabbits (Kingswheel, Columbus, Ohio) were used for standard antiserum production. They were maintained in wire cages and fed Purina Rabbit Chow (Purina, St. Louis, Mo.). Animals were housed in the College of Biological Sciences Animal Facility with temperature maintained at approximately 72°C and utilizing 12 hr day/night lighting cycles.

**Erythrocytes.** All erythrocytes were obtained from adult Sprague-Dawley rats and from sheep which were housed in the College of Biological Sciences Animal Facility.

**Preparation of teichoic acid antigen.** A purified teichoic acid (TA) antigen of polyglycerophosphate specificity was employed in all experiments. The preparation and chemical properties of this antigen were described by Decker et al. (1972). A *Bacillus species* (OSU No. 372) was cultivated in flasks of Trypticase Soy Broth (BBL, Cockeysville, Md.) supplemented with 10% yeast extract (Difco, Detroit, Mich.) from which 200 ml of a 24 hr culture was inoculated into a model MF-14 fermentation apparatus (New Brunswick Scientific Company, New Brunswick, N.J.) containing 10 liters of the medium described above. Cells were cultivated 24 hr at an optimal temperature of 32°C (Stamper and Chorpenning, 1975) with continuous stirring (200 RPM)
and aeration (10 liters air/min). All cultures were examined in Gram-
stained smears to estimate purity. A Sorvall RC2-B centrifuge (Ivan
Sorvall, Norwalk, Conn.) equipped with a "Szent-Gyorgyi and Blum"
continuous flow adaptor was employed to harvest the cells. The bac-
teria were washed three times in 0.23 M sodium chloride and resuspend-
ed to a 10% concentration in distilled water for use in the phenol
extraction method of Moskowitz (1966). An equal volume of 95% aqueous
phenol was mixed with the cell suspension and stirred for 20 min. The
pH was then adjusted to 4.7 with 1 M sodium hydroxide and continuously
monitored with a Corning pH meter for 15 minutes with constant stir-
rning. The aqueous phase of this suspension was collected by centri-
fugation, dialyzed against 4 changes of demineralized double-distilled
water, and lyophilized.

Lyophilized phenol extracts were treated with excess bovine pan-
creatic ribonuclease type III-B (Sigma Chemicals, St. Louis, Mo.) for
48 hr at 37 C in phosphate-buffered saline (0.15 M NaCl, 0.06 M phos-
phate, pH 7.4) (PBS) containing 0.02% sodium azide. The extract was
then treated with crystalline trichloroacetic acid at 10% concentra-
tion followed by precipitation with cold 95% ethanol. Ethanol pre-
cipitates were redissolved in PBS and passed through a 2.5 x 40 cm
column of Sephadex G-100 (Pharmacia, Piscataway, N.J.). Those frac-
tions collected from the column having the ability to coat erythro-
cytes for passive hemagglutination were pooled and further purified
on a Bio-Rad 50WX4 cation exchange column (Bio-Rad, Richmond, Calif.).
Passively hemagglutinating fractions were pooled, dialyzed against 3
changes of demineralized double-distilled water and lyophilized.
Chemical analysis for glycerol content was performed by the method of Hagan and Hagan (1962) and phosphorous tests by the method of Ma and McKinley (1953), both under the conditions described by Decker et al. (1972).

**Passive hemagglutination test.** Activity of the teichoic acid antigen was determined by a checkerboard titration using the passive hemagglutination (PHG) method suggested by Chorpenning and Stamper (1974). Rat erythrocytes were washed 3 times in PBS. A 5% suspension of the cells was prepared volumetrically and 0.6 ml of the suspension added to 3.4 ml distilled water. Triplicate tubes were prepared and their optical densities measured on a Bausch and Lomb Spectronic 20 at 550 nm. All rat erythrocyte suspensions subsequently used were standardized at an optical density of 0.848. All quot of different concentrations of TA antigen were added in equal volumes (1 ml) to the standardized erythrocyte suspension and incubated for 25 min at 35 C. The TA-coated cells were washed thrice in cold PBS and diluted to one-fifth the original concentration. Equal volumes (0.05 ml) of coated cells from each antigen concentration and chilled dilution of standard antiserum (prepared by injection of Bacillus sp. (OSU No. 372) into a rabbit, harvesting the immune serum, and adsorbing with rat erythrocytes) were mixed, incubated 30 min at 5 C, and spun 15 sec in a serofuge (Clay-Adams, N.Y.). Results were read microscopically and the degree of agglutination scored as follows:

<table>
<thead>
<tr>
<th>Value</th>
<th>Description</th>
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<tbody>
<tr>
<td>4+</td>
<td>100% agglutination</td>
</tr>
<tr>
<td>3+</td>
<td>75% agglutination</td>
</tr>
<tr>
<td>2+</td>
<td>50% agglutination</td>
</tr>
<tr>
<td>1+</td>
<td>25% agglutination</td>
</tr>
<tr>
<td>0</td>
<td>no agglutination</td>
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</tbody>
</table>
Titers were expressed as the reciprocal of the last dilution showing agglutination. The TA concentration giving the highest PHG titer was considered optimal and a slight excess over this amount was routinely employed thereafter.

The passive hemagglutination inhibition test was used to estimate the degree of antigen purity. Microgram quantities of teichoic acid or synthetic polyglycerophosphate (PGP) prepared by the method of McCarty (1959) were mixed with the standard rabbit antiserum and incubated 30 min at 5°C. Erythrocytes coated with teichoic acid were then added and the PHG test performed as described above. The degree of inhibition produced by the teichoic acid antigen was then compared to a previously characterized preparation.

Standardization of the passive hemolysis test. The basic protocol for the immune hemolysis assay is described in Table 1.

Table 1. Protocol for the immune hemolysis assay.

<table>
<thead>
<tr>
<th>Sensitization</th>
<th>Mix equal volumes of rat serum and teichoic acid coated erythrocytes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysis</td>
<td>Add 5 hemolytic units of complement and incubate at 37°C.</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Five min in a serofuge.</td>
</tr>
<tr>
<td>Results</td>
<td>Read spectrophotometrically and calculate 50% hemolysis titer.</td>
</tr>
</tbody>
</table>

Since this investigation was to deal entirely with rats, it was desirable to attempt employment of rat erythrocytes and rat complement for the hemolysin assay to eliminate any involvement of heteroantibodies.
Combinations of rat erythrocytes or sheep erythrocytes with rat or guinea pig complement were tried using the protocol in Table 1. All reagents used in this assay were standardized as described below.

Erythrocytes were spectrophotometrically standardized by the method of Kent and Fife (1963). A volumetric 2% suspension of washed erythrocytes was prepared in veronal-buffered saline, pH 7.2. Triplicate 0.6 ml aliquots were added to 13 x 100 cuvettes containing 3.4 ml distilled water. The lysates were read spectrophotometrically at 550 nm. Sheep erythrocyte suspensions were then routinely standardized at 0.7 optical density and rat erythrocytes at 0.56 optical density with each usage. Cells were adjusted to the standard concentration by the formula:

\[ V_2 = \frac{V_1 \cdot \text{O.D.}_1}{\text{Std. O.D.}} \]

Complement and hemolysin titrations were performed by the method of Kent and Fife (1963). Commercial guinea pig serum (complement) (G.I.B.C.O, Lot No. A843121) and rabbit anti-sheep hemolysin (G.I.B.C.O, Lot No. A913712) were employed for the entire experiment. Rat complement was prepared by pooling fresh sera from 12 rats and storing at -70 C. Complement titration was performed as described in Table 2. A standardized 2% suspension of sheep erythrocytes was sensitized with an equal volume of 1/500 hemolysin for 10 min at room temperature. Dilution \( D_1 \) was a 1/25 dilution of guinea pig serum and \( D_2 \) was 1/10 of \( D_1 \). The results were plotted on log scale paper by the method of Kabat and Mayer (1961) and the 50% hemolytic endpoint (\( C_{H_{50}} \) unit) read directly from the graph. The number of \( C_{H_{50}} \) units/ml undiluted guinea pig serum was then calculated by the formula:
Table 2. Standardization of Complement.

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>Titration</th>
<th>Controls</th>
<th>Blank</th>
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<tbody>
<tr>
<td>1 2 3 4 5 6</td>
<td>7 8 9 10</td>
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</table>

<table>
<thead>
<tr>
<th>Complement</th>
<th>ml</th>
<th>0.25</th>
<th>0.30</th>
<th>0.35</th>
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<tr>
<td>$D_1$</td>
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<td></td>
<td></td>
<td>0.40</td>
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<tr>
<td>$D_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.45</td>
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<tr>
<td>Chilled VBSa</td>
<td></td>
<td>0.65</td>
<td>0.60</td>
<td>0.55</td>
<td>0.50</td>
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<tr>
<td>Sensitized erythrocytes</td>
<td></td>
<td>0.60</td>
<td>0.60</td>
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Mix well and incubate in a water bath for 30 min at 37 C.

Add 1 ml chilled VBS to each tube.

Mix well and centrifuge 5 min in a serofuge.

Read optical densities using tube 10 as a blank $b$.

Calculate the % hemolysis in tubes 1-6 using the mean O.D. value of tubes 7-9 as the 100% hemolysis value.

---

a veronal-buffered saline, pH 7.2.
b supernate should be transferred to a clean tube before using as a blank since red cells tend to adhere to glass in the absence of serum protein and this could interfere with light transmittance.
The hemolysin titration was performed as described in Table 3, and the complement titration repeated with the optimal dilution of hemolysin. Complement titrations using the teichoic acid specificity were performed in a similar manner using sheep erythrocytes coated with TA and rat anti-TA serum adsorbed with sheep erythrocytes. Titration of rat complement was performed as described above.

The result of the complement titration was confirmed by preparing a $1 \text{ C}_{50}$ unit solution of complement and allowing it to stabilize 30 min at 5°C. Triplicate samples of 0.06 ml volume were added to tubes containing 0.6 ml sensitized sheep erythrocytes previously prepared for the complement titration. The suspensions were incubated at 37°C for 30 min. The complement titration was considered satisfactory if the hemolysis was in the range of 45-55%.

Once the basic protocol for hemolysis with TA-coated erythrocytes was developed, a checkerboard hemolysis test was performed in a similar manner to that previously described for PHG.

Complement was stored at -70°C and reconstituted as needed by the addition of cold veronal-buffered saline. Anti-sheep cell hemolysin and other antisera were stored at -20°C. Erythrocytes were maintained in a 1/4 dilution of acid-citrate-dextrose (ACD) anticoagulant at 5°C. Cells were aged 5 days before use and discarded after 4 weeks.
Table 3. Standardization of hemolysin.

<table>
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<th>Tube Number</th>
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<table>
<thead>
<tr>
<th></th>
<th>Complete Lysis</th>
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<tbody>
<tr>
<td>Hemolytic antiserum diluted in VBS 1:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>3000</td>
<td>4000</td>
</tr>
<tr>
<td>5000</td>
<td>6000</td>
<td>7000</td>
</tr>
<tr>
<td>8000</td>
<td>9000</td>
<td>10000</td>
</tr>
</tbody>
</table>

Complement

\[ D_{50}^{14} C \text{H}_{50}/0.3 \text{ ml} \]

<table>
<thead>
<tr>
<th></th>
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VBS

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<tr>
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Sensitized cells

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Mix well and incubate for 30 min in a 37°C water bath.

Add 1 ml VBS to each tube and centrifuge for 5 min in a serofuge.

Read optical densities using tube 13 as a blank and calculate the % hemolysis in tubes 1-9 using the mean O.D. value of tubes 10-12 for 100% hemolysis. The optimal concentration of hemolysin is then determined graphically.
RESULTS AND DISCUSSION

Teichoic acid preparation. A ten liter 24 hr culture of Bacillus species (OSU No. 372) yielded approximately 130 grams of packed wet cells. Phenol extraction of this quantity of bacteria produced 150-200 mg of TA antigen. The final recovery of the purified TA antigen was 10-15% of the phenol extract by weight, however this figure was variable due to losses during dialysis and trichloroacetic acid treatment. Chemical analysis of the batch of TA used in this work showed a 1:1 molar ratio of glycerol to phosphorous with 75% of the antigen weight being attributed to glycerol phosphate (Cooper and Alexander, 1975). The results of the passive hemagglutination inhibition assay are shown in Figure 1. Complete inhibition was obtained with 0.15 ug antigen and 1000 ug of synthetic PGP. The above assays compared favorably with those of Decker et al. (1972) who reported 0.1 ug of antigen and 860 ug synthetic PGP completely inhibited the passive hemagglutination test. The large amount of synthetic PGP required for inhibition was due to its very short chain length (probably less than 5 residues long) as compared to the very long PGP polymer in the natural TA preparation.

The optimal concentration of teichoic acid needed for erythrocyte coating in the passive hemagglutination assay was 50 ug/ml (Figure 2), however some agglutination was detected with erythrocytes coated with one ug/ml of the antigen. The optimal concentration of teichoic acid for passive hemolysis was 10% of the amount needed for erythrocyte coating in passive hemagglutination assays (Figure 3).
Figure 1. Passive hemagglutination inhibition assay indicating the specificity and purity of the teichoic acid antigen used in this investigation. TA-coated rat erythrocytes and the standardized rabbit antiserum were employed. The antiserum was previously absorbed with 2% rat erythrocytes for 30 min.
Figure 2. Checkerboard titration determining the optimal concentration of TA required for red cell coating in the passive hemagglutination assay. TA-coated rat erythrocytes and a standardized rabbit serum (previously absorbed with 25% rat erythrocytes) were employed.
Figure 3. Checkerboard titration determining the optimal concentration of teichoic acid for red cell coating in the passive hemolysis assay. TA-coated sheep erythrocytes and an immune rabbit antiserum (absorbed with 25% sheep erythrocytes for 30 min) were utilized.
Five ug/ml was sufficient for the batch of antigen employed in this investigation. The low concentration of teichoic acid needed for hemolysis was advantageous since it alleviated the problem of agglutination during the sensitization process which occurs prior to the addition of complement.

Hemolysis tests. The guinea pig complement titration results are shown in Figure 4. This lot of guinea pig complement contained 581 C H₅₀ units. Similar C H₅₀ titers were obtained when the complement was titrated with the sheep erythrocyte and the TA specificity (Table 4), indicating both were equally sensitive hemolytic systems.

Table 4. Complement titration results with both sheep erythrocyte and teichoic acid hemolytic systems. The titration was performed three times with the average hemolysis value for each tube expressed below.

<table>
<thead>
<tr>
<th>Complement ml</th>
<th>D₁</th>
<th>0.25</th>
<th>0.30</th>
<th>0.35</th>
<th>0.40</th>
<th>0.45</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₂</td>
<td>for 100% hemolysis blanks</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep hemolysin</td>
</tr>
<tr>
<td>TA hemolysin</td>
</tr>
</tbody>
</table>

The rat complement contained 322 C H₅₀ units (Figure 5). The sheep hemolysin titration results are expressed in Table 5. A 1:1000 dilution of anti-sheep hemolysin was found to be optimal.
Figure 4. Results of the complement titration plotted by the method of Kabat and Mayer (1961). Data from both teichoic acid and sheep erythrocyte specificities produced the same plot. $100y = \%$ hemolysis at serum dilution $'x'$. At 50% hemolysis $y = 0.5$ and therefore $\frac{y}{1-y}$ is equal to 1. Since $'x'$ is a 1/250 dilution and the amount of $'x'$ needed for 50% hemolysis is 0.43 (from graph), there are 581 C H$_{50}$ units in 1 ml of undilute guinea pig serum.

\[\log \frac{y}{1-y}\]
Figure 5. Results of the rat complement titration plotted by the method of Kabat and Mayer (1961). D_2 was 1/200 in this experiment. See Figure 4 for explanation.
Table 5. Results of sheep erythrocyte hemolysin titration.

<table>
<thead>
<tr>
<th>Tube No. (Table 3)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent hemolysis</td>
<td>50</td>
<td>48</td>
<td>46</td>
<td>43</td>
<td>40</td>
<td>37</td>
<td>34</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>Hemolysin dilution</td>
<td>2000</td>
<td>3000</td>
<td>4000</td>
<td>5000</td>
<td>6000</td>
<td>7000</td>
<td>8000</td>
<td>9000</td>
<td>10000</td>
</tr>
</tbody>
</table>

A dilution of 1/1000 was routinely used so that slight excess of antibody would be present to compensate for any day to day variability of fragility in a given bottle of erythrocytes.

The confirmation of the complement titration in which 1 C₅₀ unit was added to sensitized erythrocytes resulted in the triplicate tubes having values of 54.3%, 46.5%, and 50% hemolysis, which were within the acceptable range as described by Kent and Fife (1963). Routine complement and hemolysin titrations produced consistent results, indicating that the guinea pig serum (complement source) used in this work remained stable under the storage conditions utilized.

The results of the passive hemolysis tests using TA-coated cells of different species, anti-TA antibody, and complement of different species are summarized in Table 6.

Table 6. Summary of attempts to develop a passive hemolysis assay for anti-TA antibodies in the rat.

<table>
<thead>
<tr>
<th>Coated erythrocytes (species)</th>
<th>Antibody</th>
<th>Complement</th>
<th>Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>rat</td>
<td>rat</td>
<td>no</td>
</tr>
<tr>
<td>Rat</td>
<td>rat</td>
<td>guinea pig</td>
<td>no</td>
</tr>
<tr>
<td>Sheep</td>
<td>rat</td>
<td>rat</td>
<td>yes</td>
</tr>
<tr>
<td>Sheep</td>
<td>rat</td>
<td>guinea pig</td>
<td>yes</td>
</tr>
</tbody>
</table>
The TA-coated rat erythrocytes failed to hemolyze in the presence of rat or guinea pig complement. Fresh unfrozen rat serum, known to contain antibodies to TA, would not lyse coated rat erythrocytes but would lyse TA-coated sheep erythrocytes after the serum had been absorbed with uncoated sheep cells. TA-coated sheep erythrocytes were lysed in the presence of rat antibody and guinea pig or rat complement. The findings described above made it necessary to use TA-coated sheep cells and to absorb all rat sera with 25% sheep erythrocytes for 30 min at room temperature before testing. The following protocol was employed for subsequent assays of anti-TA antibodies by passive hemolysis (Table 7). All dilutions were performed in veronal-buffered saline. Titers were expressed as that serum dilution producing 50% hemolysis ($EP_{50}$). Calculation of this value from the spectrophotometric readings of three tubes is dependable provided readings between 20% and 80% hemolysis are obtained and an accurate optical density for the 100% hemolysis blank is utilized in the calculations. The percent hemolysis was calculated for each serum dilution and three results between 20% and 80% hemolysis were used to calculate the $EP_{50}$ according to the formula:

$$\text{Volume of serum producing 50\% hemolysis} = \frac{\text{Vol. of serum} \times \% H.}{50\% \text{ hemolysis (H.)}}$$
Table 7. Passive hemolysis test for antibodies to teichoic acid.

| Tube 1 | 0.1 ml rat serum dilution previously heat inactivated and adsorbed with 20% sheep erythrocytes.  
|        | 0.1 ml standardized sheep erythrocytes (1%) coated with TA.  
|        | Sensitize 15 min at room temperature.  
|        | Add 0.1 ml of 5 unit guinea pig complement previously adsorbed with sheep cells.  
|        | Incubate 30 min in a 37 C waterbath.  
|        | Add veronal-buffered saline to 2.5 ml.  
|        | Spin 5 min in a serofuge.  
|        | Read optical density at 550 nm.  

| Tube 2 | Same as 1 except no complement added. This is the zero hemolysis blank for each dilution.  

| Tube 3 | Same as tube 1 except that uncoated sheep erythrocytes and unadsorbed test serum are used to detect hemolytic antibodies to sheep cells.  

| Tube 4 | 100% hemolysis blank.  
|        | 0.1 ml 1% sheep cells  
|        | 0.1 ml undiluted serum  
|        | 0.1 ml 5 unit complement  
|        | Add water to 2.5 ml.  

| Controls | Coated erythrocytes + complement  
|          | Coated erythrocytes + serum (adsorbed)  
|          | Coated erythrocytes + saline  
|          | Erythrocytes + complement + adsorbed serum  

\* A large number of controls were needed because of the multi-species components of the test. One must be confident that the absorptions were 100% effective and no heteroantibodies were being detected in the assay.
SUMMARY OF PRELIMINARY RESULTS

1. The teichoic acid preparation used in this work had properties similar to those described by Decker et al. (1972), however it did not have as much PHG activity per microgram of antigen as they reported.

2. The optimal concentration of teichoic acid for rat erythrocyte coating in the PHG test was 50 µg/ml.

3. The optimal concentration of teichoic acid for sheep erythrocyte coating in the passive hemolysis test was 5 µg/ml or 10% of the values obtained for the PHG test.

4. The passive hemolysis assay required the use of teichoic acid-coated sheep erythrocytes and absorbed rat sera since rat cells would not hemolyze under the experimental conditions employed. Neither guinea pig or rat complement would lyse the teichoic acid coated rat erythrocytes in the presence of specific antibody.

5. The Kent and Fife method for standardization of reagents resulted in a reproducible hemolysis test for antibodies to sheep cells and to teichoic acid. When proper care was utilized, the guinea pig complement remained stable and did not lose hemolytic activity when stored at -70°C for lengthy periods of time.

6. Pooled guinea pig serum had a higher C₅₀ titer than did rat serum, and was chosen for use in this work because of this fact as well as the availability of a single commercial pool in sufficient quantity for the entire experiment. The high-titered commercial guinea pig complement allowed sufficient dilution so that the preparation was free of natural heteroantibodies to
teichoic acid, however the serum was absorbed with sheep cells
to remove any natural antibodies of this specificity.
MATERIALS AND METHODS

Animals. Conventional Sprague-Dawley rats (Lab. Supply, Indianapolis, Ind.) were maintained in stainless steel cages and fed Purina Laboratory Chow (Purina, St. Louis, Mo.) and water ad libitum. Germ-free rats (ARS, Madison, Wis.) were maintained in flexible plastic isolators (Trexlar, 1959) and in an isolator made of rigid plastic. These animals were given sterile water and Purina 5010 Laboratory Chow ad libitum. Environmental conditions were similar to those described in Part I.

Gnotobiotic procedures. Isolators were washed thoroughly with soap and then sterilized with 2% peracetic acid. All internal surfaces of the isolators and any materials to be placed inside were sprayed. Finally, the isolator was sealed, filters attached, and the unit fogged and inflated with the peracetic acid spray. Masks and gloves were worn at all times during this procedure. The isolator was allowed to stand 24 hr before aeration to assure sterilization and to test for leakage. Air and acid vapor were exhausted from the isolators for one week before animals were placed inside. Air was sterilized by passage through four layers of fiberglass medium (American Air Filter, Louisville, Ky.). Food and water were sterilized by the OSU Gnotobiotic Facility which utilized a Reynier's type vacuum autoclave.
(1959), and passed via sterile air lock into the isolators. The air locks were sterilized with peracetic acid by spraying all surfaces and by fogging. A 40 min waiting period was always observed after spraying to allow time for sterilization. Passage between isolators was performed in a similar manner.

Thioglycollate cultures were taken routinely from each isolator. Wet swabs of feces, animals, bedding, food, water, and filter ports were taken. The tubes were incubated at 37°C for 24 hr, then inoculated onto blood agar plates, trypticase soy broth tubes, and Sabouraud's maltose agar slants. All media were incubated 2 weeks before being considered negative. The above culturing method conforms with guidelines published by the National Academy of Science (1972).

Serial studies. Normal rats were bled by cardiac puncture bi-weekly from 4 to 32 weeks of age, with a final bleeding at 52 weeks. Sera were frozen for later testing by the teichoic acid and sheep erythrocyte hemolysis assays described previously. Individual bleedings from each rat were tested and results recorded so that the development of responses could be followed in individuals as well as in the entire population.

Group studies. Normal conventional rats (littermates) of selected ages were examined for both cell-mediated and humoral responses to glycerol-teichoic acid antigen. Within a given age group some rats were skin tested while others were examined for production of macrophage migration inhibitory factor (MIF). Sera were collected at sacrifice and frozen for later characterization and hemolysis testing. At least 10 rats were included in each group.
Skin tests. Skin tests were performed as described by Bolton and Chorpenning (1974). A 10 μg sample of sterile teichoic acid antigen was injected intradermally into a shaved side of the rat along with saline controls. Test sites were observed at 1 hr, 12 hr, 24 hr, and 48 hr after injection. Indurated lesions over 4 mm in diameter after a lapse of 24 or more hr were considered positive tests for delayed hypersensitivity. Representative lesions were excised, fixed in alcohol-acetic acid-chloroform (60:10:30, v/v/v) for 24 hr, and embedded in Epon 812. Thin sections (100-150 nm) were prepared with an ultramicrotome, fixed with xylene, and stained with Pyronine-Methyl Green. Slides were then examined microscopically for cell infiltration.

MIF production. Rats were injected intraperitoneally with 20 ml sterile fluid thioglycollate 48 hr prior to sacrifice. The animals were exsanguinated by cardiac puncture and 40 ml cold Hanks Balanced Salt Solution (HBSS) containing 2 I.U. heparin/ml (Connaught, Toronto, Can.) was injected into the peritoneal cavity. The abdomen was disinfected with ethanol and merthiolate and a 15 mm opening was cut to allow passage of a sterile perforated tube into the peritoneal cavity. Peritoneal exudate cells (PEC) were harvested with a sterile Pasteur pipette and spun at 1000 x G for 3 min. Erythrocytes were lysed by addition of 1 ml sterile water to the packed cells followed by 4-5 sec vortexing with the immediate addition of 10 ml HBSS followed by centrifugation. PEC were resuspended to 10% concentration (v/v) in Spinners Modified Medium (Difco, Detroit, Mich.) supplemented with 10% fetal calf serum, 5% L-glutamine, and 100 μg/ml gentamycin sulfate.
Cells were then examined for viability by trypan blue dye exclusion.

MIF tests were performed by drawing the PEC into 0.9-1.1 mm inner diameter capillary tubes and sealing one end with Seal-Ease Clay (Clay-Adams, N.Y.). The tubes were spun 30 sec in a serofugo and each set broken off so that equal tube lengths of packed cells were obtained. Capillary tubes were then wiped with ethanol and cotton and placed in sterile 15 x 60 mm glass petri dishes (4 tubes/dish). The tubes were held in position with sterile silicone grease (Dow-Corning, Midland, Mich.) and overlayed with 4 ml Spinners Medium or 4 ml of the medium containing 100 ug/ml TA antigen. After 24 hr incubation in 5% CO₂ at 37 C, the medium was removed by aspiration and the area of adherent cell migration measured by tracing the halos on a 50X Nikon projectorscope and weighing the tracing paper on an analytical balance. The percent inhibition of migration was calculated by the formula:

\[ 1 - \frac{\text{area of migration in test (weight)}}{\text{area of migration in control}} \times 100 \]

Only those tests producing greater than 20% inhibition were considered positive (Pick and Turk, 1972).

Natural responses in germfree rats. The MIF assay was performed germfree rats because of logistical problems involved in skin testing within the isolators. Rats were removed from the germfree environment immediately before sacrifice. Only uninjected rats were examined, with age groups selected to match those of the conventional animal studies. All procedures were identical to those described above for studies of conventional animals.
Antibody characterization. One ml serum samples were passed through a 2.5 x 70 cm column of Sephadex G-200 (Pharmacia Chemicals, Piscataway, N.J.). Samples were monitored by an I.S.C.O. UA-5 absorbance monitor (Instrumentation Specialties, Lincoln, Neb.) and collected in 2 ml fractions on an I.S.C.O. Model 328 fraction collector. Tubes under each of the first 2 absorbance peaks were pooled and concentrated back to 1 ml under pressure filtration using an XM-100A diaflow membrane (Amicon, Lexington, Mass.). Purity of each fraction was tested by immunoelectrophoresis against goat anti-rat serum (Microbiological Associates, Bethesda, Md.) using an LKB electrophoresis apparatus (LKB, Stockholm, Sweden). Special Noble agar was layered onto glass microscope slides and allowed to dry 30 min. Two wells and a trough were cut in each slide. Serum or fractions to be tested were placed in the wells and electrophoresed for 1 hr at 250 V using a high resolution barbitol buffer (pH 8.6, ionic strength 0.1). The anti-globulin serum was then placed in the troughs, the slides incubated 20 hr in a humidity chamber, and examined for precipitation arcs.

Some serum fractions and the corresponding whole serum were treated with 2-mercaptoethanol by the method of Deutsch and Morton (1957). Samples were diluted 1/4 and an equal volume of 0.2 M 2-mercaptoethanol (Eastman Kodak, Rochester, N.Y.) added. After 30 min incubation at 37 C the samples were concentrated back to the original starting volume using an XM100-A diaflow membrane and tested for hemolytic activity along with untreated controls. Complete and incomplete anti-D sera (Hyland Labs., Costa Mesa, Calif.) were used as positive
and negative controls. All sera and their fractions were heat-inactivated at 56°C for 30 min prior to hemolysis testing.

Close interval parallel dilutions were employed throughout this investigation. Those dilutions giving 20-80% hemolysis were utilized in calculation of EP$_{50}$ titers. This type of dilution combined with the spectrophotometric determination of hemolysis minimized subjectivity and permitted reproducibility within 5%.
RESULTS

Serial studies. Seventy-five rats were included in this experiment with 25 surviving after 52 weeks, however only 50% of the losses were directly related to the bleeding procedure. All animals were not represented at each bleeding date because of technical difficulties in obtaining sufficient amounts of blood from some rats. Naturally-occurring hemolytic antibodies of TA specificity were found in the serum of all rats subjected to serial bleeding, however not all animals produced such antibodies at each sampling time as illustrated by the response frequency variation in Table 8 and also by examination of the antibody levels in individual rats (Figure 6). Mean titers rose significantly up to 12 weeks, however the rise and fall observed as the population aged was not statistically significant because of the wide range of antibody levels in individual rats at each bleeding (Table 8). Examination of responses in individual rats revealed a cyclical production of natural anti-TA antibodies (Figure 6), a phenomenon which is mentioned here only as an explanation for the wide range of antibody titers observed in the population of rats at any one time.

The frequency of natural antibody responses to TA rose from 25% at 4 weeks to 93% at 14 weeks (Figure 7). Chi-square analysis produced a p value of <.005 indicating the rise was significant. The initial rise was followed by a drop in frequency at 20 weeks (p < .05). Two additional peaks at 24 (p < .025) and 52 (p < .005) weeks were also observed. Chi-square analysis of all data points from 14 to 52 weeks
using an 11 x 2 contingency table reinforced the significance of the peaks and valleys represented in Figure 7 ($p<.005$).

Natural hemolysins to sheep erythrocytes were also found in the rats described above. Titers remained at comparatively low levels throughout the experiment (Table 9), dropping to near zero at 52 weeks. Antibody cycling was also observed in the sheep hemolysin response of individual rats (Figure 8), again resulting in the wide range of antibody levels observed at each bleeding date (Table 9). The frequency of natural sheep hemolysin production in this population of rats reached peak levels at 14 weeks (Figure 9). A second peak occurred at 22 weeks and was followed by a continuous drop to 4% at 52 weeks. Chi-square analysis revealed that the two peaks were real ($p<.005$).
Table 8. Summary of serial studies on the ontogeny of natural antibody production to teichoic acid in rats.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. bled</td>
<td>12</td>
<td>29</td>
<td>35</td>
<td>46</td>
<td>46</td>
<td>45</td>
<td>41</td>
<td>42</td>
<td>40</td>
<td>39</td>
<td>37</td>
<td>35</td>
<td>25</td>
<td>27</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>Mean EP&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.5</td>
<td>6.1</td>
<td>4.7</td>
<td>12.5</td>
<td>33.1</td>
<td>65.0</td>
<td>40.7</td>
<td>11.7</td>
<td>22.5</td>
<td>28.8</td>
<td>32.5</td>
<td>34.3</td>
<td>58.5</td>
<td>31.3</td>
<td>71.0</td>
<td>256</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Frequency %</td>
<td>25</td>
<td>34</td>
<td>51</td>
<td>65</td>
<td>87</td>
<td>93</td>
<td>83</td>
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<td>89</td>
<td>80</td>
<td>74</td>
<td>77</td>
<td>100</td>
</tr>
</tbody>
</table>

*a* Represents the range of observed EP<sub>50</sub> titers at each bleeding time as determined by the passive hemolysis assay.
Figure 6. Naturally-occurring hemolytic antibody of teichoic acid specificity in representative individual rats which were subjected to serial bleeding.
Figure 7. Frequency of natural anti-teichoic antibodies in rats (serial studies). Each bleeding age is represented by a minimum of 25 serum samples which were tested by passive hemolysis.
Table 9. Summary of serial studies on the ontogeny of naturally-occurring sheep hemolysins in rats.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>52</th>
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<tbody>
<tr>
<td>No. Bled</td>
<td>15</td>
<td>39</td>
<td>47</td>
<td>50</td>
<td>47</td>
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<td>37</td>
<td>30</td>
<td>28</td>
<td>25</td>
<td>52</td>
</tr>
<tr>
<td>Mean EP&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.1</td>
<td>5.1</td>
<td>1.4</td>
<td>8.4</td>
<td>10.3</td>
<td>2.9</td>
<td>4.8</td>
<td>3.3</td>
<td>6.4</td>
<td>11.1</td>
<td>7.6</td>
<td>6.0</td>
<td>1.4</td>
<td>3.9</td>
<td>0.9</td>
<td>0.4</td>
</tr>
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<td>Range&lt;sub&gt;a&lt;/sub&gt;</td>
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<tr>
<td>Frequency %</td>
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<td>4</td>
<td>250</td>
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<td>31</td>
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<td>64</td>
<td>16</td>
<td>16</td>
<td>8</td>
<td>0.4</td>
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</tbody>
</table>

*a Represents the range of observed EP<sub>50</sub> titers at each bleeding time as determined by the hemolysin test.*
Figure 8. Naturally-occurring sheep hemolysins in representative individual rats which underwent serial bleeding.
Figure 9. Frequency of natural sheep erythrocyte hemolysins in rats (serial studies). Each bleeding age is represented by a minimum of 25 serum samples.
Group studies. Natural antibodies to teichoic acid were found in all age groups examined (2-32 weeks), while natural sheep hemolysins did not appear until 6 weeks and remained at low levels throughout the time span of the experiment (Table 10). Natural antibodies of the two specificities appeared in Sephadex G-200 Fraction I of sera from all age groups. Antibodies in this fraction were 2-mercaptoethanol sensitive and were shown by immunoelectrophoresis to be IgM. Some rats 20 weeks of age or older produced natural antibodies to TA which appeared in Sephadex Fraction II and were 2-mercaptoethanol resistant. Immunoelectrophoresis revealed these antibodies to be IgG. Hemolysin titers were consistently higher in Fraction I than in Fraction II (Figure 10) and no rats were found which produced natural anti-TA antibodies in Fraction II and not Fraction I. Natural sheep hemolysins were found only in Fraction I, regardless of the age group examined.

The frequency of natural responses in the group studies (Figure 11) followed a similar pattern to the serial studies described above. Natural response frequencies of both sheep and TA specificities rose to peak levels by 12 weeks. The TA response frequency dropped and showed a second significant peak (p<.025) at 26 weeks. The sheep hemolysin response also exhibited two frequency peaks with a dip at 18 weeks (p<.01). The anti-TA frequency remained relatively high (70-90%) in older rats while the sheep hemolysin levels dropped significantly after 26 weeks, a situation similar to that described above in the serial studies.
Table 10. Occurrence and class of naturally-occurring antibodies to teichoic acid and to sheep erythrocytes (age group studies).

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>No. Tested</th>
<th>Teichoic Hemolysis</th>
<th>Sheep Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WS&lt;sub&gt;a&lt;/sub&gt; FI&lt;sub&gt;b&lt;/sub&gt; FII&lt;sub&gt;c&lt;/sub&gt;</td>
<td>WS</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>12.0 1.1 0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>6.4 2.1 0</td>
<td>5.1</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>33.1 5.0 0</td>
<td>10.3</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>40.7 6.0 0</td>
<td>4.8</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>11.7 3.1 0</td>
<td>6.4</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>22.5 2.4 0.8</td>
<td>6.4</td>
</tr>
<tr>
<td>26</td>
<td>10</td>
<td>16.5 3.1 1.4</td>
<td>5.9</td>
</tr>
<tr>
<td>28</td>
<td>10</td>
<td>4.8 1.0 1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
<td>114.0 10.0 3.1</td>
<td>3.9</td>
</tr>
<tr>
<td>32</td>
<td>10</td>
<td>71.2 4.5 2.6</td>
<td>.9</td>
</tr>
</tbody>
</table>

a Whole serum  
b Sephadex Fraction I  
c Sephadex Fraction II
Figure 10. Fractionation of rat serum on Sephadex G-200. Bars indicate relative levels of hemolytic antibody to teichoic acid in animals over 20 weeks old.
Figure 11. Frequency of naturally-occurring antibody responses to teichoic acid and to sheep erythrocytes in age group studies. Each point represents ten animals.
Naturally-occurring cell-mediated responses to TA were found only in rats 20 weeks of age or older (Table 11). Skin tests and MIF assays were not performed on the same animal, however the two appeared well correlated since similar results were obtained with each. Skin tests showed induration, but little erythema at 48 hr post-injection (Figure 12), which is consistent with the delayed hypersensitivity response described for this species by Flax and Waksman (1962). Thin sections of lesions revealed considerable mononuclear cell infiltration. No immediate skin reactions were observed.

The frequencies of naturally-occurring cellular and humoral responses to TA are compared in Figure 13. Results of MIF and skin tests were pooled since they appeared well correlated and were shown by Ferraresi et al. (1969) to be of equal sensitivity. The IgM frequency remained high throughout the time span of the experiment while production of IgG and cell-mediated responses did not appear until 20 weeks. The onset and frequency of IgG and cell-mediated responses to TA appeared well correlated since chi-square analysis indicated no significant difference between the two curves in Figure 13. Natural cell-mediated and IgG antibody responses did not necessarily occur together in the same animal. Some rats over 20 weeks old produced both responses and some produced neither, however no animal examined produced a naturally-occurring cell-mediated response to TA without producing natural IgM antibody of the same specificity. Some rats did produce cell-mediated responses to TA without producing IgG while some produced IgG but no cell-mediated response.
Table 11. Naturally-occurring cell-media responses to TA in rats of selected ages.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>MIF</th>
<th></th>
<th>Skin Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Tested</td>
<td>No. Pos.</td>
<td>Tested</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 12. Naturally-occurring delayed hypersensitivity skin reaction in a rat which was previously injected intradermally with 10 ug of teichoic acid.
Figure 13. Frequency of naturally-occurring cell-mediated and humoral responses to teichoic acid in age group studies. Each point represents 10 animals.
Gnotobiotic studies. Germfree rats produced natural antibodies to TA but not to sheep erythrocytes (Table 12). These antibodies were found only in Sephadex G-200 Fraction I and were lower in titer when compared with those from conventional uninjected rats (Figure 14). However at 6 weeks there was no significant difference between the mean titers of the two populations. No cell-mediated responses to TA were found in any of the germfree rats examined, regardless of age.

The frequency of natural antibody responses to TA in germfree rats rose to high levels at 3 weeks \( (p < .05) \) when compared with conventional (Figure 15), however they converged at 6 weeks and followed similar patterns for the duration of the experiment. The germfree response frequency peaked at 12 weeks \( (p < .01) \), reaching 100%. This corresponded to the first peak found in conventional animals, however the second frequency peak \( (p < .05) \) observed in the germfree population occurred several weeks sooner than in conventional rats (Figure 7, Figure 11).
Table 12. Natural responses to teichoic acid and sheep erythrocytes in germfree rats.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>No. Tested</th>
<th>MIF Pos.</th>
<th>Mean EP&lt;sub&gt;50&lt;/sub&gt; Titer</th>
<th>Teichoic Acid</th>
<th>Sheep Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>10</td>
<td>NT</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>NT</td>
<td>2.1</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0</td>
<td>5.0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>0</td>
<td>1.8</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>0</td>
<td>4.0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>13</td>
<td>0</td>
<td>3.7</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>0</td>
<td>7.0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>9</td>
<td>0</td>
<td>3.0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 14. Comparison of natural anti-teichoic acid antibodies in germfree and conventional rats. Each point represents 10 conventional rats and 5-13 germfree.
Figure 15. Frequency of naturally-occurring anti-TA antibodies in germfree and conventional rats.
Weanling rats were orally immunized with a Bacillus species (OSU No. 372) by adding the bacteria to their drinking water. Each rat received approximately $10^9$ cells/day. There were 46 rats in the experiment with 1/4 of the population started on the immunization at weaning. The remainder of the population was started on the immunization at weekly intervals so that when all of the rats were examined at 12 weeks of age there were 4 different time spans of immunization (6, 7, 8, 9 weeks). All rats were skin tested with teichoic acid and were exsanguinated 48 hr later. Sera were frozen for later fractionation and hemolysis testing.

A second experiment entailed oral immunization of rats for 5 weeks prior to sacrifice at 16 and 18 weeks age. Both MIF and skin tests for cell-mediated immunity to TA were performed on these groups but not on the same animal. Sera were also collected and frozen for later use. All assays were performed in an identical manner to those described in Part I for normal rats.
RESULTS

Oral immunization with *Bacillus* species (OSU No. 372) significantly increased the response of the rat to teichoic acid. Rats which were 12 weeks old and immunized for 8 and 9 weeks produced significantly higher levels of hemolytic antibody to TA than did normal controls ($p < .01$) (Table 13). These antibodies were found in Sephadex G-200 Fractions I and II whereas control rats produced antibody only in Fraction I. A 100% response frequency was observed in serum Fraction I of rats immunized for 8 and 9 weeks (Table 15), whereas in Fraction II those immunized for 9 weeks responded with a 100% frequency while those fed bacteria for only 8 weeks had a 50% response frequency. These rats also produced positive delayed hypersensitivity skin tests to TA (Table 13) with a similar frequency to that found in normal animals over 20 weeks old (Table 15). Those rats which were 12 weeks old and fed bacilli for 6 and 7 weeks produced anti-TA antibodies at levels similar to those of the controls (Table 13) however activity was still present in both Sephadex G-200 Fractions I and II. The frequency remained at 100% for Fraction I but dropped significantly in Fraction II (Table 15). Positive skin tests were also observed in these rats (Table 13) but with lower frequencies than those immunized for longer periods (Table 15).

Rats which were 16 and 18 weeks old and orally immunized for 5 weeks produced antibodies to TA with a 100% frequency (Table 15), but only in Sephadex G-200 Fraction II and at lower levels than found in normal controls, which produced natural antibodies to TA only in
Sephadex Fraction I (Table 14). These animals produced both positive skin tests and MIF to TA, whereas controls did not. The 18-week-old immunized rats produced cell-mediated responses to TA with a 100% frequency (Table 15), whereas those only 16 weeks old responded with a lesser frequency.
Table 13. Responses to teichoic acid in 12 week old rats which were orally immunized with a *Bacillus* species (OSU No. 372) for various periods of time prior to testing.

<table>
<thead>
<tr>
<th>Length of immunization</th>
<th>No. Tested</th>
<th>No. Pos.</th>
<th>Skin Test</th>
<th>Mean EP$_{50}$ Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 weeks</td>
<td>12</td>
<td>6</td>
<td></td>
<td>WS$_a$ 17.1 FI$_b$ 9.1</td>
</tr>
<tr>
<td>8 weeks</td>
<td>12</td>
<td>7</td>
<td></td>
<td>WS$_a$ 13.2 FI$_b$ 3.1</td>
</tr>
<tr>
<td>7 weeks</td>
<td>11</td>
<td>5</td>
<td></td>
<td>WS$_a$ 9.0 FI$_b$ 1.1</td>
</tr>
<tr>
<td>6 weeks</td>
<td>11</td>
<td>1</td>
<td></td>
<td>WS$_a$ 2.0 FI$_b$ .5</td>
</tr>
<tr>
<td>Controls (unimmunized)</td>
<td>10</td>
<td>0</td>
<td></td>
<td>WS$_a$ 5.0 FI$_b$ 0</td>
</tr>
</tbody>
</table>

a Whole serum (tested by passive hemolysis)
b Sephadex G-200 Fraction I
c Sephadex G-200 Fraction II
Table 14. Responses to teichoic acid in rats orally immunized for 35 days with a Bacillus sp.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>MIF No. Tested No. Pos.</th>
<th>Skin Test No. Tested No. Pos.</th>
<th>Mean EP\textsubscript{50} Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>5 4</td>
<td>7 3</td>
<td>3.3 0.8</td>
</tr>
<tr>
<td>18</td>
<td>5 5</td>
<td>7 7</td>
<td>5.4 0 1.6</td>
</tr>
<tr>
<td>Unimmunized controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5 0</td>
<td>7 0</td>
<td>40.7 4.8 0</td>
</tr>
<tr>
<td>18</td>
<td>7 0</td>
<td>7 0</td>
<td>11.7 1.8 0</td>
</tr>
</tbody>
</table>

\(a\) Whole serum  
\(b\) Sephadex G-200 FI  
\(c\) Sephadex G-200 FII
Table 15. Frequency of responses to teichoic acid in rats fed $10^9$ *Bacillus* sp. (OSU No. 372) per day.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Length of Immunization</th>
<th>Skin Test</th>
<th>MIF</th>
<th>$W_{s}$</th>
<th>$F_{Ib}$</th>
<th>$F_{IIc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>9 weeks</td>
<td>50</td>
<td>NT</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>8 weeks</td>
<td>58</td>
<td>NT</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>7 weeks</td>
<td>45</td>
<td>NT</td>
<td>100</td>
<td>100</td>
<td>27</td>
</tr>
<tr>
<td>12</td>
<td>6 weeks</td>
<td>9</td>
<td>NT</td>
<td>100</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>16</td>
<td>5 weeks</td>
<td>43</td>
<td>80</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>5 weeks</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

**Controls (unimmunized)**

| 12          | none                   | 0         | 0   | 87      | 87      | 0        |
| 16          | none                   | 0         | 0   | 83      | 83      | 0        |
| 18          | none                   | 0         | 0   | 79      | 79      | 0        |

a  Whole serum  
b  Sephadex G-200 FI  
c  Sephadex G-200 FII
Animals. Germfree and conventional Sprague-Dawley rats were housed as described previously. Test animals were given the TA-deficient diet and drinking water ad libitum.

Teichoic acid-deficient diet. Sterile Similac Liquid was supplied in cans by Ross Laboratories (Columbus, Ohio). A diet, which appeared to be nutritionally adequate for rats, was formulated by mixing Similac, water, and corn oil in a 75:24:1 ratio. The formula was prepared daily for the conventional rats since coagulation of the preparation occurred after 24 hr. The cans of Similac were sprayed with peracetic acid during transfer into the germfree isolators via sterile air lock as described in Part II. Water and corn oil were autoclaved before being placed in the isolator. The formula was prepared several days in advance for the germfree rats since spoilage was no problem.

The Similac and corn oil were examined microscopically for the presence of bacteria. The corn oil was emulsified with saline and the aqueous phase examined for bacterial bodies with a phase contrast microscope. The Similac was centrifuged at 25000 x G and the sediment examined microscopically. Both corn oil and Similac were
cultured in thioglycollate and blood agar medium at room temperature and at 37 C. A phenol extraction was performed on the Similac by the method described in Part I for extraction of TA from bacteria. The aqueous phase of the extract was dialyzed and lyophilized. The phenol extract and the intact formula were examined for TA content by the passive hemagglutination inhibition test described in Part I using TA-coated erythrocytes and a standard antiserum.

**Serological assays.** Sera from normal rats, germfree and conventional, fed the TA-deficient diet were examined for antibodies to TA by the passive hemolysis test previously described in Part I. Rats on this diet were also immunized orally \(10^9\) cells/day or intraperitoneally \(10^9\) cells with a *Bacillus* species (OSU No. 372) and their sera examined for antibodies to TA.
RESULTS

Microscopic examination of the TA-deficient diet revealed no bacterial bodies in the corn oil and only very few in the Similac. No growth was observed in the cultures of either component. Neither the Similac nor its phenol extract inhibited the passive hemagglutination test and therefore did not contain polyglycerophosphate at detectable levels (0.08 ug).

Neither germfree nor conventional rats which were reared on the TA-deficient diet produced naturally-occurring hemolytic antibodies to TA (Table 16), whereas control animals of the same age (16 weeks) which were fed a regular diet produced such antibodies.

Table 16. Natural antibodies to teichoic acid in germfree and conventional rats fed a teichoic acid-deficient diet.

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. Tested</th>
<th>Mean EP50</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional-Regular Diet</td>
<td>44</td>
<td>40</td>
<td>83%</td>
</tr>
<tr>
<td>Conventional-Deficient Diet</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Germfree-Regular Diet</td>
<td>11</td>
<td>4</td>
<td>46%</td>
</tr>
<tr>
<td>Germfree-Deficient Diet</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Titer obtained by passive hemolysis

Conventional rats on the TA-deficient diet did produce natural hemolysins to sheep erythrocytes at levels which were similar to those described in Part II, however the germfree rats on the diet did not produce such responses (Table 17).

Germfree and conventional animals on the TA-deficient diet which were given 3 intraperitoneal injections of $10^9$ Bacillus species (OSU No. 372) produced antibodies to TA with a 100% frequency ($p < .01$).
Table 17. Natural sheep hemolysins produced by rats on the teichoic acid deficient diet.

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. Tested</th>
<th>Mean EP50 Titer</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional-Regular Diet</td>
<td>44</td>
<td>4.8</td>
<td>67%</td>
</tr>
<tr>
<td>Conventional-Deficient Diet</td>
<td>20</td>
<td>4.0</td>
<td>70%</td>
</tr>
<tr>
<td>Germfree-Regular Diet</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Germfree-Deficient Diet</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(Table 18), Baseline bleeding revealed that these rats were not producing antibodies to TA prior to injection. Animals on this diet which were orally immunized with $10^9$ bacilli per day for 8 weeks also produced antibodies to TA with a 100% frequency ($p < .01$) (Table 19). The germfree animals responded more readily to this method of stimulation since serum samples collected after six weeks of oral immunization revealed responses to TA in the germfree, but not in the conventional rats, on the deficient diet. All animals utilized in the immunization studies were 12 weeks old at sacrifice.

Table 18. Responses to teichoic acid following intraperitoneal immunization$^a$ in individual germfree and conventional rats fed the teichoic acid deficient diet.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Status</th>
<th>EP50 Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Conventional</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>Germfree</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$ Mean = 6.4

Mean = 11

Three injections of $10^9$ bacilli at one week intervals prior to sacrifice.
Table 19. Responses to teichoic acid in orally immunized rats fed the teichoic acid deficient diet.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Status</th>
<th>EP50 Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Conventional</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>3 Mean = 6.66</td>
</tr>
<tr>
<td>7</td>
<td>Germfree</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
<td>7 Mean = 8.25</td>
</tr>
</tbody>
</table>

* Fed $10^9$ bacilli/day for 8 weeks.
Animals and Immunization. Adult Sprague-Dawley rats (14 weeks old) were bled and then immunized intracardially with $10^9$ Bacillus sp. (OSU No. 372). Animals were exsanguinated at regular time intervals up to 32 days post-injection. Serum was collected and frozen for later testing. The hemolytic plaque assay was performed on cells from the spleen, mesenteric lymph nodes, and bone marrow of each rat in order to determine the number of cells producing antibody to TA. A second group of uninjected rats were serially bled every 4 days and serum samples examined for natural antibodies to TA by passive hemolysis.

Hemolytic plaque assay. A modification of the technique described by Jerne and Nordin (1963) was employed. Lymphoid tissue was collected in cold Hanks Balanced Salt Solution (HBSS), teased with dissecting needles, and filtered through 4 layers of gauze. Viable cells were enumerated by Trypan Blue dye exclusion and adjusted to $10^7$ cells/ml HBSS. Sheep erythrocytes were prepared as described for the hemolysis tests in Part I. Rat cells would not hemolyze and therefore were inadequate for this procedure. A 10% suspension of sheep erythrocytes (SRBC) and TA-coated SRBC was utilized. The assay was performed with both cell suspensions so that SRBC hemolytic
could be enumerated and the values used to calculate the number of TA plaques.

The standardized erythrocytes (0.2 ml) and lymphoid cells (0.2 ml) were thoroughly mixed into 2 ml of 0.6% agarose (Sargent-Welch, Cincinnati, Ohio) which was previously dissolved in HBSS and maintained at 45 C. The suspension was poured into a 15 x 60 mm petri dish which contained 3 ml agarose (1.2%) base layer. Twelve plates were prepared with the lymphoid cells from each organ. SRBC were used in 6 plates and TA-coated SRBC in the remainder. All plates were incubated at 37 C in 5% CO₂ for 2 hr. Goat anti-rat IgG (Microbiological Associates, Bethesda, Md.) was added to one-half of the plates in each group. One ml of a 1/75 dilution was used. The remaining plates received 1 ml of the HBSS diluent. After an additional 1 hr incubation the anti-IgG or diluent was poured off and 1 ml of guinea pig complement (Grand Island Biological, Grand Island, N.Y.) added to all plates. The complement was previously adsorbed with 25% SRBC and 25% Bacillus sp. (OSU No. 372) with a final 1/10 dilution being used for the tests. Plates were further incubated for 30 min, the complement poured off, and refrigerated overnight. Plaques were enumerated in a blind fashion before results were recorded. Two control plates were always included with each group of rats; one containing erythrocytes and complement and the second containing erythrocytes and lymphoid cells but no complement.

The number of direct TA plaque-forming cells (PFC) was determined by subtracting the mean number of direct SRBC PFC from the mean number of PFC found on the plates which utilized TA-coated SRBC. The
number of indirect (anti-IgG facilitated) PFC of TA specificity was
determined by subtracting the mean number of direct TA and indirect
SRBC PFC from the mean number of PFC on the anti-IgG facilitated
plates which utilized TA-coated SRBC. TA PFC were considered to be
present only if the calculated number of plaques was greater than the
standard deviation for the triplicate set of TA-coated SRBC plates
and the SRBC background plates for each lymphoid organ examined.

Serology. The passive hemolysis test which utilized TA-coated
SRBC was performed as described in Part I. All sera were tested on
the same day with a single preparation of TA-coated erythrocytes and
standardized guinea pig complement, thus minimizing technical vari-
bility which could influence test sensitivity and reproducibility.
RESULTS

The rats which received a single injection of Bacillus sp. (OSU No. 372) showed cycling in both their PFC and circulating antibody responses to TA. Figure 16 illustrates the bimodal peaks observed in the serum antibody levels in which close interval parallel dilutions were used to determine EP\(^{50}\) titers. Each point on the graph represents 5 animals, all of the same age. A drop from base-line levels was observed at day 4, which was followed by a rise in titer up to day 16. The antibody level dropped by day 20 and was followed by a second peak at day 28.

Plaque-forming cells to TA in the spleens of the rats described above showed bimodal responses, with both direct and indirect PFC peaking at 4 and 16 days (Figure 17), although the peaks were much more pronounced in the indirect plaques. Direct PFC of TA specificity in the mesenteric lymph node showed a poorly defined peak between 4 and 16 weeks (Figure 18) which was followed by a sharp drop at 24 weeks. A second rise was apparent, but no peak was observed before termination of the experiment. Indirect PFC in the mesenteric nodes exhibited a slow rise with a single peak at 24 weeks. The bone marrow yielded a persistent number of direct PFC to TA after an initial peak at 4 days (Figure 19), however indirect plaques showed peaks at 4-8 days, 16 days, and 28 days.

Uninjected rats which were bled every 4 days exhibited cycling of their natural antibody responses to TA (Table 20). The number of cycles and duration of peaks and dips varied immensely among
individuals, with titers dropping to zero in several rats. These animals were under 18 weeks old and produced only IgM natural antibody to TA as determined by Sephadex G-200 gel filtration and 2-mercaptoethanol treatment of Fraction I, where all of the hemolytic activity was found.
Figure 16. Cycling of hemolytic antibodies to teichoic acid in rats given a single injection of Gram-positive bacteria. Five animals were examined every four days post-injection.
Figure 17. Plaque-forming cells to teichoic acid in spleens of rats which received a single injection of Bacillus sp. (OSU No. 372).
Plaque-forming cells to teichoic acid in the mesenteric lymph nodes in rats which received a single injection of *Bacillus* sp. (OSU No. 372).
Figure 19. Plaque-forming cells to teichoic acid in bone marrow of rats which received a single injection of Bacillus species (OSU No. 372).
Table 20. Cycling of natural antibodies to teichoic acid in individual rats which were serially bled for 4 weeks.

<table>
<thead>
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<th>Day 0</th>
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<th>8</th>
<th>12</th>
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<td>30</td>
<td>15</td>
<td>65</td>
<td>60</td>
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</tr>
</tbody>
</table>

\(^a\) Obtained by using close interval parallel dilutions which yielded reproducibility within 5\%. 

DISCUSSION

The occurrence of natural responses to glycerol-teichoic acid in rats reported here confirms the earlier work of Frederick (1972) and of Bolton and Chorpenning (1974), however some of the results were slightly different although not contradictory. The serial studies of normal rats revealed that all animals in the experiment produced naturally-occurring antibodies to glycerol-teichoic acid. However antibody cycling (discussed below) in individual rats resulted in less than 100% response frequencies at any one time and in a wide range of titers at each bleeding date, which provided an explanation for the 95% frequency reported by Bolton and Chorpenning (1974) and the 50-60% frequency observed by Frederick (1972), who used only single bleedings from small groups of rats in their determinations. In the present investigation antibody frequencies and titers from group studies (measured by passive hemolysis) were well correlated with those from the serial studies.

Natural antibodies to glycerol-teichoic acid were found in the serum of rats which were only 2 weeks old. A drop in EP50 titers was observed at 6 weeks age and was followed by a significant rise. The drop at 6 weeks suggests that maternal antibody was being measured since the half-life of such antibody is 5.5 days (Bangham and Terry, 1957) and the rat is apparently not fully immunocompetent until 4-5
weeks after birth (Rowley and Fitch, 1965), (Solomon et al., 1972). The maternal antibody of teichoic acid specificity could have inhibited natural antibody production by several mechanisms (reviewed by Greaves et al., 1973b), however by the sixth week it would have been catabolized to negligible quantities thus allowing antibody synthesis to begin. Such antibody suppression of the immune response has been demonstrated in artificial situations by Graf and Uhr (1969). Natural antibodies to teichoic acid reached peak frequencies and titers by 12 weeks age in the populations examined in this work, and fluctuated at high levels for the remainder of the experiment, with the exception of a sharp drop in frequency and mean titers at 18 weeks age. This drop was followed by the spontaneous and well-correlated onset of naturally-occurring cell-mediated immunity and IgG antibody responses to teichoic acid, while prior to this age the rats produced only IgM antibodies of this specificity. This is characteristic of many other natural antibodies (Rosen, 1963) and of a primary immune response (Uhr, 1964). The relative amounts of natural IgM and IgG were not compared in this investigation since 100% recovery of Sephadex fractions was not possible and the serological assay which was used favored detection of IgM (Borsos and Rapp, 1965b). The spontaneous shift to IgG production and cell-mediated immunity in natural responses to teichoic acid in the rat was previously reported by Bolton and Chorpenning (1974), who used only small groups of animals in poorly defined age groups. Frederick et al. (1972) reported natural cell-mediated responses to teichoic acid in guinea pigs and Frederick and Chorpenning (1973) found a spontaneous shift to natural IgG
production in guinea pigs over 20 weeks old. Unfortunately, no ontogeny data is available in guinea pigs in which cell-mediated and humoral responses to teichoic acid were examined in the same animals, such as reported in the present investigation.

Several theories may be offered in explanation of the spontaneous shift in natural responses to teichoic acid observed in this investigation. The immune system of the neonatal rat is poorly developed and tolerance can be induced quite readily (Nossal, 1958). At birth the rat becomes immediately exposed to small amounts of environmental antigens which would include teichoic acid (discussed below). Since small amounts of persisting antigen readily induce T-cell tolerance which has been shown to persist for extended periods (reviewed by Weigle, 1973), a split tolerance involving the T-cell population of teichoic acid specificity could ensue. Asherson and Stone (1966) first reported such a phenomenon in which delayed hypersensitivity, but not antibody production, was inhibited following injection of antigen. As a result of the T-cell tolerance to teichoic acid no cell-mediated or IgG antibody responses could occur, assuming a T-B cooperation is necessary for IgG production (Katz and Benacerraf, 1972). One might refute such a theory by suggesting that teichoic acid, by nature of its repeating polyglycerophosphate structure, is probably a T-cell independent antigen, similar to pneumococcal polysaccharide (Howard et al., 1971), and therefore no T-B cell cooperation is needed for these low level IgG responses. However more recent work has shown that pneumococcal polysaccharide can elicit an IgG response (Barthold et al., 1974) and that such responses are
regulated by T-cells (Mullen, 1974). If glycerol-teichoic acid were a T-cell independent antigen, the form in which it is presented to the animal under natural circumstances may convert it to a T-cell dependent antigen, as was done by coupling pneumococcal polysaccharide to erythrocytes (Mullen, 1975). The teichoic acid antigen, being an integral part of bacterial cells, which could conceivably remain coupled to a carrier when presented to the immune system. The work of Frederick (1972) lends support to such a contention. Humoral responses to teichoic acid were not detected when guinea pigs were immunized with polyglycerophosphate whereas extracts of Bacillus sp. (OSU No. 372) as well as whole organisms produced such responses. Cell-mediated responses were produced only if whole bacteria were used for immunization. One may conclude from his work that immunization by teichoic acid may involve a more complex molecule than the polyglycerophosphate backbone characterized by Decker et al. (1972) and utilized in the present investigation for detection of responses.

Assuming that a partial tolerance to teichoic acid may exist in normal rats under 20 weeks of age, one must explain how this unresponsiveness is spontaneously broken. Mauel et al. (1970) and Parish (1971) suggested that the specificity requirements were broader for T-cell mediated functions than for those mediated by B-cells, possibly because of the lower density of determinants on T-cells (Engers and Unanue, 1974). It is well known that tolerance can be broken by use of cross-reacting antigens (reviewed by Weigle, 1973). Teichoic acid, like other polysaccharide antigens, should persist in the tissues for long periods of time (Kaplan et al., 1950) and by 20 weeks...
sufficient cross-reacting antigen may be present to break tolerance. Considering the ubiquity of this antigen, it is reasonable to expect that an animal may be exposed to a number of different and possibly cross-reacting forms over a 20 week period. The initial T-cell tolerance proposed may have been induced by teichoic acid contained in the rats' food (discussed below). As the animal matures, the absorptive properties of the gut change (Wostmann et al., 1971a), possibly allowing a variety of conjugated forms of teichoic acid to enter via mucosal surfaces.

The above argument implied that dosage is an important factor in the breaking of partial tolerance to teichoic acid with the subsequent production of naturally-occurring IgG and cell-mediated immunity of that specificity. The oral immunization studies described in Part III illustrate the significance of dosage in this situation. Rats less than 20 weeks of age, when fed bacteria for lengthy time periods, produced IgG and cell-mediated responses of teichoic acid specificity, whereas controls did not produce such responses until they were 20 weeks old, as discussed above. The lengthy oral immunization required to produce an effect on responses to teichoic acid is supported by the work of Rothberg et al. (1973), who found that 4–8 weeks of oral immunization with a protein antigen was necessary for stimulation. The role of dosage in the induction of naturally-occurring cell-mediated and IgG antibody responses to teichoic acid is further illustrated in germfree rats. These animals produced only IgM natural antibodies to teichoic acid and no cell-mediated responses, regardless of age.
A second explanation for the spontaneous development of naturally-occurring cell-mediated responses to teichoic acid in rats over 20 weeks of age may involve an inter-relationship between cell-mediated responses and antibody production. A number of years ago several investigators suggested that delayed hypersensitivity was merely an initial stage of the immune response which later subsided as antibody formation began (Dienes and Mallory, 1932), (Salvin, 1958), (Sell and Weigle, 1959). This early work also suggested that delayed hypersensitivity and antibody responses could not occur simultaneously, however Gell and Benacerraf (1960) cast doubt on these theories by showing the two responses were independent, although coincident at times. The present work further refutes such ideas since antibody formation preceded the development of delayed hypersensitivity. Natural antibody responses to teichoic acid in the rat may suppress cell-mediated responses of the same specificity, a phenomenon first reported by Neta and Salvin (1973). They later found that such suppression was not dependent upon circulating antibody but was caused by a suppressor B-cell (Neta and Salvin, 1974). The possibility of this mechanism operating in the present investigation is further supported by the work of Miller and Jackson (1973), who reported active immuno-suppression of sheep erythrocyte responses by glycerol-teichoic acid.

A third explanation for the appearance of naturally-occurring cell-mediated and IgG responses to glycerol-teichoic acid at 20 weeks age may be solely a dose-related phenomenon and exclusive of the other factors mentioned above. The absorptive properties of the gut
gradually change as the rat matures allowing larger quantities or different complex forms of teichoic acid to gain entrance via gut mucosal surfaces, thus effecting a change in the natural responses. The oral immunizations described in Part III established that dosage of teichoic acid by this route can significantly affect responses. Rats which were 16 and 18 weeks old and fed bacteria for 5 weeks prior to examination produced cell-mediated responses to teichoic acid while antibody responses of this specificity shifted entirely to IgG. Control rats produced only IgM and no cell-mediated responses of teichoic acid specificity. Antibody titers were significantly lower in the immunized rats as compared to normals of the same age, however no significance may be attached to this observation since the passive hemolysis test favors IgM (Borsos and Rapp, 1965a, 1965b). The shift to IgG, resulting in the lower hemolysin titers observed in the oral immunization experiments may also explain the drop in frequency and mean titer observed in the serial studies of responses to teichoic acid in normal rats (Part II). The apparent drop at 18 weeks may have been caused by the spontaneous shift to natural IgG production which resulted in an artificial drop in titer and frequency because of the partiality of the hemolysis test for IgM.

The ontogeny study of natural responses to teichoic acid in germ-free rats revealed that these animals produced natural antibodies of teichoic acid specificity with a frequency similar to that observed in conventional rats, but at much lower levels and only of the IgM class. No naturally-occurring cell-mediated responses to teichoic acid were observed. Apparently these rats were still being exposed
to teichoic acid antigen present in the dead Gram-positive bacteria associated with the autoclaved food and bedding (Frederick and Chorpenning, 1973), however the dosage could conceivably be less than that received by conventional animals, accounting for the low levels of antibody and lack of IgG and cell-mediated responses to teichoic acid. Such observations are supported by the work of Wagner (1959), who found dead bacteria in food retained antigenicity and by Landy and Weidanz (1964), who observed lower levels of natural antibodies in germfree animals. An alternative explanation for the lack of naturally-occurring cell-mediated and IgG responses to teichoic acid in germfree rats is that these animals may have a deficiency in T-cell mediated responses as reported by Pollard and Nordin (1971) in germ-free guinea pigs. Lending further support to such an argument is the fact that all rats produce peculiar cell-mediated responses (Waksman, 1968). The lower levels of natural antibodies to teichoic acid in germfree rats is in contrast with the findings of Frederick and Chorpenning (1973), who reported higher levels of natural antibodies to teichoic acid in germfree guinea pigs than in conventional ones. One may attribute the difference to species variation in ability to respond to polysaccharide antigens.

Naturally-occurring sheep hemolysins were produced by all conventional rats examined, but at much lower levels than those of teichoic acid specificity and only of the IgM class. These antibodies were not detected until the rats were 6 weeks old, as compared to antibodies of teichoic acid specificity which were found in animals as young as two weeks. Germfree rats did not produce natural sheep
hemolysins although a small number of plaque-forming cells were found in the spleens of these animals (Rozmiarek, 1976). These results suggest that cross-reacting antigens in the gut flora may be a major source of stimulation for these responses, as suggested by the work of other investigators (Michael et al., 1962), (Landy and Weidanz, 1964), (Cheng and Trentin, 1967), (Crabbe et al., 1968). Such a phenomenon was described by Springer et al. (1959), who found germfree chicks lacked anti-human 'B' isoagglutinins and Muschel and Osawa (1959), who demonstrated cross-reactivity of the 'B' substance with several enteric bacteria. That sheep hemolysins were also produced in rats fed the teichoic acid deficient diet further supports the contribution of gut flora in stimulation of these natural sheep hemolysin responses.

Antibody cycling was observed in individual rats which were examined for natural antibodies to teichoic acid by serial bleeding. The cycles varied in intensity, duration, and number with each animal examined during the 32 week period. This observation, considered along with Weigle's suggestion (1975) that more work was needed on antibody cycling utilizing the more persistent polysaccharide antigens, prompted further investigation. Utilization of teichoic acid provided a well-characterized polysaccharide antigen of polyglycerophosphate specificity in contrast to the complex antigens used in previous studies. Cycling of antibodies to teichoic acid was observed after a single injection of Bacillus species (OSU No. 372) as well as in a second group of normal rats which were serially bled every 4th day. The antibody cycling observed in the injected rats was probably
the result of an anamnestic response to teichoic acid since baseline bleedings revealed these animals were producing natural antibodies of this specificity. The high levels of plaque-forming cells in the bone marrow of these animals further suggests anamnesis since the work of other investigators has shown bone marrow cells to be poorly responsive to primary antigenic challenge (Benner and Van Oudenaren, 1975). The cycling observed in the indirect plaque-forming cells was much more pronounced than with direct PFC, analogous to the secondary response cycling reported by Romball and Weigle (1973). The serum antibody peaks lagged several days behind the PFC peaks, again consistent with the findings of Romball and Weigle (1973).

Weigle (1975) suggested a correlation between route of injection, organ of antigen localization, and cycling of the PFC response. In his work a single intravenous injection of labelled protein antigen persisted in the spleen, but not in the mesenteric lymph nodes. Cycling of PFC was also observed in the spleen, but not in the nodes. In the present investigation, direct and indirect PFC produced bimodal peaks in the spleen, however in the mesenteric lymph nodes single peaks were observed. The direct PFC peak occurred at 8 days and the indirect at 24 days. A second rise in direct PFC number was evident at 32 days after injection, suggesting the possibility of a second peak. Antigen persisted in the spleen, mesenteric lymph nodes, and bone marrow for the duration of this experiment (Rozmiarek, 1976). This differed from the previous work of Weigle (1975) probably because the animals were already primed and were producing natural antibodies to teichoic acid. Nossal and Ada (1971) found a definite affect of
natural antibodies on antigen localization and Rothberg et al., (1973) showed that oral immunization affected both antigen localization and responses, especially in the mesenteric lymph nodes.

The cycling of antibodies to teichoic acid in the normal rats which were bled every fourth day confirmed the initial observation of cycling in the 32 week serial studies. The results indicated that cycling varied immensely among individual normal rats and any attempt to pool data from these animals would prove erroneous. The initial 32 week serial studies also revealed cycling of natural sheep erythrocyte hemolysins, however the matter was not pursued since the sheep erythrocyte represents a mosaic of antigenic determinants.

The antibody cycling observed in the immunized rats can be readily explained by antibody feedback suppression of B cell activity (Uhr and Moller, 1968), however the situation becomes more complicated when one attempts explanation of natural antibody cycling. Part of the work in this thesis provided conclusive evidence that natural antibody production of teichoic acid specificity is the result of constant dietary stimulation via gut mucosal surfaces. Rozmiarek (1976) found that radiolabelled teichoic acid, when administered orally to rats, localized in the mesenteric lymph nodes. Considering this source of stimulation, one may utilize the theory proposed by Weigle (1975) in suggesting a mechanism for cycling of natural antibodies to teichoic acid. Antibody regulation of the response may occur locally in the mesenteric lymph nodes. Lymphoid cells in this tissue would have a constant source of antigenic stimulation from environmental teichoic acid. Cells in the appropriate phase of differentiation would be
stimulated to produce antibody. This locally produced antibody may cause lymphoid cells of teichoic acid specificity to become arrested in a stationary phase of the cell cycle. A precise latent period may then be required before the arrested cells could be stimulated by incoming antigen, thus resulting in a cyclical production of antibody. A certain number of contacts with antigen may then be required to stimulate the arrested cells therefore further enhancing the apparent cycling, as suggested by Weigle (1975). This theory is based upon the well-documented phenomenon of B cell regulation by antibody (Uhr and Moller, 1968), however one must consider a possible role for helper and suppressor T cells, which are known to regulate B cell activity (reviewed in Greaves et al., 1973). Unfortunately we have no current evidence implicating these cells in antibody cycling and one can not, at present, make any valid statements concerning the T cell dependency of the teichoic acid antigen. Therefore it would not be of value to speculate in this discussion. A third course of argument in explanation of antibody cycling suggests that it is the result of lymphoid cells responding to different antigenic determinants at different times (Wortis et al., 1966), however this is unlikely in the teichoic acid system since the assays used detected only a single specificity (Decker et al., 1972). Antibody cycling such as reported in this investigation may play an important role in immunoregulation, however its mechanism and importance has not been clarified as of this writing. Cycling is certainly a more widespread phenomenon than first realized, especially with the present report of its occurrence in normal animals.
Having examined and discussed a number of parameters associated with naturally-occurring responses to glycerol-teichoic acid including ontogeny, cycling, and the effect of germfree environments, one is compelled to investigate possible sources of stimulation for such responses. The teichoic acids, being widely distributed in cell wall or membrane components of Gram-positive bacteria (Archibald and Baddiley, 1966), are widespread in the environment. The importance of such antigens in the elicitation of natural antibodies has been demonstrated in germfree animals by Springer et al. (1959) and further supported by the work of Wagner (1959), Sterzl et al. (1962), Landy and Weidanz (1964), and Ikari (1964). The present investigation as well as the work of Frederick and Chorpenning (1973) demonstrated naturally-occurring responses to teichoic acid in both germfree and conventional animals, which were determined to be of polyglycerophosphate specificity (Decker et al. 1972). The PGP backbone of teichoic acid is quite stable, thus allowing it to be immunogenic when presented as part of either living or dead bacteria via the gastrointestinal route (Frederick and Chorpenning, 1973). Such stimulation may account for the natural responses observed in both germfree and conventional rats examined in this investigation, however we had no direct proof of this assumption. Therefore an attempt was made to characterize the environmental nature of glycerol-teichoic acid by removal of the antigen from the animals' environment. The Similac diet was chosen since it appeared nutritionally adequate for the rat. Examination of this diet revealed few bacterial bodies and no detectable teichoic acid, which was rather unusual since it was a milk base
formula. The filtering, which had previously been considered, became unnecessary. Not only did the germfree rats on the diet fail to produce natural responses to teichoic acid, but neither did conventional controls. These results suggested that food was the major source of antigenic stimulation for responses to teichoic acid and that the gut flora contributed very little, which is supported by the observation that oral immunization with Gram-positive bacteria produced responses to teichoic acid in both germfree and conventional animals which were reared on the TA-deficient diet.

The environmental nature of the glycerol-teichoic acid antigen and its impact upon the immune response was clearly demonstrated in this investigation, especially with regards to the source of stimulation for natural responses of PGP specificity. Considering these results, one may disregard Wilson and Miles' definition (1955) of natural antibodies which described them as arising without apparent immunization or infection. It has been shown that antibodies to teichoic acid arise as the result of oral immunization and are not unlike immune antibodies of the same specificity. Future communications concerning natural antibodies to teichoic acid should contain an explanation describing the source of stimulation for such responses.
SUMMARY AND CONCLUSIONS

1. Production of natural antibodies to glycerol-teichoic acid begins at a very young age in the rat and continues throughout the first year of life. Only IgM is produced until 20 weeks of age is reached, when some rats begin production of natural IgG and cell-mediated responses of teichoic acid specificity.

2. Natural sheep hemolysins were found in very low levels in the rat and were only of the IgM class. These antibodies were present in all animals tested during the first year of life.

3. Antibody cycling was observed in individual rats included in the serial studies, a phenomenon which accounted for the wide range of titers observed in both natural sheep and teichoic hemolysins.

4. Feeding of Gram-positive bacteria for long time periods stimulated responses to teichoic acid. Cell-mediated and IgG antibody responses occurred in these animals at a much earlier age than found in normal rats.

5. Germfree rats produced natural IgM antibodies to teichoic acid with a similar frequency to that found in conventional animals, but of lower titer. No natural IgG or cell-mediated responses to teichoic acid were found in these animals and they produced no sheep erythrocyte hemolysins.
6. Antibody cycling was observed in rats given a single intravenous injection of Gram-positive bacteria. Serum antibody cycles lagged several days behind plaque-forming cell cycles. The high numbers of plaque-forming cells found in the bone marrow, combined with the large number of indirect plaques in the spleens of these animals, suggested that a secondary responses occurred after a single injection of antigen. That the animals were already producing natural antibodies to teichoic acid prior to injection supports the suggestion.

7. Conventional and germfree rats which were reared on a teichoic acid-deficient diet produced no antibodies to teichoic acid, but continued producing sheep hemolysins. Rats on this diet which were fed Gram-positive bacteria did produce antibodies to teichoic acid.

8. The environmental nature of the glycerol-teichoic acid antigen was clarified. The results suggest that food was a major source of stimulation for natural responses to teichoic acid while gut flora appeared to be the major source of stimulation for natural sheep hemolysin production.
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