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The humoral immune response to streptococcal cell wall-induced arthritis in the rat

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The University of Arizona, 1989
THE HUMORAL IMMUNE RESPONSE TO STREPTOCOCCAL CELL WALL-INDUCED ARTHRITIS IN THE RAT

by

Bernard Stephen Effertz

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Arthritis in the Rat.

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I dedicate my dissertation to Dr. Robert "Bogart" DePond, my best friend, who taught me how to not take life so seriously. I'll miss you.

Thank you mom and dad for always being there for me. I'll always be there for you.

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ABSTRACT

I investigated the humoral immune response to streptococcal cell walls (SCW) in arthritis susceptible Lewis and resistant Fisher rats. All rats were given a single intraperitoneal injection of either SCW or saline (controls). Rats were sacrificed, three rats per time point, over an eleven week period and serum was collected for ELISA. SCW injected Lewis rats produced anti-SCW antibody, whereas control rats did not. Anti-SCW antibody was significantly elevated over controls between days 14-28 (post injection). Both saline and SCW injected Fisher rats produced anti-SCW antibody, but with different kinetics. Anti-SCW antibody increased by day 7 and remained elevated over controls till day 21, after which there was no difference. ELISA were designed to determine the SCW epitope(s) recognized by anti-SCW antibody. Formamide extracts of SCW, peptidoglycan and polysaccharide, were investigated along with the terminal epitope of polysaccharide, N-acetyl-D-glucosamine, and the peptidoglycan precursor peptide. The data revealed that anti-SCW antibody was directed against a combined SCW epitope, given the lack of significant binding to any of
the SCW epitopes tested. Isotype analysis of anti-SCW antibody revealed that the Lewis response was composed primarily of IgG2a whereas the Fisher response was composed primarily of IgM. Binding of rat IgG isotypes to whole streptococcus, SCW, peptidoglycan, and polysaccharide was investigated, given the possibility of background binding by the streptococcal Fc-receptor. Streptococcal binding of rat IgG was specific for IgG2c and the polysaccharide portion of SCW was necessary for binding. Passive immunization of naive Lewis rats with antibody from rats with active arthritis was ineffective at transferring the disease. However, subcutaneous injection of affinity purified anti-SCW antibody or IgG into Lewis rats, followed twenty-four hours later by a single intraperitoneal injection of SCW, suppressed the acute phase and inhibited the chronic disease. IgM rheumatoid factor (RF) was present in the serum of both saline and SCW injected Lewis and Fisher rats. However, SCW injection only induced a significant increase in IgM RF (between days 3-7) in Lewis rats. Passive immunization of Fisher rats with affinity purified IgM RF (from Lewis serum), three days post SCW injection, was ineffective at inducing arthritis.
CHAPTER 1: INTRODUCTION

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease of unknown etiology characterized by its effects on the joints. RA was first described in the medical literature in 1800 by Landre-Beauvias, who described it as a variant of gout. In 1819 Brodie defined RA as a slowly progressing synovitis, and in 1858 the term "rheumatoid arthritis" was coined by Garrod (Short 1959). RA has a worldwide distribution and its prevalence in the U.S. is estimated at 2-3% of the population. The incidence of RA increases with age and women are afflicted two to three times more than men. The mean age of onset is between 30 and 50 years. The afflicted population in Arizona is twice the national average (Birch 1986).

CLASSIFICATION AS AN AUTOIMMUNE DISEASE

Classification of RA as an autoimmune disease began with the discovery of rheumatoid factor (RF), independently by Rose and Waaler in the 1940s by observing the agglutination of sensitized sheep erythrocytes with rheumatoid sera (Rose 1948, Waaler 1940). RF has since been identified as antibody which binds to the second and
third heavy chain constant region of IgG with low affinity and is heterologous in its binding (Sasso et al 1988, Roitt and Cooke 1987, Weisbart et al 1987). Based upon the presence of RF, RA patients are divided into seropositive and seronegative groups with seropositive patients inclined to develop a more severe form of the disease (Carson et al 1981, Koopman and Schrohenloher 1985). However, RF are not specific to RA as other autoimmune and inflammatory diseases as well as healthy individuals display varying levels of RF. Immune complexes containing IgG and RF from synovial fluid of patients with RA support the argument in favor of an inflammatory autoimmune pathology (Winchester et al 1970).

Evidence of autoimmunity to collagen is also present in RA. Anti-collagen antibody in RA was first reported by Steffen and Timpl (Steffen and Timpl 1963). Since then anti-collagen antibody and immune complexes have been detected in the synovial fluid (Menzel et al 1978). However, once again immunity to collagen is not unique to RA, as it also occurs in pulmonary fibrosis, scleroderma, chronic liver disease, plus a percent of normals (Kravis et al 1976, Mackel et al 1982, Menzel et al 1980, Steffen 1978).
GENETIC PREDISPOSITION

Genetic factors have been shown to predispose individuals to RA. Interest in the genetic associations between disease and the presence of histocompatibility markers began in 1973 when the HLA-B27 marker was correlated with an increased risk of developing ankylosing spondylitis (Schlosstein 1973). Although less dramatic, the HLA-DR4 marker was shown by Stastny in 1978 to place its carriers at a four-fold relative risk of developing RA (Stastny 1978). Monozygotic twins exhibit a higher incidence of RA when similarly paired with dizygotic twins (Christiansen et al 1984). Racial factors also appear to play a role as several North American Indian tribes have a higher incidence of RA while American blacks, native Japanese, and Chinese have a lower incidence relative to whites (Cunningham and Kelsey 1984, Beasley et al 1983).

Sex hormones have been implicated in the pathogenesis of RA and its animal models (Allen et al 1983b). The mitogen properties of estrogens on B cells was recently investigated. The results indicate that under the influence of estrogens there is increased production of autoantibodies without a proportionate increase in the Ly1+ subset, a subset committed to autoantibody production (Ahmed et al 1988). This hormonal phenomenon may be due to differences in lymphocyte reactivity in females as compared
with males. Women with RA experience a remission of symptoms during pregnancy and pregnancy has been associated with decreased numbers of T cells (Hench 1949, Birkeland and Kristofferson 1977, Tallon et al 1984). The decline in prevalence of RA among women between 1960-1974 has been linked to a possible protective effect of oral contraceptives and the use of estrogens in post-menopausal women (Wingrave and Kay 1978, Linos et al 1983).

**CYTOKINES AND GROWTH FACTORS**

Recombinant DNA technology has made investigations into the role of cytokines and growth factors in the arthritic process feasible. There is an imbalance of cell regulation in the rheumatoid synovium caused by a complex picture of autocrine and paracrine regulation. The two most prevalent and most studied cytokines in the rheumatoid synovium are interleukin-1 and tumor necrosis factor-alpha (Nouri et al 1984, Yocum et al 1989). TNF-a and IL-1 are pleiotropic cytokines with numerous proinflammatory properties. Both have been shown to stimulate collagenase and PGE2 production (Dayer et al 1985, Mizel et al 1981), induce bone and cartilage resorption (Bertolini et al 1986, Saklatvala 1986), and enhance fibroblast proliferation (Sugarman et al 1985, Vilcek et al 1986, Gowan et al 1984). Prostaglandin-E2 (PGE2) which is elevated in RA...
synovial fluid has been reported to inhibit antigen and mitogen driven T cell activation, suppress the release of IL-2 and gamma-IFN, and suppress the clonal expansion of activated B cells (Ellner and Spagnuolo 1979, Goodwin et al 1977, Rappaport and Dodge 1982, Hasler et al 1983). However, RA synovial tissue has enhanced expression of class II MHC antigens which suggests the presence of interferon-gamma (IFN-g), yet IFN-g is only detectable in trace amounts (Janossy et al 1981). Furthermore, the typical RA profile of elevated immunoglobulin titer and local production by plasma cells is contrary to the above reported action of PGE2.

In the milieu of the rheumatoid synovium, chondrocytes and fibroblast-like synoviocytes exhibit characteristics not unlike a localized, non-metastatic neoplasm. On a gross histologic level, synoviocytes have large pale nuclei and enlarged nucleoli (Harris 1985, Fassbender et al 1980). The synoviocytes are highly proliferative and invasive. In culture, synoviocytes do not contact inhibit and grow under anchorage independent conditions (Lafyatis et al 1989). Further evidence of the transformed phenotype is demonstrated by the expression of transformation-associated genes. For example, the transin gene, which translates a 51 Kd neutral metalloprotease, as well as the nuclear expressed myc oncogene, exhibit high level
expression in RA (Case et al 1989).

The transformed phenotype is likely regulated by paracrine factors, given their return to normal growth after a few passages in vitro. Platelet-derived growth factor (PDGF) has been implicated in synoviocyte anchorage independent growth in vitro. Epidermal growth factor (EGF) has a synergistic effect with PDGF synoviocyte proliferation, whereas transforming growth factor-beta (TGF-β) and all-trans-retinoic acid are down regulatory (Remmers et al 1988, Lafyatis et al 1989).

ETIOLOGY OF RA

BACTERIAL. Ever since Kaplan demonstrated the link between rheumatic fever and streptococcal infection, the inflammatory and autoimmunogenic properties of bacterial cell walls have continued to interest scientists (Kaplan 1963). Bacteria and/or their cell walls are felt to play important roles in human diseases such as Reiter's, the spondyloarthopathies, and RA (Catterall 1975, Kinsella 1985, Bennett 1978). High levels of antibodies to peptidoglycan-polysaccharide, a major constituent of bacterial cell walls, have been measured in both seropositive and seronegative RA and juvenile onset arthritis (Heymer 1976, Burgos-Vargas et al 1986, Johnson et al 1984, Pope et al 1979, Braun and Holm 1970). Despite
clinical and pathological features of an infectious etiology in RA, no single bacterium has been consistently isolated or associated with the disease (Bennett 1978). The inability of investigators to consistently isolate a single microbe from RA synovial fluid may be explained, assuming an infectious etiology, by the finding in experimental arthritis that following a suboptimal injection SCW, the arthritis may be reactivated using fragments from unrelated bacteria...ie, E. coli (van der Broek et al 1989).

VIRAL. Evidence of a viral etiology in RA, like that for bacteria, is controversial and suffers from the lack of a consistently isolated pathogen. RA has been associated with rubella virus infection and rubella antibody has been detected in synovial fluid. The pathophysiology is believed to arise from the deposition of circulating immune complexes in the joints leading to the activation of complement and the initiation of inflammation (Hollander et al 1962, Cremer et al 1974). Hepatitis B has been associated with cases of persistent synovitis and rheumatoid factor production (Duffy et al 1976, Bouchier et al 1964). Epstein-Barr virus (EBV) was linked to RA through the high frequency of RA patients expressing a nuclear antigen, rheumatoid arthritis-associated nuclear antigen (RANA), which is typically seen in the early stages
of EBV transformation of human B cells (Alspaugh et al 1978, Catalano et al 1980). In addition, EBV is a polyclonal B cell activator and RA patients have defective immune regulation of EBV-infected lymphocytes (Tosato et al 1983). Another viral candidate is the parvovirus RA-1 which was implicated in RA by the finding of virus-like particles in the synovium using electron microscopy (Simpson et al 1984). Cytomegalovirus (CMV) has been isolated from synovial cells, and some patients with early disease have elevated antibodies to CMV (Hamerman et al 1982, Male et al 1982). The evidence presented here for a viral etiology of RA is only a small fraction of that reported in the literature and does not include those reports which contain contrary (or conflicting) evidence.

In summary, when considering a bacterial or viral etiology for RA one must keep in mind that the data may represent the isolation of an innocent passenger in vivo, acquisition of a laboratory contaminant in vitro, or result from false-positives, ie, cross-reacting antibodies. It is the belief of this author that the development of RA requires both a genetic predisposition (ie, an immunoregulatory defect) and an initiation factor (ie, bacterial or viral infection).
Chapter 2: MODELS OF ARTHRITIS

There are three commonly used animal models for the study of human RA. Adjuvant-induced arthritis (AA), collagen-induced arthritis (CIA), and streptococcal cell wall-induced arthritis. There is also a model in MRL/lpr mice in which an arthritis-like condition occurs spontaneously. This model is largely used for the study of systemic lupus erythematosus.

ADJUVANT ARTHRITIS

In 1956, the first animal model of RA was proposed by Pearson. Susceptible strains of rats given a single intradermal (ID) injection of a water-in-oil emulsion containing mycobacterium developed arthritis 2-3 weeks later. The disease followed a remittant course characterized by an influx of polymorphonuclear leukocytes and monocytes resulting in proliferative synovitis, ossification and ankylosis of tarsal joints and the lower spine (Pearson 1956). A mild form of the disease may be passively transferred to naive recipients via lymph node or spleen cells (Pearson and Wood 1964). Evidence pointing to a cell-mediated immunopathology include suppression of

COLLAGEN ARTHRITIS

Central to the destructive process in RA is the assault on the cartilagenous integrity of the joint, apparently through an autoimmune mechanism. The cellular and humoral immunity to collagen observed in RA, lead Trentham et al to develop an animal model in 1977. The model they developed involved an ID injection of soluble native type II collagen emulsified in incomplete Freund's adjuvant, followed by a booster injection at one week. After two weeks, 40% of rats will develop an inflammatory polyarthritis which progresses over the next 2-3 months, resulting in erosion of periarticular bone and cartilage (Trentham et al 1977). Susceptibility has been linked to the Ir genes of the major histocompatibility complex in the mouse and rat (Wooley et al 1981, Griffiths et al 1981).
The immunizing antigen must be homologous or heterologous type II collagen; denatured, or types I and III collagen are not effective (Trentham et al 1978a). The arthritis may be transferred to naive recipients using lymph node or spleen cells, or serum from arthritic donors (Trentham et al 1978b, Stuart et al 1982a). Anti-collagen antibodies elevate in concordance with disease onset, and whereas injected collagen does not localize to the joints, anti-collagen antibody does (Stuart et al 1982b).

**STREPTOCOCCAL CELL WALL ARTHRITIS**

The search for a suitable animal model for the study of RA has yielded several models, but none of them possess all the features of the human disease. The streptococcal cell wall model, developed by Cromartie in 1977, is probably the best overall model and will be discussed in detail in Chapter 4. Briefly, arthritis is induced in genetically susceptible rats following a single intraperitoneal (IP) injection of an aqueous suspension of streptococcal cell wall fragments. Within 2 days rats develop an acute arthritis which evolves into a chronic, erosive, proliferative polyarthritis with destruction of periarticular cartilage and bone. The arthritis is clinically, radiologically, and histologically similar to human RA (Cromartie et al 1977, Clark et al 1979).
CHAPTER 3: STREPTOCOCCUS AND THE IMMUNE RESPONSE

This chapter will begin with some general information about group A Streptococcus pyogenes, the bacteria most commonly used in the induction of streptococcal cell wall-induced arthritis. In addition, the structure of the streptococcal cell wall (SCW) will be discussed in detail. And in order to better understand why SCW induce arthritis upon injection into rats, I will address the effects of SCW on the immune system with emphasis on B cells.

GROUP A STREPTOCOCCUS

Group A Streptococcus pyogenes is a gram positive, facultative anaerobe which may become gram variable with age. The bacterium has a spherical-ovoid shape, 0.6-1.0 microns in diameter. It requires complex minimal media (Todd Hewitt is preferred) with optimal growth at pH 7.4-7.6 at 37 C under reduced oxygen tension. In primary culture it is B-hemolytic on sheep blood agar and may produce a hyaluronic acid capsule during early growth.

Streptococci have been a major human pathogen especially in the preantibiotic era. Pharyngitis and
impetigo often result from acute streptococcal infection (Joklik et al 1984). Lipotechoic acid and M protein are the main adherence mechanisms used in attachment to epithelial cells (Ofek et al 1982, Mattingly and Johnston 1987).

M protein is a major virulence factor and is the only known antigen to evoke protective antibody against itself (Lancefield 1959). M protein may, in addition, prevent opsonization via the alternate complement pathway (Cleary et al 1975). M protein is a dimeric molecule, two alpha-helical chains in a coiled-coil conformation, which extends as a fibril and is attached to the streptococcal surface by its carboxy terminus (Phillips et al 1981, Fischetti et al 1985). More than 80 immunologically distinct M protein antigens are recognized (Lancefield 1962), with diversity resulting from antigenic drift and its expression subject to phase switching (Facklam and Edwards 1979, Maxted and Valkenburg 1969, Spanier et al 1984, Simpson and Cleary 1987). Furthermore, antigenic diversity may be the result of tandem repeats, repetitive regions of DNA known as hotspots for duplication and deletion events (Hollingshead et al 1986). M protein has been implicated in the etiology of such autoimmune diseases as glomerulonephritis and rheumatic fever. Goroncy-Bermes et al identified a monoclonal antibody which cross-reacted with both human
renal glomeruli antigen and M protein (Goronyc-Bermes et al 1987). Similarly, M protein epitopes have been shown to cross-react with sarcolemmal membrane proteins and cardiac myosin, providing a possible immunologic basis of rheumatic fever (Dale and Beachey 1982, Krisher and Cunningham 1985).

Group A streptococci express on their surface receptors which bind specifically to the Fc-region of human IgG (Myhre and Kronvall 1977, Myhre and Kronvall 1979). The binding specificity of this FcR with respect to rat IgG isotypes is unknown. The role of streptococcal FcRs in SCW-induced arthritis will be addressed in later chapters. In addition, most strains of group A streptococci produce pyrogenic exotoxins, DNases, streptokinase, hyaluronidase, and various proteinases.

**STRUCTURE OF SCW**

The common denominator of bacterial cell wall-induced arthritis is the peptidoglycan-polysaccharide (PG-PS) complex, common to gram positive bacteria. Peptidoglycan is composed of a repeating disaccharide backbone of N-acetylglucosamine and N-acetylmuramic acid joined by a β,1-4 linkage and amino acid side chains attached to the lactyl ether group on the N-acetylmuramic acid (Krause and McCarty 1961). Group A streptococcus contains a pentapeptide (L-Ala,D-Glu,L-Lys,D-Ala,(D-Ala)), which is common to
staphylococcal and streptococcal PG (Schleifer 1972). Variations in the amino acids and the extent of crosslinking between the peptide chains accounts for bacterial differences (Schleifer 1972). The polysaccharide portion is composed of rhamnose (60\%) and N-acetylglucosamine (40\%) attached to the sixth carbon of N-acetylmuramic acid (Krause and McCarty 1961).

**GROUP A STREPTOCOCCAL CELL WALL STRUCTURE**

\[
\begin{align*}
\text{GlcNAc} & \quad \text{GlcNAc} \\
\text{Rh} & \quad \text{Rh} \quad \text{(Rh-Rh)}_n \\
\text{L-Ala} & \quad \text{L-Ala} \\
\text{D-Glu} & \quad \text{D-Glu} \\
\text{L-Lys} & \quad \text{L-Lys-L-Ala-L-Ala-D-Ala} \\
\text{D-Ala} & \quad \text{D-Ala} \\
\text{D-Ala} & \quad \text{(D-Ala)} \\
\text{MurNAc} & \quad \text{N-acetylmuramic acid} \\
\text{GlcNAc} & \quad \text{N-acetylglucosamine} \\
\text{Rh} & \quad \text{rhamnose}
\end{align*}
\]

Given the ubiquity of PG-PS, it is not surprising that many different bacteria have arthropathic potential. For example, *Lactobacillus casei* (part of the normal enteric flora in man), *Lactobacillus plantarum*, and *Staphylococcus aureus* are all capable of inducing chronic erosive

IMMUNOLOGIC PROPERTIES OF SCW

SCW exhibit several proinflammatory properties in vitro. PG and PG-PS have been shown to function as a B cell mitogen in the absence of T cells (Babu and Zeiger 1983, Damais et al 1975) and cause polyclonal B cell activation (Zeiger et al 1981, Dziarski 1980, Heymer and Rietschel 1977). However, SCW induce mitogenic anergy in T cells as measured by a decreased proliferative response to Con A or PHA (Ridge et al 1986, Regan et al 1988). This mitogenic anergy may be reversed by the removal of phagocytes and may be caused by the secretion of TGF-B by SCW-activated macrophages (Regan et al 1988, Wahl et al 1988). SCW activate both classical and alternate complement pathways as well as the formation of complement-SCW complexes in the presence of specific antibody (Greenblatt et al 1978, Eisenberg and Schwab 1986). The induction of the respiratory burst in PMNs, as measured by increased oxygen consumption and hexose monophosphate shunt activity (Leong et al 1984a). In addition, SCW act in a serum dependent manner to inhibit PMN mediated phagocytosis without being cytotoxic to them (Leong and Cohen 1984b). PG has been shown to function as an immunologic adjuvant
(Adam et al 1981) and stimulates monocytes and macrophages, resulting in the release of interleukin-1 (IL-1) and colony stimulating factor (Chedid and Audibert 1977, Takada et al 1979, Nauciel et al 1974, Gold et al 1985, Wilder 1987). Furthermore, SCW-activated monocytes stimulate synovial fibroblast plasminogen activator (PA) activity (Hamilton et al 1982). Plasmin, a biproduct of PA interaction with plasminogen, has been shown to degrade cartilage and activate latent collagenase by synovial cells (Lack and Rogers 1958, Werb et al 1977). The effects of SCW on Lewis and Fisher rats in vivo will be discussed in Chapter 4.
CHAPTER 4: THE SCW MODEL

The purpose of this chapter is to provide a detailed characterization of SCW-induced arthritis in rats. Briefly, the effects of SCW in animals other than the rat will be discussed. The remainder of the chapter will deal with the rat model in the following sequence: (a) induction of arthritis, (b) the importance of particle size, (c) acute phase response, (d) the chronic disease, and (e) the polygenetic nature of susceptibility.

As the arthropathic properties are not an exclusive characteristic of only SCW, so too the pathologic effects of SCW are not exclusive to the rat. The effects of streptococcal cell walls (SCW) in various animal species are profound and have found numerous research applications over the years. The intravenous (IV) or IP injection of SCW into mice induces a pancarditis (Cromartie and Craddock 1966, Ohanian et al 1969) while the intradermal injection of SCW into rabbits induces recurrent multinodular skin lesions (Schwab and Cromartie 1957, Ohanian and Schwab 1967) and the injection of rat gingival tissues with SCW induces periodontitis (Hunter et al 1979). However, injection of Rhesus monkeys with SCW resulted in chronic
erosive arthritis similar to that observed in rats (Yount et al 1989). The characteristic pathology of these various animal models is based on intrinsic properties governing the regional deposition of SCW antigen and the immunologic competence of the host.

INDUCTION OF ARTHRITIS

The classic method of induction in this model is via a single aqueous suspension of sonicated SCW injected IP. However, some investigators use an intraarticular (IA) or IV injection. IA injection of SCW polymers, LPS, or muramyl dipeptide (MD) induce only a transient inflammation with no chronic, relapsing arthritis (Esser et al 1986). This observation appears to implicate the importance of systemic exposure to regional lymph nodes, resulting in antigen presentation. Reactivation of joint inflammation initially induced via IA injection of PG-PS occurs upon IV injection of normally subarthropathic doses of SCW, MD, LPS, or IL-1 (Esser et al 1985, Stimpson et al 1987, Stimpson et al 1988). These data suggest that joints initially injured by PG-PS have increased sensitivity to subsequent antigenic challenge.

SCW SIZE REQUIREMENT

Groups A, B, and D streptococci vary in their
arthropathic potential depending on the manner in which they are prepared (i.e., whole, sonicated) prior to injection. Whole groups B and D induce arthritis but group B requires a latent period of 6-8 days and group D induces only a transient response. Whole group A is ineffective. Sonicated cell walls from groups A and B induce similar biphasic responses, however sonicated group D is ineffective (Spitznagel et al 1983). Even the relative severity induced by sonicated group A cell walls varies as a function of particle size. Small fragments (5 megadaltons) induce the most severe acute inflammation with only slight chronic disease, while large fragments (500 Md) induce only slight acute inflammation and moderate, late chronic disease. However, intermediate size fragments (50 Md) induce moderate acute inflammation and the most severe chronic disease (Fox et al 1982).

FIG. 6. Effect of particle size of PG-APS fragments isolated from group A streptococcal cell walls upon acute and chronic joint diseases. All rats were injected i.p. with PG-APS fragments (30 µg of rhamnose per g of body weight. □ (large fragments), 5 rats; △ (intermediate fragments), 12 rats; ○ (small fragments), 6 rats. Mean joint score ± standard error of the mean. Total of four legs; maximum score is 16. The level of significance between the three groups was calculated for each day that scores were recorded. For clarity, the standard error of the mean is only shown at 3-day intervals. Fraction 100±60 is significantly greater than 100±60 or 100±30 at days 2 through 18 and significantly less than fraction 100± from days 38 to 92 (P ≤ 0.05).

(Fox et al 1982)
These results suggest that arthrogenicity depends on the ability of phagocytes to process the various streptococcal forms. Isolated PG and MD, the smallest immunologically active PG fragment, induce only moderate acute inflammation with no chronic disease (Fox et al 1982). It is the PS portion which protects the PG from biodegradation by inhibiting lysozyme from cleaving the B,1-4 linkages of the disaccharide backbone, thereby allowing PG to persist in the tissues (Abdulla and Schwab 1966). Work done by Chetty et al makes a case for PS as the SCW component responsible for translocation to the joints following IP injection. This migration requires that PS contain terminal N-acetyl-D-glucosamine (NADG) and suggests the presence of sugar receptors in the synovial lining (Chetty et al 1983). In short, the development of arthritis depends on the deposition and persistence of the appropriate size SCW fragments in the joints.

STREPTOCOCCAL CELL WALL-INDUCED ARTHRITIS

Following a single IP injection of an aqueous suspension of sonicated SCW into susceptible strains of rats, an acute inflammatory reaction develops, subsides, then is followed by a chronic, relapsing, proliferative polyarthritis which eventually leads to the destruction of periarticular bone and cartilage (Cromartie et al 1977).
SCW are dispersed primarily to the spleen, liver, and peripheral joints (Dalldorf et al 1980, Wilder et al 1983). The development of arthritis depends on the deposition and persistence of SCW in the joints (Eisenberg et al 1982), and disease severity correlates with the amount of SCW within the joints (Eisenberg et al 1982).

**ACUTE PHASE.** After the IP injection of SCW, an acute peritonitis develops within 3 hours, followed by entrance into the bloodstream and lymphatics. During the first 3 days, SCW are associated with an intense PMN response, which gradually subsides over the next 2 weeks. SCW are present in splenic, Kupffer, and synovial macrophages and lymph nodes on day 1, is maximal on day 3, then progressively decreases to trace amounts by day 90. SCW were not found in the lungs, kidneys, or heart (Dalldorf et al 1980). The acute phase reaction is complement dependent. While depletion of serum complement by treatment with cobra venom factor resulted in reduced acute joint inflammation, it does not affect the chronic arthritis (Schwab et al 1982).

**THE CHRONIC DISEASE.** The chronic phase becomes clinically apparent at 3-4 weeks and lasts for months. This phase is characterized by the formation of hepatic and splenic granulomas and chronic erosive polyarthritis of peripheral joints, and is T cell dependent (Wahl et al 1986, Cromartie
et al 1977). The degree of granuloma development and intensity of Ia antigen expression in the liver are correlated with disease severity (Allen and Wilder 1987). Congenitally athymic (rnu/rnu) Lewis rats develop an acute phase reaction to SCW injection, but do not develop the chronic disease. However, reconstitution of nude rats with T cells causes a return to chronic phase susceptibility (Ridge et al 1985). Rats appropriately treated with cyclosporin A, a fungal metabolite that blocks T cell proliferation and lymphokine production, develop only an acute inflammatory reaction which resolves completely (Yocum et al 1986). These data strongly implicate T cells as having a pivotal role in this model. In addition, the chronic disease may also be suppressed by diethyldithiocarbamate (DTC) in a dose-related manner through unknown mechanisms (Seterah and Yocum 1989). The oral administration of retinoids (4-hydroxyphenyl retinamide) prior to SCW injection suppressed both acute and chronic phases (Haraqui et al 1985).

As seen in RA, the chronic phase synovium of SCW-injected Lewis rats develop neoplastic characteristics. On a histologic level the synoviocytes appear transformed given their spindle shape and prominence along invasive sites. These fibroblast-like cells are characterized by large pale nuclei, and prominent nucleoli. In culture,
these cells do not contact inhibit and grow under anchorage-independent conditions (Yocum et al 1988). Furthermore, the synovium demonstrates positive staining for transformation-associated cell markers i.e., stromolysin, vimentin, and myc (Case et al 1989, Yocum et al 1988, Wilder seminar '89). The transformed state as defined by stromolysin expression is present during the acute phase, but its persistent expression is T cell dependent.

THE POLYGENETIC NATURE OF SUSCEPTIBILITY

The susceptibility to development of the chronic, proliferative and erosive arthritis characteristic of this model is under polygenetic regulation (Anderle et al 1985, Wilder et al 1982). To date, strain, sex, and stress hormone response have each been found to play key roles in susceptibility. Interestingly, each has human correlates.

First, the relative susceptibility (or resistance) to arthritis is strain specific. Outbred Sprague-Dawley rats, which were used in the original model of Cromartie eleven years ago, have been replaced by inbred Lewis rats as the strain of choice. Out of sixteen inbred rat strains tested, the incidence of chronic polyarthritis ranged from 4 to 100% with Lewis females as the most susceptible and Fisher females as the most resistant (Wilder et al 1982). The resistance of certain rat strains to the development of
chronic arthritis appears to be a result of genetic differences in regulation of the inflammatory response and not to differences in the localization of SCW in the joints, given that SCW are deposited in the synovial tissue, spleen, liver, and mesenteric lymph nodes of Fisher rats (Anderle et al 1985). In addition, resistance is not due to an unresponsive state to the proinflammatory properties of SCW as Fisher rats develop an acute inflammatory reaction upon ID injection (Allen et al 1983a).

However, the arthritis resistant Fisher rats do not develop

Table 3. Incidence and severity of chronic polyarthritis in various inbred rat strains

<table>
<thead>
<tr>
<th>Rat strain*</th>
<th>No. of animals injected and observed &gt;60 days†</th>
<th>Incidence of chronic polyarthritis (%)</th>
<th>Maximum articular indices observed‡ (mean = SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEW/N</td>
<td>38</td>
<td>100</td>
<td>12.8 ± 2.0</td>
</tr>
<tr>
<td>LOU/MN</td>
<td>5</td>
<td>100</td>
<td>10.5 ± 3.3</td>
</tr>
<tr>
<td>LA/N</td>
<td>5</td>
<td>100</td>
<td>9.7 ± 1.8</td>
</tr>
<tr>
<td>RHA/N</td>
<td>16</td>
<td>100</td>
<td>9.7 ± 3.5</td>
</tr>
<tr>
<td>NSD/N</td>
<td>6</td>
<td>100</td>
<td>9.2 ± 3.1</td>
</tr>
<tr>
<td>MS20/N</td>
<td>5</td>
<td>100</td>
<td>8.1 ± 1.6</td>
</tr>
<tr>
<td>A28807/N</td>
<td>5</td>
<td>100</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td>BN/N</td>
<td>6</td>
<td>100</td>
<td>7.2 ± 1.6</td>
</tr>
<tr>
<td>ODU/N</td>
<td>11</td>
<td>63</td>
<td>3.8 ± 4.5</td>
</tr>
<tr>
<td>MR/N</td>
<td>5</td>
<td>80</td>
<td>4.4 ± 3.0</td>
</tr>
<tr>
<td>BUF/N</td>
<td>6</td>
<td>50</td>
<td>2.6 ± 3.1</td>
</tr>
<tr>
<td>MNR/N</td>
<td>6</td>
<td>67</td>
<td>2.3 ± 2.4</td>
</tr>
<tr>
<td>SHR/N</td>
<td>9</td>
<td>22</td>
<td>1.0 ± 1.2</td>
</tr>
<tr>
<td>ACI/N</td>
<td>17</td>
<td>12</td>
<td>0.3 ± 0.8</td>
</tr>
<tr>
<td>F344/N</td>
<td>53</td>
<td>4</td>
<td>0.1 ± 0.5</td>
</tr>
</tbody>
</table>

* Female rats that weighed approximately 100 gm at the time of injection.
† Intraperitoneal injection of an aqueous suspension of streptococcal cell walls at 60 µg of cell wall pergram body weight.
‡ Maximum articular indices assigned on basis of chronic polyarthritis, i.e., arthritis observed 14 to 90 days after injection with streptococcal cell walls.

(Wilder et al 1982)
SCW-specific T cell responses or T cell dependent SCW-induced chronic erosive polyarthritis, whereas Lewis rats do. It has been suggested that Fisher rats develop immunological tolerance to the arthritogenic epitopes of SCW. Removal of OX8+ (CD8 analogue) cells results in SCW-specific T cell responses in Fishers (van der Broek et al 1988a). It was also shown that this tolerance to SCW may be prevented using germ-free Fisher rats which following SCW injection develop an arthritis equal in severity to Lewis rats. The authors suggest that the tolerance seen in conventional Fisher rats is neonatally induced and sustained via the bacterial flora (van der Broek et al 1989).

SCW injection induces a splenic IL-2 deficiency and decreased mitogenic responses to concanavalin A in both Lewis and Fisher rats (Ridge et al 1986). This observed IL-2 deficiency may contribute to the impaired cell-mediated responses observed in this model (Hunter et al 1980). However, there is immunohistological evidence of positive staining for Ia antigens within the pannus (Yocum et al 1988). This splenic anergy may be mediated by the secretion of TGF-β, a polypeptide with immunosuppressive activity, by SCW-activated macrophages (Wahl et al 1988).

The sex-linked factors determining susceptibility to arthritis are largely related to sex hormones rather than
sex-linked immune response genes (Allen et al 1983b). Castrated Lewis male rats, which normally develop chronic polyarthritis at a 33% incidence compared to females at a 100% incidence, developed chronic arthritis almost equal to females (Wilder et al 1982). Treatment of castrated males with high doses beta-estradiol four days prior to SCW injection further exacerbated the arthritis (Allen et al 1983b).

Recent work in the laboratory of Dr. Wilder suggests that the susceptibility of Lewis rats to experimental arthritis may be due, in part, to a defective stress response along the hypothalamic-pituitary-adrenal axis. Lewis rats exhibit a deficient stress response to SCW when compared with Fisher rats as measured by decreased mRNA levels of corticotropin releasing hormone, ACTH, corticosterone, and enkephalin (Sternberg et al 1989). Furthermore, arthritis may be suppressed in Lewis rats by the injection of corticosteroids (dexamethasone) and induced in Fisher rats by the injection of the glucocorticoid receptor antagonist, RU 486 (Sternberg et al 1989).
CHAPTER 5: SPECIFIC AIMS

SCW have been described as a polyclonal B cell activator and mitogen. While T cells play an important role in the arthritogenic properties of SCW, little is known about the role of B cells. The hypothesis of this project was that SCW induce the production of RF and anti-SCW antibody of restricted heterogeneity and defined kinetics which may be directly involved in the pathogenesis of the chronic polyarthritis. The goal of this research was to investigate this hypothesis by defining the kinetics and specificity of the humoral immune response to SCW in arthritis susceptible (Lewis) and resistant (Fisher) rats.

The specific aims of this project were as follows:

1. Measure total anti-SCW antibody in serum samples collected from Lewis and Fisher SCW injected and saline injected (control) rats. This was accomplished using an ELISA.

2. Define the SCW epitope(s) specific for anti-SCW antibody. Serum from Lewis and Fisher rats were tested for antibody reactivity against the following SCW components
using various ELISAs:
   a. formamide extracted PG (peptidoglycan)
   b. formamide extracted PS (polysaccharide)
   c. PG precursor peptide
   d. NADG (N-acetyl-D-glucosamine)

3. Isotype the antibody response to SCW in Lewis and Fisher serum samples. Anti-SCW antibody was isotyped against all known rat isotypes (IgG1, IgG2a, IgG2b, IgG2c, IgM, IgA, and IgE) using an ELISA technique.

4. The binding specificity of the streptococcal FcR is unknown with respect to rat IgG subclasses. Therefore, the binding of affinity purified rat IgG isotypes to WS (whole streptococcus), SCW, PG, and PS was measured using an ELISA. The purpose of this experiment was to investigate any possible background (Fc-FcR) binding of serum immunoglobulin in specific aims 1-3.

5. Measure IgM RF in the serum of Lewis and Fisher rats. This was accomplished using an ELISA.

6. In order to evaluate the role anti-SCW antibody and RF in the pathogenesis of this model, the following passive immunization experiments were performed:
a. Can arthritis be passively transferred via anti-SCW antibody? Naive Lewis rats were immunized with concentrated antibody taken from Lewis rats with SCW-induced arthritis.
b. Does passive immunization provide protection to subsequent SCW injection? Affinity purified anti-SCW antibody obtained from Lewis rats with active arthritis (day 21) was injected SQ into naive Lewis rats. One day later the rats were given a single IP injection of SCW.
c. Can arthritis be induced in Fisher rats via passive immunization with Lewis RF? Affinity purified RF was obtained from Lewis rats 3 days post SCW injection and injected IP into Fisher rats which had been given a single IP injection of SCW 3 days earlier.
ANIMALS. Specific pathogen-free inbred LEW/N (Lewis) and F344/N (Fisher) female rats were obtained from Harlan Sprague-Dawley, Inc., Walkersville, Md. The rats were approximately 90 grams and 6 weeks old at the time of cell wall injection.

PREPARATION OF CELL WALLS. Streptococcal cell walls (SCW) were isolated using a modified version of another method (Cromartie et al 1977). Group A *Streptococcus pyogenes* type 3, strain D58 (American Type Culture Collection, Rockville, MD) were grown to log phase in Todd-Hewitt media (Microbio, Tempe, AZ) at 37 C under aerobic conditions. Bacteria were heat killed at 60 C for 2 hours then collected via centrifugation (20 minutes at 10,000 rpm) in sterile 250 ml Sorvall containers. The bacterial pellet was susupended in 200 ml of 4% sodium dodecyl sulphate (SDS), then disrupted by sonication (W-380 ultrasonic processor with a 3/4" high gain Q horn, Heat Systems-Ultrasonics Inc., Farmingdale, NY) for 3 minutes at full power and left for 18 hours at room temperature (RT). The SDS was removed from the SCW by a series of washes and centrifugations, followed by resuspension in sterile
phosphate buffered saline (PBS), pH 7.4. The KCl precipitation test was used to indicate the presence of SDS. The SDS-free pellet was then suspended in 100 ml of PBS containing 30 mg each of DNase (Sigma Chemical Co., St. Louis, MO), and RNase (Sigma) and incubated at RT for 4 hours. The SCW were washed twice with PBS then incubated for 4 hours with trypsin (Sigma; 30 mg/100 ml PBS). The SCW were washed 2 times with PBS, suspended to 35 ml with PBS and sonicated for 2 hours at full power on a discontinuous 2 second cycle. The SCW were centrifuged for 30 minutes at 10,000 rpm to remove any residue and frozen at -20 C till use.

RHAMNOSE ASSAY. Rhamnose concentration was determined, in order to standardize the amount of SCW for injection (dose: ug Rh/g body weight), using a method previously reported (Kennet et al 1980). Samples and rhamnose standards were set up in glass tubes in triplicate, diluted with H2O to 1 ml as follows: samples...1:10, 1:20, 1:30, 1:40, 1:50; standards...blank, 1:4, 2:3, 3:2, 4:1. Tubes were placed in an ice bath where 4.5 ml of diluted sulphuric acid (6:1 with H2O) was added to each tube, vortexed, and allowed to cool to RT. Tubes were immersed in boiling water for 10 minutes, then cooled to RT. 100 ul of 3% cysteine was added to all tubes, vortexed, then incubated for 2 hours at RT. Absorbance at 396 nm and 430 nm was used to calculate
the rhamnose content of the SCW.

INDUCTION OF ARTHRITIS. Induction of the polyarthritis was carried out as previously described in detail (Cromartie et al 1977). Briefly, a sterile aqueous suspension of sonicated SCW (9.8 mg/ml) in PBS, pH 7.4, was injected intraperitoneally (IP) into 27 female Lewis and 27 female Fisher rats at a dose equivalent to 50 ug of cell wall rhamnose per gram of body weight. This dose of SCW has previously been shown to induce severe chronic polyarthritis with nearly 100% incidence (Wilder et al 1982). Control rats (15 Lewis and 15 Fisher) were injected with sterile PBS.

SAMPLE COLLECTION. Serum was collected from 3 rats per time point (days post SCW injection 1, 3, 7, 14, 21, 28, 42, 56, 77; days post saline injection 0, 7, 21, 42, 77) for both Lewis and Fisher rats. Rats were sacrificed at each collection point by CO2 asphyxiation. Blood was collected by cardiac puncture, diluted 1:2 with PBS, and allowed to clot at RT for 2 hours. The cellular portion was removed by centrifugation. Serum samples were labeled and frozen at -70 C till needed.

FORMAMIDE EXTRACTION. Group A polysaccharide (PS) and peptidoglycan (PG) components were extracted from SCW using methods reported previously (Fuller 1938, Krause and McCarty 1961, Chetty et al 1983). SCW, prepared as
described above but without the 2 hours of sonication, were pelleted (20 minutes at 10,000 rpm), supernatant discarded, and the pellet resuspended in formamide (Sigma) at 5 mg/ml. The SCW suspension was placed in an oil bath at 150 C for 20 minutes, removed and allowed to cool overnight at 4 C. The mixture was centrifuged at 36,000 g for 30 min. at 4 C and the pellet discarded. Two volumes of acid alcohol (95 parts anhydrous alcohol plus 5 parts 2N HCl) was added to the supernatant, allowed to precipitate at RT, then centrifuged at 12,000 g for 30 min. at 4 C. The pellet, containing PG, was set aside and the PS in the alcoholic supernatant was precipitated using 5 volumes of acid-acetone (99% acetone, 1% HCl plus a few crystals of sodium acetate). The PS was allowed to precipitate for 72 hours at 4 C, then centrifuged at 12,000 g for 30 min. at 4 C. The acid-alcohol and acid-acetone steps were then repeated. PG and PS fractions were suspended in dH2O and dialyzed against dH2O for 24 hours at 4 C.

ELISA PROCEDURES. The enzyme-linked immunoassay techniques used were adapted from methods previously reported (Engvall and Perlmann 1972). Binding of antigen and antibody are described below. All washings were done as follows: 3 times with PBS-Tween (0.5% Tween-20 from Sigma), pH 7.4, then 2 times with PBS. All assays were performed using Immulon II plates (Dynatech Laboratories Inc., Alexandria,
VA). All steps involving the addition of reagents to wells were in the amount of 100 ul/well. The substrate was ABTS-citrate (Sigma; 2,2'-azinobis[3-ethylbenz-thiazolone sulphonic acid] diammonium salt, 54.8 mg plus citric acid monohydrate, 2.10 g, in 100ml H2O, pH 4.2) plus 1 ul/ml of 30% H2O2 added just prior to use. Absorbance at 405 nm was measured on a Titertek Multiskan 310-C (Flow Laboratories Inc., McLean, VA).

A. MEASUREMENT OF ANTI-SCW ANTIBODY. Plates were coated with sonicated SCW (11.7 mg/ml; total protein) diluted 1:100 in carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4 C. After washing, serum diluted 1:200 in PBS-T was added and incubated at RT for 1 hour. Plates were washed and incubated for 1 hour at RT with goat anti-rat IgG heavy and light chain specific horseradish peroxidase (HRP) conjugate (American Qualex International, La Mirada, CA; 1 mg/ml) diluted 1:1,000 in PBS-T. The plates were then washed, substrate added and incubated at RT for 10 minutes before reading.

B. MEASUREMENT OF ANTI-PG, PS, PPP, AND NADG ANTIBODY. These assays were performed identically to the anti-SCW antibody ELISA with the following exceptions. The PG (9.1 mg/ml; total protein) and PS (13.7 mg/ml; rhamnose) formamide extraction products were diluted 1:100, PPP (PG precursor peptide 5 mg/ml; Sigma) diluted 1:2,000, and NADG
(N-acetyl-D-glucosamine 1 mg/ml; Sigma) diluted 1:1,000. Plates were incubated with substrate for 20 minutes.

C. ISOTYPE OF ANTI-STREPTOCOCCUS ANTIBODY. Plates were coated with SCW as described above. Serum was diluted 1:40 in PBS-T and incubated for 1 hour at RT. After washing, mouse anti-rat isotype specific monoclonal antibodies (Bioproducts for Science, Indianapolis, IN; 1 mg/ml) were diluted 1:1,000 in PBS-T and incubated for 1 hour at RT. The plates were then washed and incubated for 1 hour with goat anti-mouse IgG-HRP conjugate (American Qualex; 1 mg/ml) diluted 1:2,000 in PBS-T. After washing, substrate was added for exactly 15 minutes before reading.

D. CONTROLS. Rabbit anti-Group A streptococcus antibody (Ventrex, Portland, ME; 1 mg/ml) diluted 1:1,000 in PBS-T was used to confirm binding of SCW, PG, and PPP to the plates. Griffonia Simplicifolia II-HRP conjugate (E-Y Labs, San Mateo, CA; 1 mg/ml) diluted 1:500 in PBS-T was used to confirm PS and NADG binding. Since the NADG was purchased covalently bound to BSA (bovine serum albumin: Sigma), a 3% BSA only control was also included. Nonspecific binding of the goat anti-rat IgG/HRP (or the mouse anti-rat monoclons/goat anti-mouse; isotype ELISA) was determined using wells incubated with PBS-T only (no serum).

Fc-GAMMA RECEPTOR ELISA. WS, SCW, PG, and PS were screened
for the presence of functional Fc-gamma receptors using affinity purified rat Ig isotypes. SCW, PG, and PS were bound to plates at a 1:50 dilution in carbonate/bicarbonate buffer, pH 9.6, and incubated overnight at 4 C.

WS was glutaraldehyde-fixed as described by (Kennet et al 1980). Briefly, 50 ul of poly-l-lysine (Sigma; 1 mg/100ml in PBS, pH 7.4) solution was added to the wells of 96 well, flat bottom Immulon II plates, incubated for 30 minutes at RT, then washed (2X PBS only). WS was prevashed in PBS and suspended to a concentration of 0.25% (W/V). Cells were added at 50 ul per well and centrifuged 5 minutes at 2000 rpm in microplate carriers. 50 ul/well of 0.5% glutaraldehyde (Sigma) in PBS was added and incubated for 15 minutes at RT. After washing (2X PBS only), 200 ul of 100 mM glycine (Sigma) in 0.1% BSA/PBS was added (to block excess glutaraldehyde), and incubated for 30 minutes at RT. The plates were then washed (2X PBS only) and incubated with 100 ul of 1% BSA in PBS for 1 hour at RT to block nonspecific protein binding. Plates were stored at minus 20 C till needed and washed 2 times with PBS-T before use.

Affinity purified rat IgG isotypes (Bioproducts for Science, 0.1 mg/ml) were diluted 1:1,000 in PBS-T and incubated for 1 hour at RT. The plates were then washed and incubated with goat anti-rat IgG-HRP (American Qualex)
diluted in PBS-T at 1:1,000 for 1 hour at RT. After washing, substrate was added and incubated for 10 minutes before reading.

**TOTAL IgM RHEUMATOID FACTOR ELISA.** Rat IgG (Sigma; 1 mg/ml) was aggregated by heating at 63°C for 20 minutes. Heat aggregated IgG (HAg-IgG) at 5 ug/ml in carbonate/bicarbonate buffer, pH 9.6, was added to the wells and incubated at RT for 2 hours. Binding of HAg-IgG was confirmed using goat anti-rat IgG (American Qualex). After washing, serum was diluted 1:40 in PBS-T and incubated at RT for 1 hour. The plates were then washed and rabbit anti-rat IgM biotin conjugate (Zymed Laboratories, South San Francisco, CA; 0.25 mg/ml) was diluted 1:1,000 in PBS-T and incubated at RT for 1 hour. After washing, avidin-HRP conjugate (Vector, Burlingame, CA; 1 mg/ml), diluted 1:5,000 in PBS-T, was added and incubated at RT for 30 minutes. Substrate was added after washing and incubated for exactly 15 minutes at RT before reading.

**ARTICULAR INDEX.** The severity of arthritis was scored daily on a scale of 0 (no inflammation) to 4 (severe inflammation) based on observations of erythema, edema, and size of the carpal and tarsal joints. A maximum score of 16 is possible.

**PASSIVE IMMUNIZATION.** Eighteen female Lewis rats were
injected with SCW and 6 were injected with saline as described earlier. Rats were sacrificed in 4 groups (with 6 rats per group) on days 5, 21, 88, and 90 (saline) post injection. Serum, collected as described earlier, was mixed with saturated ammonium sulphate (Sigma; 1,000 g /l dH2O stirred at RT for 8 hours, pH 7.0) at a 1:2 dilution (50% final concentration), swirled, and allowed to precipitate for 1 hour on ice. The precipitate was collected by centrifugation at 48,000 g for 30 minutes at 4 C, resuspended in minimal dH20, and dialyzed (Spectrapor, Pittsburgh, PA; 6,000-8,000 mwco) against several changes of PBS at 4 C. The soluble concentrate was then adjusted to 50% of the original pooled volume with PBS. The concentrated antibody (CA) from days 5, 21, 88, and 90 was injected IP into naive 4 week old female LEW/N rats. Rats were injected with CA from day 5 (19.8 mg/ml) at doses of 2 and 3 ml, CA from days 21 (26.6 mg/ml) and 88 (25.6 mg/ml) were injected at doses of 2, 3, and 4 ml, and the control, CA day 90 (24.3 mg/ml), was injected at a 3 ml dose. Arthritis was assessed using the articular index over a four week period.

ISOLATION OF RHEUMATOID FACTOR AND ANTI-SCW ANTIBODY. Rat IgG (Sigma) and SCW were coupled separately to CNBr-activated Sepharose 4B (Pharmacia, Princeton, NJ) according to the manufacturer's recommendations. IgG was heat
aggregated at 63°C for 20 minutes and coupled at 10 mg/ml of wet gel as was SCW (11.7 mg/ml). 3 g of sepharose was used for each column. The sepharose was washed with 1 mM HCl on a sintered glass filter. The ligand (SCW or HAg-IgG) was dissolved in 15 ml of coupling buffer (0.1 M NaHCO3, pH 8.3, plus 0.5 M NaCl) and mixed end-over-end overnight at 4°C. Excess ligand was washed away with coupling buffer and the remaining active sites were blocked with 1 M ethanolamine, pH 9, for 2 hours at RT. Afterwards, the beads were washed with 3 cycles of alternating 0.1 M acetate buffer, pH 4, and 0.1 M Tris buffer, pH 8, (each containing 0.5 M NaCl).

PASSIVE IMMUNIZATION WITH SCW INJECTION. Serum from 15 female Lewis rats injected IP with SCW was collected 21 days post SCW injection and passed over (3 times) a SCW-sepharose affinity column. Ant-SCW antibody was eluted using 0.2 M glycine/HCl, pH 2.5 (unbound serum proteins were eluted by washing the column with PBS until no protein could be detected by measuring absorbance at 280 nm on a spectrophotometer). Fractions containing anti-SCW antibody were pooled and immediately dialyzed against PBS for 24 hours at 4°C. Unbound serum immunoglobulin was also collected and dialyzed for use as non-SCW specific control Ig. Anti-SCW antibody and Ig (non-SCW specific) were ammonium sulphate precipitated as described above.
Twenty-two female Lewis rats received subcutaneous (SQ) injections (half dose in each thigh) of either anti-SCW antibody (group 1), Ig (group 2), or saline (group 3). In group 1, ten rats were injected SQ with anti-SCW antibody at 20 mg/ml (5 rats) and 50 mg/ml (5 rats). Group 2 contained eight rats injected with Ig at 20 mg/ml (4 rats) and 50 mg/ml (4 rats). Group 3 contained 4 rats injected with saline. One day later all 22 rats were given a single IP injection of SCW (50 ug Rh/g BWT). All rats were clinically scored using the articular index, over a 4 week period, then sacrificed for pathologic evaluation (ie, granuloma formation).

PASSIVE IMMUNIZATION WITH RHEUMATOID FACTOR. Serum from 15 SCW-injected female Lewis rats was collected 3 days post SCW injection and passed over (3 times) an IgG-sepharose affinity column. Rheumatoid factor was eluted using 0.2 M glycine/HCl, ph 2.5 (unbound serum proteins were eluted by washing the column with PBS until no protein could be detected by measuring absorbance at 280 nm on a spectrophotometer). Fractions containing RF were pooled and immediately dialyzed against PBS for 24 hours at 4 C. Unbound serum immunoglobulin was also collected and dialyzed for use as non-RF control. Affinity purified RF and Ig were ammonium sulphate precipitated as described earlier.
Twenty-two female Fisher rats were given a single IP injection of SCW (50 μg rhamnose/g BWT). Three days later the rats received IP injections of either RF (group 1), non-RF Ig (group 2), or saline (group 3). In group 1, eight rats were injected IP with RF at 10 mg/ml (4 rats) and 20 mg/ml (4 rats). Group 2 contained eight rats injected with non-RF Ig at 10 mg/ml (4 rats) and 20 mg/ml (4 rats). Group 3 contained 6 rats injected with saline. All rats were clinically scored using the articular index, over a 4 week period, then sacrificed for pathologic evaluation (i.e., granuloma formation).

Ouchterlony. The binding specificity of affinity purified RF was determined using an ouchterlony technique. Plates were poured using 1% unpurpure agarose (BRL, Gaithersburg, MD) in dH2O, then allowed to cool to RT and refrigerated overnight. Wells were cut using a standard seven hole ouchterlony punch, and the cut agarose was suctioned away. RF was added to the center well in each assay. In the first gel, mouse anti-rat isotype (IgGα & IgM) specific monoclonal antibodies (Bioproducts for Science) were used to determine the isotype of the RF. In the second gel, affinity purified rat IgG isotopes (Bioproducts for Science) and HAg-IgG (Sigma) were used to determine the binding specificity of the RF. Each gel also included 3% BSA in a single well as a control. Diffusion was at RT and
the plates were read after 72 hours.

STATISTICS. ELISA graphs were developed using Sigmaplot (Jandel Co) and the graph statistics (mean, standard error (SEM)) were done by the program. Computation of statistical significance (95% confidence intervals) was done using Statgraphics (STSC, Rockville, MD), and was based on comparison of 2 samples (T test).
CHAPTER 7: RESULTS

On day 0, 42 of each female Lewis (susceptible) and female Fisher (resistant) rats were injected IP with a single aqueous suspension of SCW (50 μg Rh/g BWT). This dose has been shown previously to induce severe arthritis at near 100% incidence (Wilder et al. 1982). Control rats were injected with sterile saline. Rats were sacrificed 3 per time point over an eleven week period. None of the Fisher rats developed chronic arthritis and their acute phase reaction was limited to ascites fluid build-up with no observable joint involvement. None of the Fisher rats developed hepatic or splenic granuloma; however, there was mild fibrosis throughout the peritoneum. No peritonitis serous was observed in the Fisher controls or in any of the Lewis rats. SCW injection induced classic acute and chronic phase responses in the Lewis rats. Acute inflammation was apparent between days 3-7, then subsided. Hepatic and splenic granuloma developed between days 21-28 and by day 42 the spleens were almost entirely consumed by fibrosis. The tarsus was severely affected by day 28 and mobility was greatly reduced as the joints became fibrotic due to extensive erosive damage and ankylosis. Control
rats developed none of these arthritic conditions.

Analysis of the humoral response to SCW injection was done using various ELISA techniques on serum samples collected throughout the eleven week period. Three serum samples (one per rat) for each time point were collected separately and frozen till after all samples were collected.

**ANTI-SCW ANTIBODY.** My first goal was to determine the effect of SCW injection on serum anti-SCW antibody in Lewis versus Fisher rats. Sonicated SCW were bound to the wells as the primary antigen, then reacted with the various serum samples. As shown in Figure 1, Lewis control serum contained very low levels of anti-SCW antibody (0.069 ± .055 SEM) throughout the eleven weeks. However, SCW injection induced an increased serum titer, which was significantly higher (p < .05) than controls on day 21 with a mean of 0.871 ± .311 SEM between days 14-28. Serum antibody returned to control levels between days 42-56, then increased again on day 77 (0.615 ± .131 SEM). It is interesting that the serum antibody titer was elevated in the serum just before the onset of the chronic disease.

The response to SCW injection was different in the Fisher rats as can be seen in Figure 2. First, control antibody titer increased with time (and independent of SCW) between days 42-77. SCW injection induced a significant (p
< .05) increase as early as day 7, one week earlier than that measured in the Lewis rats. Between days 7-28 the antibody titer in SCW-injected rats (0.419 ± 0.118 SEM) was elevated over controls (0.084 ± 0.051 SEM), afterwards the two groups contained similar antibody titers.

**ANTI-PEPTIDOGLYCAN (PG) ANTIBODY.** After having measured anti-SCW antibody in both rat strains, my next goal was to determine the epitope(s) binding specificity of the anti-SCW antibody. As discussed in the background section, the SCW may be roughly divided into two components...PG and PS. These two components were extracted using a formamide extraction procedure (Fuller 1938, Krause and McCarty 1961, Chetty et al 1983) for use in measuring anti-PG and anti-PS antibodies. In order to further elucidate any possible epitope preference of anti-SCW antibody, the PG and PS components were pursued in more detail. Affinity for the terminal monosaccharide of PS, NADG, was investigated because of its reported importance in the translocation of SCW to the joint (Chetty et al 1983). NADG was purchased (Sigma) covalently bound to BSA in order to facilitate its binding to the microtiter plates. Affinity within PG component was investigated using a PG precursor peptide (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala), PPP, purchased from Sigma. This strategy allowed me to differentiate PG binding directed against the
disaccharide backbone versus the peptide side chain, or both. Binding of PG and PPP to the microtiter plates was confirmed using a polyclonal rabbit anti-strep A antibody, while the adherence of PS and NADG was confirmed using the lectin, Grifonia Simplicifolia II, which binds specifically to NADG.

Serum anti-PG antibody was measured in both rat strains as shown in Figures 3 and 4. Only trace amounts of anti-PG antibody was detectable in Lewis and Fisher serum, and there was no significant (p > .05) difference between SCW and saline treated animals.

**ANTI-PG PRECURSOR PEPTIDE (PPP) ANTIBODY.** Serum samples were assayed for the presence of anti-PPP antibody. As shown in Figures 5 and 6, the affinity for PPP was negligible in all serum samples tested from both Lewis and Fisher rats. There was no significant difference between SCW and saline treated animals.

**ANTI-POLYSACCHARIDE (PS) ANTIBODY.** Serum samples were assayed for the presence of anti-PS antibody. Lewis serum exhibited modest increases in antibody titer between days 21-77 in both SCW and saline injected rats, although there was no significant (p > .05) difference between the two groups (Figure 7). As illustrated in Figure 8, both SCW and saline injected Fisher rats produced a similar increase in antibody titer between days 21-77, but it too was not
attributable to the SCW and the time points were not significant.

ANTI-N-ACETYL-D-GLUCOSAMINE (NADG) ANTIBODY. Lewis serum, whether from SCW or saline injected rats, contained no anti-NADG antibody over the eleven week period (Figure 9). Fisher serum followed a similar pattern, except for SCW-injected day 56 (0.461 ± .624 SEM) and 77 (1.073 ± .634 SEM) serum (Figure 10). Anti-NADG antibody was detected on these days, however, large binding variability between samples resulted in standard error values which rendered the time points insignificant relative to controls.

ISOTYPE OF ANTI-SCW ANTIBODY. After having measured antibody against SCW and having characterized it as clonally restricted to a "combined" epitope, my next goal was to investigate whether said antibody response was isotype restricted. This was made feasible by the recent commercial availability of monoclonal antibodies specific to all rat immunoglobulin isotypes. Anti-SCW antibody from Lewis and Fisher serum was isotyped for all known rat immunoglobulins (IgG1, IgG2a, IgG2b, IgG2c, IgM, IgA, and IgE), however only IgM and IgG2a were present in detectable quantities.

Isotyping of Lewis arthritic and control serum is shown in Figures 11 and 12. IgM anti-SCW antibody (Fig. 11) was detectable in both SCW and saline injected serum.
There was no significant ($p > .05$) increases in IgM anti-SCW antibody in arthritics relative to the corresponding controls; however, IgG2a anti-SCW antibody production was increased by SCW injection. IgG2a anti-SCW antibody from days 14-28 (0.548 ± .204 SEM) was elevated over controls (0.154 ± .062 SEM), afterwards it returned to control levels at day 42. A second antibody peak in arthritics occurred on day 77 (0.816 ± .143 SEM). The kinetics of this response was similar to that measured for total anti-SCW antibody, Figure 1.

Isotyping data for the Fisher anti-SCW antibody are shown in Figures 13 and 14. IgM anti-SCW antibody was significantly elevated over controls (days 0-42: 0.076 ± .072 SEM) as early as day 7 (0.538 ± .006 SEM) and remained elevated through day 42 (0.583 ± .041 SEM). Control antibody increased on day 77, equal to that from SCW-injected serum. IgG2a anti-SCW antibody, on the other hand, increased in both SCW and saline injected rats with similar kinetics. Therefore, the observed increase in IgG2a anti-SCW antibody occurs independent of SCW injection in the Fisher rats. The IgM anti-SCW antibody response followed the same kinetics as seen in Figure 2 (total anti-SCW antibody). Furthermore, it is probable that the anti-SCW antibody detected in Fisher controls was the same as that measured for anti-PS antibody, Figure 8.
It is interesting that whereas both rat strains were induced to produce anti-SCW antibody, Fisher rats were isotype restricted to IgM and Lewis to IgG2a.

**STREPTOCOCCAL FcR BINDING.** Group A *Streptococcus pyogenes*, as well as other bacteria, express on their surface receptors for IgG-Fc region (FcR), the binding specificity with respect to rat IgG isotypes was previously unknown. Since my measurements of serum antibody relied on the binding of antibody to bound SCW, it was necessary for me to differentiate between binding via the Fab region versus background binding to the Fc region via the putative streptococcal FcR. To investigate this possibility WS, SCW, PS, and PG were bound to microtiter plates, then incubated with affinity purified rat IgG isotypes. As shown in Figure 15, there was a low affinity attraction for IgG1, IgG2a, and IgG2b, whereas IgG2c was bound with high affinity. Sonication diminished binding of IgG2c as demonstrated by the decreased binding by SCW (0.395 ± .057 SEM) as compared with WS (1.055 ± .050 SEM). Given the lack of IgG attachment to PG, the Fc-binding region appeared to reside with the PS (IgG2c: 0.412 ± .023 SEM) component. Since the anti-SCW antibody response was restricted to IgM and IgG2a and the fact that IgG2c was present in very low titer in the serum, I conclude that background binding to SCW (or PS) was not a significant
factor in the results.

IgM RHEUMATOID FACTOR. RF is a common clinical feature in RA, and although it is present in other autoimmune diseases as well as normal individuals, it is positively correlated with RA severity (Carson et al 1981, Koopman and Schrohenloher 1985). The effect of SCW injection on IgM RF had not been investigated in this model. In order to investigate this, HAg-IgG was bound to plates and incubated with serum samples. HAg-IgG binding was confirmed using a polyclonal goat anti-rat IgG antibody. As seen in Figure 16, both Lewis arthritic and control serum contained IgM RF, however, SCW injection induced a significant (p < .05) increase in serum titer from day 3-7 (0.387 ± .044 SEM) over controls (0.187 ± .033 SEM), afterwards it returned to baseline level for the remainder of the experiment. Fisher controls also produced IgM RF, but SCW injection had no effect on its serum titer, Figure 17. Since there was no paired control/SCW samples for day 3 and to determine the relative difference in SCW-induced RF production between rat strains, a separate ELISA was done comparing day 3 SCW-injected serum. In Figure 18, the day 3 comparison reveals that Lewis rats (0.630 ± .022 SEM) produced significantly more IgM RF than Fisher rats (0.345 ± .061 SEM). Day 0 and 77 represent control serum included for a reference.

PASSIVE IMMUNIZATION. Nine Lewis rats were injected IP
with concentrated antibody (day 5, 21, 88, and 90) obtained from SCW-injected Lewis rats. The passively immunized rats were observed over a 4 week period during which time there was no clinical signs of arthritis. Pathologic observation was also negative (ie, no fibrosis or granuloma).

**Passive Immunization with SCW.** In some of my previous experiments, immunoglobulin from Lewis rats with active arthritis was protective if injected prior to SCW inoculation. In order to investigate this phenomenon in detail, 3 groups of female Lewis rats were injected SQ. Group 1 rats were injected with 20 mg (5 rats) and 50 mg (5 rats) of affinity purified anti-SCW antibody obtained from Lewis rats with arthritis (day 21). Group 2 rats were injected with 20 mg (4 rats) and 50 mg (4 rats) of control immunoglobulin (no anti-SCW activity). Group 3 contained 5 rats which were saline injected. The next day all rats were injected IP with a single dose (50 ug Rh/g BWT) of SCW. As shown in Figure 19, groups 1 and 2 rats developed slight acute responses with day 3 joint scores of 0.5 ± 0.48 SEM and 1.38 ± 0.96 SEM, respectively. In addition, groups 1 and 2 rats developed serous peritonitis which was clinically apparent by day 1 and lasted till days 9-12. There were no signs of chronic phase joint involvement in group 1 rats, however, group 2 rats developed a low level response (day 28: 1.13 ± .93 SEM). Group 3 control rats
developed both acute (day 3; 7.12 ± 2.63 SEM) and chronic phase (day 28, 8.83 ± 2.09 SEM) responses. Pathologic examination on day 28 revealed the formation of hepatic and splenic granuloma in group 3 rats only. These data suggest that passive immunization with anti-SCW antibody or IgG twenty-four hours prior to SCW injection results in suppression of acute inflammation and inhibition of the chronic disease.

PASSIVE IMMUNIZATION WITH RF. Comparison of serum RF titer between Fisher and Lewis rats (Figures 16 & 17) revealed that SCW injection induced an increase in RF above controls in Lewis rats only. RF peaked on day 3, remained elevated until day 7, then returned to baseline. The purpose of this experiment was to investigate the pathologic importance of RF by simulating the day 3 RF peak (seen in Lewis rats) in Fisher rats. RF was affinity purified from the serum (day 3) of SCW injected Lewis rats and injected IP into Fisher rats 3 days post SCW injection. Group 1 rats received 10 mg (4 rats) and 20 mg (4 rats) of RF. Group 2 rats received 10 mg (4 rats) and 20 mg (4 rats) of control immunoglobulin which was void of RF activity. Group 3 contained 6 rats injected with saline.

An analysis of RF isotype and binding specificity was performed using an Ouchterlony method. In the first gel, RF was reacted against monoclonal antibodies to rat IgM and
IgG subclasses. After 24 hours, RF was precipitated by anti-IgM only, indicating that the RF was of the IgM class. In the second gel, RF was reacted with IgG subclasses and HAg-IgG. RF did not bind (ppt) monomeric IgG, but did precipitate IgG in the heat aggregated form.

None of the rats in any of the three groups developed any clinical symptoms of arthritis as measured by the articular index over a four week period. However, all of the rats developed sereous peritonitis beginning on day 1 and continuing till days 9-12. Pathologic evalution revealed no evidence of hepatic or splenic granuloma and no arthritis. The Fisher rats did have remnants of peritonitis as evidenced by extensive fibrin deposition throughout peritoneum. It is interesting that this response was identical to the response observed in Lewis rats (groups 1 & 2) which were protected via SQ injection of immunoglobulin.
Figure 1
Figure 2
Figure 3

ANTI-PEPTIDOGLYCAN ANTIBODY
LEWIS

○ ○ CONTROL
○○○ SCW INJ

ABSORBANCE
0.0 0.2 0.4 0.6 0.8

DAYS POST INJECTION
0 20 40 60 80
Figure 5

ANTI-PPP ANTIBODY
LEWIS

O—O CONTROL
○—○ SCW INJ

ABSORBANCE

DAYS POST INJECTION
Figure 6

ANTI-PPP ANTIBODY
FISHER

ABSORBANCE

O——O CONTROL

O——O SCW INJ

DAYS POST INJECTION
Figure 7
Figure 8
Figure 9
Figure 10

ANTI-NADG ANTIBODY FISHER

O - O CONTROL SCW. INJ

ABSORBANCE

DAYS POST INJECTION
Figure 11
Figure 12

IgG2a ANTI-SCW ANTIBODY

LEWIS

O O CONTROL

SCW INJ

ABSORPTION

DAYS POST INJECTION
Figure 13
Figure 14
BINDING OF RAT ISOTYPES TO STREPTOCOCCAL FcR

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- IgG1
- IgG2a
- IgG2b
- IgG2c
Figure 16

IgM RHEUMATOID FACTOR
LEWIS

O—O CONTROL
○—○ SCW INJ

ABSORBANCE

DAYS POST INJECTION
IgM RHEUMATOID FACTOR
FISHER

Figure 17

- - O CONTROL
- - - SCW INJ

ABSORBANCE

DAYS POST INJECTION
Figure 18
PASSIVE IMMUNIZATION WITH SCW–INJ.
LEWIS RATS

- O—O SALINE
- □—□ ANTI–SCW (20mg)
- ◆—◆ ANTI–SCW (50mg)
- △—△ Ig (20mg)
- ▼—▼ Ig (50mg)

ARTICULAR INDEX

DAYS POST SCW INJECTION
CHAPTER 8: DISCUSSION

ANTIBODY TO SCW

Despite the mitogenic properties of SCW for B cells, the humoral immune response of rats to SCW has been investigated in only one study (Greenblatt et al 1980). Serum anti-PS and anti-PG antibodies were measured in outbred Sprague-Dawley rats after a single IP injection of sonicated SCW (60 ug rhamnose/g body weight). PG and PS were obtained using a similar formamide extraction procedure. However, antibody levels were quantitated using an antigen binding assay in which serum antibody and radiolabelled PG and PS were precipitated and the percent antigen bound determined by comparison with a standard curve of known antibody concentration. Anti-PG and anti-PS antibodies showed similar kinetics. Antibody reached maximal levels 1-2 weeks post immunization, then returned to baseline by day 63. No antibody to PG or PS was present in controls throughout the experiment. Although antibody to PS was 10 to 100-fold greater than anti-PG antibody, only anti-PG antibody was correlated with chronic joint lesions.

In my experiments inbred Lewis rats were used instead
of outbred Sprague-Dawley. The use of outbred animals, which are more variable in their immune responses, could explain some of the differences between our respective data. In addition, I also investigated the antibody response in arthritis resistant Fisher rats. The kinetics of the anti-PS and anti-PG responses as reported by Greenblatt were similar to that found for anti-SCW (Figure 1) in my experiments. My finding of minimal anti-PG and anti-PS antibody was supported by the measurements of anti-PPP and anti-NADG, respectively, which also remained low and insignificant compared to controls. The different methods employed to assess antibody titer may have also played a role.

The epitope mapping experiments suggest that SCW stimulated a restricted humoral response against a "combined" epitope. It can also be concluded that the antibody response directed against SCW was clonally restricted which contrasts with reports of polyclonal stimulation of B cells by SCW in vitro (Zeiger et al 1981, Dziarski 1980, Heymer and Rietschel 1977). On the other hand, the B lymphocytes may have been polyclonally stimulated but it was not reflected in the response directed against SCW, suggesting a limited genetic repertoire with respect to SCW.

In addition to investigating the kinetics and epitope
specificity of the anti-SCW antibody response, I also looked at isotype restriction. I conclude that Lewis anti-SCW antibody was composed primarily of IgG2a, whereas the Fisher response was composed primarily of IgM and elevated 1 week earlier than did the Lewis response. Both IgM and IgG2a are capable of fixing complement, Clq (Bazin '83), which if activated may induce changes in vascular permeability and leukocyte chemotaxis. Considering the principles of affinity maturation, the Fisher response was characteristic of a primary reaction, and the Lewis response of a secondary reaction (with respect to isotype, not kinetics). Another interesting finding was that the peak (day 14-28) Lewis anti-SCW antibody response occurred during the nadir of the biphasic response (based of the articular index). It is unknown whether the chronic phase suppressed the anti-SCW response or if its onset correlated with the deposition of antibody from the circulation to the joints.

STREPTOCOCCAL FcR

Since the discovery of bacterial cell wall receptors which bind specifically to the Fc-region of IgG, the role of bacterial Fc-receptors (FcR) in infection has become a topic of considerable interest. Although numerous streptococcal (groups A, C, G) and staphylococcal FcR have been identified (Kronvall 1973, Langone 1982),
Staphylococcus aureus protein A (SPA) is the best characterized bacterial FcR and has found numerous applications in immunology. Five distinct types of FcR have been classified: Type I is SPA; Type II is on a few group A streptococci; Type III is on streptococci groups C and G; Type IV is on certain bovine group G streptococci; and Type V is contained on certain strains of Streptococcus zooepidemicus (Myhre and Kronvall 1980, Myhre and kronvall 1981, Langone 1982). Whereas the affinity of SPA for rat IgG isotypes has been elucidated (IgG2c >> IgG1 >> IgG2b- >> IgG2a; Medgyesi et al 1978, Rousseaux et al 1981), the affinity of the streptococcal (Type II) FcR for rat IgG isotypes had not been investigated previously.

As demonstrated in Figure 15, the streptococcal (type II) FcR demonstrated a binding preference for rat IgG2c. Sonication appeared to diminish binding capacity, but its selectivity for IgG2c was retained. The Fc-binding activity appeared to reside within the PS fraction. In a separate study, the streptococcal type II FcR was isolated in two forms (56 & 38 Kd) and the 56 Kd form exhibited a higher binding capacity (Yarnall and Boyle 1986). Taken together, these data suggest the possibility of two Fc-receptors on streptococcus...high affinity (IgG2c) and low affinity (IgG1, IgG2a, IgG2b).
Since the streptococcal FcR bound only IgG2c at low serum concentration, then based on the isotyping of anti-SCW antibody (Fig. 2) in which IgG2a was the sole IgG isotype involved, it is unlikely that the ELISA data was complicated by the background (FcR-Fc) binding of rat IgG to the streptococcal FcR.

**Passive Immunization**

After having defined the antibody response my next goal was to try and passively transfer the disease using antibody from Lewis rats with active arthritis. In an earlier study, anti-collagen antibody isolated from rats with collagen-induced arthritis caused an acute reaction when passively transferred to naive recipients (Stuart et al 1982b). Rats developed a mild form of arthritis within 18-72 hours which resolved completely within 3-5 days and caused no chronic lesions or permanent damage. Despite the fact that Stuart et al could transfer the disease with as little as 12 mg of anti-collagen antibody, I was unable to passively transfer arthritis using up to 10-fold more antibody. These data lend further support to the hypothesis that SCW-induced arthritis requires the deposition of SCW within the joints.

While performing the passive immunization experiments it was fortuitously discovered that passive immunization
followed by SCW injection was protective. This observation lead to a second round of experiments aimed at investigating this prophylactic effect in more detail. In related work by other investigators, arthritis has been prevented from progressing into its chronic state by a variety of methods. The acute inflammatory response was eliminated by pretreatment with cobra venom factor (Schwab et al 1982); however, this had no effect on the chronic phase. The chronic phase, on the other hand, may be eliminated by agents which have no or little affect on the acute phase. Cyclosporin given prior to SCW injection or up to 12 days afterwards prevented the chronic disease (Yocum et al 1986). Diethyldithiocarbamate (DTC) effectively suppressed the chronic phase in a dose related manner, although its mechanism of action is not understood (Seterah and Yocum 1989). Arthritis may also be suppressed by pretreatment of rats with mycobacterial heat shock protein prior to SCW injection (van den Broek et al 1989, Gaston et al 1989, and Cherrie et al 1989). Suppression was via inhibition of SCW-specific T cell responses with no effect on anti-SCW antibody, and the protection could be transferred by spleen cells.

My data revealed that if Lewis rats were given a SQ injection of immunoglobulin 1 day prior to SCW injection, arthritis was prevented. There was no apparent requirement
for specific antibody, as protection was observed in rats injected with immunoglobulin which was void of RF and anti-SCW antibody. Treatment suppressed acute inflammation of the joints; however, there was substantial peritonitis serous in all rats treated. The chronic phase was inhibited as demonstrated by a lack of granuloma and joint pathology.

Intravenous gammaglobulin therapy has been successful in managing disorders characterized by abnormal Ig regulation. Silverman et al demonstrated that intravenous IgG therapy decreased both in vivo and in vitro Ig production in juvenile onset RA (Silverman et al 1989). Human immunoglobulin was shown to induce a reversal of polyarthritis in adults with hypogammaglobulinemia (Webster et al 1976). In addition, IV immunoglobulin therapy has been shown to increase T suppressor function, decrease autoantibody titers in patients with acquired autoantibodies to factor VIII, and cause phagocyte FcR blockage (Durandy et al 1981, Rossi et al 1986, Clarkson et al 1986). Intramuscular gammaglobulin therapy decreased the maturation of B cells to plasma cells, but only if the IgG was deaggregated prior to injection (Durandy et al 1981). Intraperitoneal injection of immunoglobulin increased the capacity of peritoneal exudate to opsonize bacteria and increased phagocytic activity of peritoneal
macrophages (Lamperi et al 1987).

The aforementioned data suggest several mechanisms whereby IgG therapy was effective in treating immune disorders. Although my data were inconclusive with respect to the mechanism of arthritis prophylaxis, it is possible that all or part of these mechanisms were involved. The elevated immunoglobulin titer may have helped in preventing SCW migration to the joints by opsonizing the SCW, forming immune complexes which could be cleared by the reticuloendothelial system. Exogenous IgG may have inhibited the activation of SCW-specific T helper (CD4) cells. In this scenario FcR blockade was protective via inhibition of antigen processing and presentation by mononuclear phagocytes. In addition, stimulation of peritoneal macrophage FcR (i.e., initiation of a cytokine/hormone cycle) and the induction of T suppressor cells may have been important in suppressing the chronic disease.

RHEUMATOID FACTOR

Rheumatoid factors are a commonly clinical finding in RA, and their presence is correlated with disease severity (Carson et al 1981, Koopman and Schrohenloher 1985). Rabbits immunized with streptococcus produced both IgM and IgG RF (Bokisch et al 1972), and cultured human lymphocytes
secrete RF upon exposure to peptidoglycan (Levy et al 1986). However, RF had not previously been measured in the rat-SCW model.

In addressing this possibility, I found that both Lewis and Fisher rats have IgM RF as part of their normal serum immunoglobulin pool. Injection of SCW induced an 83% increase in IgM RF on day 3 in Lewis as compared with Fisher rats.

Rheumatoid factors were originally described in patients with rheumatoid arthritis; however, the presence of RF in the serum of nonarthritic patients, commonly with chronic inflammation and bacterial infections, suggests that RF are part of the normal immune response (Egeland and Munthe 1983). RF is produced in humans and mice during early anamnestic responses and during polyclonal B cell activation (Namazee and Sato 1983, Coulie and Van Snick 1983, Koopman and Schrohenloher 1980). The type of immunizing antigen (T-dep. or T-indep.) is reflected via isotype restriction of the antibody response and in the RF elicited (Slack et al 1980). RF follows the kinetics of and is directed against the dominant IgG isotype in the anti-antigen response (Stanley et al 1987), yet this was not the case in my experiments. IgM RF elevated before anti-SCW antibody, and our ouchterlony results indicate only IgM RF with no other isotypes detected. Also the IgM
RF only bound heat aggregated IgG and not monomeric IgG, indicative of a preference for immune complexes. Immune complexes have been shown to trigger the production of RF via carrier specific helper T cells (Namazee 1985, Coulie and Van Snick 1985). It has been hypothesized that RF acts as part of the normal immune system in the clearance of immune complexes and derepression of IgG feedback inhibition of B cells (Namazee and Sato 1982, Sinclair and Panoskaltsis 1987).

Autoimmune mice have elevated levels of Ly1 +, MER + B cells (CD5, CD2 human analogs), an immature B cell population found early in ontogeny and following bone marrow transplantation (Bofil et al 1985, Ault et al 1985). These autoimmune mouse strains (ie, MRL/lpr, NZB) spontaneously produce high levels of RF (Hang et al 1982). One hypothesis is that these Ly1 + B cells represent a distinct germ line lineage which codes for autoantibodies, like RF (Lydyard et al 1987). Furthermore, recent data indicates an increase in CD5+ B cells in the circulation of RA patients (Lydyard et al 1987, Youinou et al 1984). IgM RF activity is greater in newborn than in adult peripheral blood mononuclear cells which is consistent with the hypothesis that IgM RF originates from germ line genes (Levinson et al 1987). Another possibility is that autoantibodies reactive with self antigens and normally
Immunization of BABB/C mice with IgM RF (arrow A) from seropositive RA patients induced the production of antibody which bound (arrow B) to the PG-PS fraction of SCW. Immunization with non-RF IgM did not induce an anti-PG-PS response, and binding of this putative anti-idiotypic antibody to PG-PS was inhibited by absorption with RF or PG-PS (Johnson et al. 1985). Immunization of rabbits (Bokisch et al. 1973) and rats (reported here) with PG-PS stimulated the production of IgM and IgG RF (arrow C).

Figure 20
held in check by suppressive mechanisms, may cross-react with a common idioype on antibodies produced upon immunization or infection (Cooke et al 1983).

Several theories exist to explain the presence of RF, some of which are linked to FcR and even bacterial PG. As seen in Figure 20, immunization of mice (BALB/c) with human IgM RF induced an immune response to SCW which could be absorbed out with either RF or PG-PS, suggesting idiotypic complementarity between RF and anti-PG-PS antibody (Johnson et al 1985). The autoreactivity of RF with IgG may result as a consequence of abnormal galactosylation of IgG, rendering it antigenic. Decreased galactosylation of the C-gamma-2 region of IgG in RA may be related to decreased lymphocytic galactosyltransferase activity (Axford et al 1985). Hypogalactosylation of IgG has been measured in RA (Parekh et al 1985). Synovial fluid immune complexes isolated from RAs were high in IgG which had a low ratio of terminal galactose (Sumar et al 1988). IgG deficient in terminal galactose and sialic acid has exposed N-acetylglucosamine epitopes which are a major immuno-determinant of SCW and may trigger a cross-reactive response. Recently several investigators have shown that RF and staphylococcus protein A (SPA) bind to the same region on the IgG Fc molecule (Weisbart et al 1987, Nardella et al 1985, Sasso et al 1986) and that SPA bears
an internal image of the RF idiotypic determinant (Oppliger et al. 1987). Furthermore, the increased RF present in RAs may arise following infection with FcR bearing bacteria (Oppliger et al. 1988). It is now apparent that streptococcal type II FcR also binds to the same location on IgG as do RF and SPA (Schroder et al. 1986b, Nardella et al. 1987).

According to one study, RF was shown to bind to cartilage bound immune complexes and amplify inflammation (Watson et al. 1987). Amplification of the acute phase by RF may occur in the Lewis joints and contribute to the development of the chronic disease. I investigated the possibility that the lack of chronic disease in Fishers was related to their lack of an IgM RF surge as seen in Lewis rats 3-7 days post SCW injection. This RF transfer therapy, however, did not, along with SCW, lead to the development of arthritis. I conclude that although RF may be important in the early stages of SCW-induced arthritis in Lewis rats, additional factors exist which were not accounted for in this experiment. Such factors may include strain differences and/or a deficient stress response as discussed in Chapter 4 (Wilder et al. 1982, Sternberg et al. 1989).
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