M.Sc. Faculty of Graduate Studies and Research

THE ROLE OF HOMOCYTOTROPIC ANTIBODIES IN A MODEL OF INFLAMMATORY JOINT DISEASE

by

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ABSTRACT

Rheumatoid arthritis is a chronic inflammatory joint disease marked by exacerbations of more severe inflammation that resemble hypersensitivity reactions. A rat model of passive synovial anaphylaxis (PSA) was developed to examine nomocytotropic antibody activity in synovial inflammation. This technique involves passively sensitizing rat knee joints with IgE, challenging intravenously with corresponding antigen, and monitoring for signs of inflammation. The PSA reaction was successfully induced with ovalbumin and pertussis as antigens. Histological studies and radioisotopic joint scans provided evidence of synovial mast cell degranulation and tissue edema concurrent with the measurable PSA induced joint swelling. The PSA joint inflammation correlated directly with the amount of antigen specific IgE in sensitizing serum, was partly inhibited by antihistamine pretreatment, and could still be elicited 36 days after sensitization. Under certain conditions, the PSA reaction modified collagen II and adjuvant induced joint inflammation, and was in turn modified by the underlying synovitis.

RÉSUMÉ

La polyarthrite rhumatoide est une maladie inflammatoire chronique affectant les articulations, où l'on remarque des exacerbations inflammatoires plus sévères ressemblant à des réactions d'hypersenibilité. Un modèle d'anaphylaxie synoviale passive (ASP) chez le rat fût développé afin d'examiner l'activité d'anticorps homocytotropiques dans l'inflammation synoviale. Cette technique consiste à sensibiliser passivement les articulations du genou des rats avec de l'IgE, stimuler par voie intraveineuse avec l'antigène correspondant, et enfin de surveiller pour la présence de signes inflammatoires produits. La réaction d'ASP fût induite avec succès utilisant l'ovalbumine et le pertussis comme antigènes. Concomitant au gonflement articulaire mesurable induite par l'ASP, les études histologiques et les scintigraphies articulares témoignaient de la dégranulation des mastocytes synoviales et de l'édème tissulaire. L'inflammation d'ASP était en corrélation direct avec la quantité d'IgE spécifiques aux antigènes dans le sérum provocateur, était partiellement inhibée par un prétraitment antihistaminique et pouvait encore être elicitée 36 heures après la sensibilisation. Sous certaines conditions, la réaction d'ASP pouvait modifier l'inflammation induite par le collagène II et l'adjuvant, et elle même pouvait être modifiée par une synovite sous jacente.

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CHAPTER 1--INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the articular joints. The clinical course of the disease varies from patient to patient, and is marked by spontaneous remissions and exacerbations. Much of the research into the etiology of RA has concentrated on the immunologic abnormalities prevalent within the inflamed synovium: active cellular and humoral immune responses, local production of autoantibodies, and the accumulation of immune complexes within the joint tissues. The experimental animal models of rheumatoid arthritis currently used in research can effectively reproduce many of the pathologic characteristics of human disease. However, no single model available encompasses all of the varying immunologic reactions that persist and escalate into the destructive inflammatory process of rheumatoid synovitis.

There have been numerous accounts of allergic reactions exacerbating rheumatoid symptoms (1), and even the suggestion that an allergic mechanism might be the cause of certain arthritides (2,3). The involvement of allergic mechanisms in chronic arthritis is supported by the detection of increased mast cell numbers in inflamed synovial tissues (4). Reports of elevations in serum and synovial fluid IgE in RA patients (5) provides additional evidence for the possible occurence of hypersensitivity reactions in the joint.

Considering the extensive synovial vascularity, an increase in local permeability during inflammatory responses in the joint

can facilitate membrane perfusion with circulating IgE, and contribute to sensitization of synovial mast cells. After synovial mast cells are sensitized with IgE, any subsequent contact with corresponding antigen can trigger a local hypersensitivity reaction. The allergic reaction is itself pro-inflammatory, and is capable of enhancing immune complex deposition and contributing to the on-going joint inflammation.

Activated tissue mast cells release preformed vasoactive amines such as histamine and heparin that can result in tissue edema and promote infiltration of inflammatory cells. Mast cell mediators, both the preformed mediators and the newly-synthesized leukotrienes and prostaglandins, possess immunomodulating properties (see Table 1.1). These regulatory effects of mast cell products suggest a pivotal role for the activity of synovial mast cells in joint inflammatory reactions (6,7).

This thesis describes data on a novel model of induction of joint inflammation in rats. A local allergic reaction is provoked in joints by a technique called passive synovial anaphylaxis (PSA). This involves passively sensitizing rat joints with serum containing antigen-specific IgE. The rats are challenged intravenously with the corresponding antigen, and their joints are monitored for signs of swelling or inflammation. This model is then used to study the contribution of hypersensitivity reactions to other standard models of arthritis, such as collagen type II and adjuvant-induced arthritis in rats.

TABLE 1.1
Chemical Mediators of Immediate Hypersensitivity

Category	Mediator	Function
Primary (preformed granule-associated) mediators	Histamine	Increases vascular permeability Elevation of cyclic-AMP Chemokinesis Induces T-suppressor functions Decreases T-cytotoxic and T-helper functions Inhibits immunoglobulin synthesis Activates collagenase Activates chondrocytes Decreases fibroblast synthesis of glycosaminoglycan
	Serotonin	Increases vascular permeability Activates collagenase
	Heparin proteoglycan	Anti-coagulant, anti-complement activity Angiogenesis
	Eosinophil chemotactic factor (ECF)	Eosinophil and neutrophil chemotaxis
	Neutrophil chemotactic factor (NCF)	Neutrophil chemotaxis
	Tryptase	Proteolysis Cleavage of C3 to C3a
	Chymase	Proteolysis
Secondary (unstored) mediators	Slow reacting substance of anaphylaxis (Leukotrienes C4, D4, E4)	Increases vascular permeability Contraction of human bronchioles
	Platelet activating factors (PAF)	Aggregation of platelets Induces serotonin release from platelets
	Leukotriene-B4	Eosinophil and neutrophil chemotaxis
	Prostaglandins D4 and E4	Vasodilation

CHAPTER 2--HISTORICAL REVIEW

2.1 PATHOLOGY OF RHEUMATOID ARTHRITIS

Rheumatoid arthritis is manifested by the activation of a variety of immunologic reactions that contribute to the chronic inflammatory responses in affected joints. Although no single etiological factor has been identified, there is evidence of both cellular (8,9,10) and humoral (11) immune responses, some of which are auto-reactive in nature, occurring within the inflamed synovium. Surface immunoglobulin positive cells and plasma cells capable of local antibody production have been identified in rheumatoid synovium (12,13). Inclusions containing immunoglobulin and complement are detectable in polymorphonuclear leukocytes (PMNs) isolated from the synovial fluids (12,14) of RA patients. These pathologic changes are consistent with a local activation of the immune system in RA.

Autoimmunity to collagen type II is of particular importance to the pathology of RA (15-18). Collagen, which is the major structural component of joint cartilage, is enzymatically degraded in actively inflamed joints. Both cell-mediated (15) and antibody responses to collagen type II have been described in the sera (16) and synovial fluids (17,18) of RA patients. The immune reactivity is predominantly directed against denatured as opposed to native determinants of type II collagen. Furthermore, human anti-collagen cellular immunity is significantly associated with HLA-DR4 ($P=0.03$) (18), the same haplotype that is most strongly associated with RA (19). This

suggests a genetic predisposition for individuals bearing this haplotype to mount an immune response to collagen type II. In fact, individuals with the HLA-DR4 haplotype appear to have a 6 fold greater risk (20) of developing RA than individuals of different haplotypes.

The production of anti-IgG antibodies, or rheumatoid factors (RFs), has also been linked to HLA-DR4 (21). This provides a possible genetic cause for the clinical distinction between seropositive and seronegative RA. It may be significant that the same individuals genetically predisposed to anti-collagen-II and anti-IgG autoimmunity have a greater risk of developing RA than those lacking the HLA-DR4 haplotype, these being two of the major autoantibody specificities observed in RA patients.

Other auto-reactive antibody activities observed in RA patient sera include anti-lymphocyte (22), anti-IgG (rheumatoid factors) identified in sera and synovial fluids (23-25), and anti-nuclear antibodies (26,27). Autoantibodies of these specificities may participate in immune complex (IC) formation upon binding the corresponding antigen located either within the circulation or within an accessible joint. Deposition or local formation of ICs in the joints can subsequently lead to acute synovitis (28,29) and vasculitis (30), which are common pathological features of RA.

There is evidence for the presence of ICs both in the sera (24) and synovial fluids (25) of RA patients. Complement

activation by these ICs is substantiated by a significant consumption of complement in RA patients with circulating ICs (25,31,32). Complement activation products include chemoattractants for PMNs, which engulf the complexes and at the same time, release proteolytic enzymes that contribute to the destruction of surrounding tissue (33). Complement activation also produces C3a and C5a anaphylatoxins that may cause tissue mast cells to degranulate and thereby produce a local hypersensitivity reaction. The liberation of vasoactive amines from mast cells results in increased local blood vessel permeability. Vasodilation facilitates the influx of inflammatory cells and further IC deposition (34), thus amplifying the existing immunological reactions and creating the self-perpetuating cycle of inflammatory processes characteristic of RA.

Exposed epitopes on ICs may undergo proteolytic modifications. This may promote further auto-sensitization and production of antibodies against new IgG epitopes. Rheumatoid factors are autoantibodies capable of IC formation as well as increasing the mass of existing complexes to a precipitable size (34), thus contributing to the tissue deposition and destructive synovitis (28-30).

During the later stages of progressive RA, a hyperplastic growth of the inflammatory and synovial lining cells (pannus) invades the adjacent cartilage, causing extensive destruction and remodelling of cartilage and bone (33). Fibrin deposition in

the tissues (36), granuloma formation and fibrosis are also prominent in well established disease.

The initial stimulus for the ensuing rheumatoid inflammation remains unknown, yet it is the chronicity and repeated exacerbations (2) that are characteristic of continuing disease. Some patients experience episodes of acute-onset inflammation that resemble the course primarily associated with hypersensitivity reactions, and suggest the possibility of IgE and mast cell involvement in the flare-up (3).

Recently published reports have emphasized the importance of mast cells as potential regulators of inflammatory reactions (6,7,37). The diverse functional array of mast cell mediators suggests that the cell could contribute to both immediate and more chronic phases of inflammation. Consequently, the potential role of the mast cell in rheumatoid inflammation has been recently recognized and is actively being investigated.

2.2 MAST CELL CHARACTERISTICS

Significant differences have been observed between the circulating basophils and tissue mast cell populations (38). There is also extensive heterogeneity within the tissue-bound cell population itself (39). The mucosal type mast cell is distinguished from the connective tissue type by its differing staining properties (40), functional responses to histamine liberating drugs (41), morphological characteristics (39,42), and even surface antigenic determinants (43).

The various rat mast cell populations differ in the type of neutral proteases found within the storage granules. Mucosal mast cells and basophils contain a type-II rat mast cell protease (RMCP), while connective tissue mast cells contain type-I RMCP (40). These mast cell serine proteases have similar enzyme specificities, but different molecular weights (40). Mast cell subtypes also differ in their synthesis of proteoglycans - mucosal mast cells produce only chondroitin sulfate-E proteoglycan and connective tissue mast cells produce the proteoglycan heparin (44). The levels of histamine released upon cellular activation also vary among the mast cell subtypes. Connective tissue mast cells contain a 10 fold higher concentration of histamine than do the mucosal mast cells (45).

Although mast cells demonstrate different histochemical phenotypes depending on their tissue of origin, the persistent influence of environmental factors is important in maintaining these phenotypes (46). Mucosal mast cells may be stimulated to express the connective tissue phenotype when they are co-cultured with connective tissue fibroblast cells. This phenotypic change is followed histochemically by a shift from granules containing chondroitin sulfate which do not stain with safranin-O, to granules containing heparin which stain with safranin-O (46). This evidence suggests the importance of the microenvironment in determining mast cell phenotypes, and may account for the great heterogeneity observed between cells of differing tissue source and location (41). It is also indicative of the importance of

the microenvironment in the final differentiation and maturation of mast cells after their migration from the bone marrow pool of precursor cells (39,42).

2.2.1 MAST CELLS IN HYPERSENSITIVITY REACTIONS

IgE mediated mast cell degranulation is responsible for the immediate hypersensitivity responses that occur in allergic individuals. Genetically predisposed individuals (47), respond to particular antigens (allergens) with an immune respcnse which includes antigen-specific antibodies of the IgE immunoglobulin class. The circulating IgE has a short half life (2 to 3 days), due in part to its high susceptibility to proteolysis, and its high-affinity binding via the Fc portion of the epsilon heavy chain to specific receptors located on the surface of basophils or tissue fixed mast cells.

The cross-linking of 2 adjacent receptor-bound IgE molecules by corresponding divalent antigen will activate the mast cell and bring about release of its storage granules. Preformed mediators including vasodilatory amines such as histamine (and serotonin in rat mast cells); chemotactic factors for eosinophils, neutrophils, and T- and B-lymphocytes (48); anticoagulant heparin; and proteolytic enzymes such as chymase, tryptase, and arylsulfatase-A are all released. Normal termination of local effects of these compounds is achieved by direct phagocytosis and inactivation of the granules by eosinophils or neutrophils (81).

Activation of surface receptors results in the mobilization of arachidonic acid from membrane phospholipids by the action of phospholipase-A2. In mast cells, arachidonic acid is metabolized via the cyclooxygenase pathway to produce predominantly prostaglandin-D2 (PGD2) (49), a potent vasodilator, along with low levels of prostaglandin-E2 (50). Alternately, metabolism of arachidonic acid by the lipoxygenase pathway results in the production of biologically active leukotrienes (51). These include the chemoattractant leukotriene-B4 (LTB4), and the slow reacting substances of anaphylaxis (leukotriene-C4, -D4, -E4), which are 100 times more potent than histamine at enhancing vascular permeability while at the same time causing contraction of smooth muscle in the bronchioles. Another newly-synthesized mediator released from mast cells is the platelet activating factor (PAF), which can induce serotonin release from platelets and is itself a potent vasodilator. (See Table 1.1.)

All of these newly-formed mediators, acting in synergy with heparin and histamine, contribute to the immediate hypersensitivity response seen in allergic individuals. The immediate response is characterized by local tissue edema, redness and itchiness, bronchoconstriction, and dilatation of peripheral blood vessels.

Although mast cell degranulation is primarily associated with allergic mechanisms induced by the interaction of receptor-bound IgE with specific antigen, the presence of IgE is not an absolute requirement. Other stimuli such as antibodies to the

IgE receptor (52), C3a and C5a anaphylatoxins (53), and lymphokines (54) also demonstrate histamine releasing activity. Hormone-like peptides such as substance-P and gastrin (55) are also capable of directly producing mast cell degranulation. Thus autoantibodies with specificity for IgE or the IgE-receptor, T-lymphocyte activation with the elaboration of histamine releasing activity (54), and anaphylatoxin production following complement activation can all induce the release of mediators of immediate hypersensitivity reactions. The joint pain associated with RA can itself lead to local hyperproduction of substance-P, a neurotransmitter involved in the transduction of pain perceptions from the periphery (56). Consequently, high concentrations of this neurotransmitter in painful rheumatoid joints may trigger local mast cell degranulation (55) and contribute to the prolongation of inflammation.

2.2.2 MAST CELLS IN INFLAMMATORY REACTIONS

Immunologically induced inflammatory reactions are an important part of the host response to foreign substances such as microbial organisms. However, inflammation may lead to host tissue damage if it is repeatedly induced as part of an allergic response, or part of an autoimmune response resulting from improper regulation of the immune system. Cells capable of regulating an inflammatory response are therefore of considerable importance. The location of mast cells at potential sites of foreign artigen entry into the body, such as the sub-mucosal and

sub-cutaneous connective tissues surrounding blood vessels, suggests a functional role for these cells in the primary immunologic reactions mounted against the intruding foreign material. The ability of some mast cell mediators to recruit and/or regulate other cellular immune functions further suggests a physiologically important role for the mast cell in local homeostasis during immune and inflammatory responses (6,7,37).

2.2.2.1 IMMUNOMODULATION BY HISTAMINE

Histamine has the ability to regulate the activity of immune cells by binding to either of two specific surface receptors designated as H1 or H2 receptors (57). H2 receptor activation induces intracellular cyclic-AMP levels in both PMN and lymphocytic cells, and cyclic-AMP is the secondary messenger that directs further biological changes within the target cells.

Exogenous histamine does not appear to have any effect on human lung mast cell secretory functions. However, histamine activation of H2 receptors on human basophils and skin mast cells results in a suppression of further histamine release (58). By stimulating H1 receptors and inducing cyclic-GMP, histamine also increases the number of cell-surface receptors for C3b and C4 complement components and induces the chemotaxis of eosinophils. Histamine stimulation of neutrophil H2 receptors causes decreases in chemotaxis, lysosomal enzyme release, and production of superoxide anion and peroxide within these cells (59).

Histamine also has the capacity to regulate lymphocyte activity by binding to surface H2 receptors. Exogenous histamine suppresses both T-cell (60) and natural killer cell mediated cytotoxicity in the mouse, and has an overall inhibitory effect on human lymphocyte activity. It decreases human T-helper lymphokine production and the surface expression of Ia/Dr antigens. Histamine also stimulates suppressor T-lymphocyte activity, and inhibits immunoglobulin production by B-lymphocytes (57). Thus allergic mediators such as histamine can be shown to have a variety of effects on local immunologic and inflammatory reactions.

2.2.2.2 IMMUNOMODULATION BY SECONDARY MEDIATORS

Prostaglandins and leukotrienes are newly-synthesized mast cell mediators that also exhibit immunoregulatory properties. Prostaglandin-E2 (PGE2) enhances the rate and degree of PMN infiltration, and can stimulate IgG synthesis by B-lymphocytes (61). Some of the pain associated with inflammation can also be induced experimentally by PGE2, histamine, and arachidonic acid, all of which are released by the mast cell (62). Modulation of inflammation by leukotrienes is exemplified by the ability of leukotriene-B4 to increase PMN chemotaxis (63).

Consequently, mast cells can increase PMN and lymphocyte activity in response to prostaglandins and leukotrienes, and simultaneously down-regulate their activity in response to histamine. While it may be desirable to stimulate immune cells

locally to accelerate the clearance of foreign organisms, the balance between stimulatory and inhibitory effects must be maintained to prevent excessive amplification of the inflammatory response. Therefore local secretion of mast cell mediators may be important in the modulation of tissue inflammation. The duration of the stimulus and the degree of mast cell degranulation can thus be a determinant of the severity of the inflammation.

2.2.3 MAST CELLS IN RHEUMATOID ARTHRITIS

Mast cells have been recognized as cellular components of normal synovial tissue since 1960 when Castor determined that mast cells make up 3% of the total cell population (64). Only recently has research provided significant evidence for the direct involvement of mast cells in rheumatoid synovitis (89).

Smyth and Gum (65) were one of the first groups to suggest the need to investigate the function of mast cells in the synovium because of their distribution in perivascular regions, areas widely affected in rheumatoid inflammatory synovitis. Subsequent histological examinations of rheumatoid synovium (66-68) have noted the presence of increased numbers of mast cells, but their role in inflammation was not directly addressed. The studies were mainly concerned with the variability of infiltrating cell types observed in established rheumatoid synovitis, concentrating mainly on the contribution of lymphocytes and PMNs to inflammation.

The importance of mast cell involvement in experimental synovitis was reassessed following the results of Gryfe and coworkers (69). They demonstrated that the mast cell was the first cell type to increase in the synovium during the development of experimental rat adjuvant arthritis. Previous studies of synovia from animals and humans with already well established disease, had not addressed the time course or sequence of cellular infiltrations in the development of RA. In the adjuvant model of RA, mast cell numbers were found to increase with the development of synovitis (69). The initial increase peaked after 6 days, and was followed by evidence of steady mast cell degranulation until very few granulated cells remained by day 12. The number of granulated mast cells observed at any point in time was inversely proportional to the edema in the tissue. Also, as mast cell numbers decreased, the number of infiltrating polymorphs increased with the progression of synovitis. This implies an active participation of mast cells in the early phase of rheumatoid inflammation. By increasing vascular permeability, mast cell degranulation can account for the observed tissue edema as well as the inflammatory cell infiltration of tissue in response to chemoattractants.

Gryfe's results have subsequently been corroborated by human studies demonstrating that the degree of lymphocyte infiltration into synovial tissue correlates both with tissue histamine levels (70), as well as with total mast cell numbers (71). Since mast cells also release chemoattractants for PMNs, degranulation may

contribute to cellular accumulation in the tissue. One can also conclude from these results that the degree of cellular turnover at the site of inflammation is dependent upon the extent of mast cell activity. Thus cellular turnover may account for the high variablity in cell types found between individual tissue samples (72).

Rapid cellular turnover during the progression of inflammation has been demonstrated by more recent investigations. Synovial tissue obtained during the early onset phase of arthritis or active synovitis contain increased concentrations of mast cells (73). On the other hand, tissue taken from patients with end-stage disease, which is characterized by extensive bone destruction and no synovitis, contains predominantly macrophages and fibroblasts (72) but few detectable mast cells (74). Since mast cells can only be positively identified histochemically by the presence of metachromatically staining cytoplasmic granules, degranulation could render mast cells indistinguishable from other cell types and falsely lower the mast cell count. Due to the lack of evidence for mast cell death during inflammatory responses, the apparent decrease in mast cell numbers late in rheumatoid inflammation has been attributed to cellular degranulation rather than an actual loss of viable cells. However, this point needs further clarification.

The participation of mast cells in bone metabolism has also been established. Increased mast cell numbers are found at sites of active bone degeneration (75), as well as in areas of bone

surgery (76) and in osteoporosis (77). The recent discovery that mast cell histamine (90) and PGE2 (91) can activate chondrocytes implies that these mediators might stimulate cartilage resorption.

A negative correlation between stainable mast cell numbers and fibrin deposition in tissues, which is a characteristic of late stages of arthritis, has been demonstrated (71). Thus while mast cell participation in arthritic synovitis occurs during the early flare-up reaction, chronic release of its mediators contributes to the pathologic features of rheumatoid disease of longer duration.

Some investigators have recently demonstrated a significant accumulation of mast cells within the site of pannus formation in inflamed synovial tissue of RA (4,71,72,73,75), juvenile rheumatoid arthritis and other rheumatic diseases (73). No similar increase in mast cells was found in other forms of arthritis such as osteoarthritis (77), in which immunological processes are not believed to play an important etiological role.

Increased rheumatoid synovial mast cell counts have been found to correlate positively with the severity of the synovitis (4). Furthermore, the identification of mast cells in synovial fluids (78,79) reflects extensive tissue destruction in the inflamed joints, and suggests that the number of mast cells in synovial fluids could serve as a useful index of disease activity and severity.

2.2.3.1 MAST CELL MEDIATORS IN RHEUMATOID PATHOLOGY

Mast cell degranulation can not only cause acute hypersensitivity symptoms, but the preformed mediators alone (ie. without contributions from the secondary mediators) are also capable of inducing a prolonged inflammatory response (80). The effects of mast cell mediator release are normally terminated via phagocytosis of the granules by neutrophils and eosinophils (81). Therefore, the observed phagocytosis and degradation of mast cell granules by connective tissue fibroblasts (82) may be an adaptive method for down-regulating the proinflammatory effects of mast cell granules in these tissues. It has been observed that 24 to 48 hours following mast cell granule ingestion, fibroblasts begin to secrete elevated levels of collagenase (82). This induction of collagenase production may be relevant to rheumatoid cartilage destruction since this enzyme can specifically degrade collagen type II, a structural protein in articular joints.

Since the mast cell is the major source of histamine, elevations in the urinary excretion of histamine's major metabolite, 1-methyl-4-imidazole-acetic acid (83), suggest a significant activation of mast cells in non-allergic RA patients. This is also implied by the work of Permin and coworkers (84) demonstrating that half of the 12 RA patients treated with H1 and H2 antihistamines showed improvement in clinical symptoms.

The injection of test animals with high doses of histamine (ie. daily injections of 5 mg/kg body weight) has been shown to decrease the synthesis of glycosaminoglycan (GAG) and collagen

(85) by fibroblasts. Histamine may also be involved in the activation of PMN leukocyte collagenase (86). Thus high local concentrations of histamine in the joint would tend to inhibit tissue repair which requires increased collagen and GAG synthesis (87), and at the same time, promote collagen breakdown. In contrast to the effects of high doses of histamine, low doses stimulate fibroblast growth (88), thus increasing the source of collagen and GAG. Chronic overproduction of collagen may lead to abnormal fibrosis, frequently associated with elevated mast cell numbers (89). Therefore, depending upon the concentration of histamine released, synovial mast cell activation may either promote connective tissue repair or contribute to the degradation of collagenous tissues.

Taylor (90) has shown the presence of histamine H2 receptors on human articular cartilage chondrocytes that can be stimulated with low doses of histamine to increase intracellular cyclic-AMP levels. PGE2 is also capable of stimulating adenylate cyclase in human chondrocytes (91). Since measurable quantities of histamine are found in the synovial fluid of rheumatoid joints (79), and PGE2 is usually present in diseased joints (92), both of these mast cell mediators may be involved in chondrocyte activation and hence be responsible for some of the erosive changes seen in RA.

Mast cells are the sole source of heparin in the body. This glycosaminoglycan has anticoagulant properties, which in conjuction with vasodilatory mediators such as histamine,

leukotrienes, and prostaglandins, can contribute to the increased blood flow in the joints during inflammatory synovitis. Specific binding sites for heparin have been identified on endothelial cells (93). By binding to these sites, heparin induces the migration of endothelial cells - a processs believed necessary for the phenomenon of neovascularization during tumor growth. This conclusion follows the observation of increased mast cell numbers at these sites (94), and a direct correlation between mast cell numbers and degree of angiogenesis.

Heparin's function in angiogenesis may be important in the pathogenesis of rheumatoid arthritis. Pannus formation over joint cartilage resembles an invasive neoplastic tissue growth, and such tissue growth is dependent upon its capacity to generate new capillary networks. It has been proposed that since mast cell tryptase, histamine, and heparin can also induce collagenase activity (96,86,95), partial degradation of the connective tissue matrix by mast cell mediators might serve to facilitate new capillary growth during pannus formation. The implication of these results is that synovial mast cells are directly involved in rheumatoid pannus formation.

2.3 PROTEOLYTIC ENZYMES IN RHEUMATOID PATHOLOGY

The acute phase of synovitis involves infiltration of inflammatory cells into the synovial fluid and depletion of articular cartilage. The sub-acute phase is marked by proliferation of synovial lining cells and the formation of

invasive pannus which causes progressive destruction of the cartilage matrix. In both cases, degradation of cartilage is associated with a rapid loss of proteoglycan, possibly due to increased leukocyte proteolytic activity (97,98).

PMN leukocyte elastase has been shown to degrade and release proteoglycans from bovine articular cartilage in vitro (99), producing erosions that resemble those seen in arthritic cartilage. Synovial mast cells release chemoattractants for PMNs, and the subsequent local release of lysosomal enzymes may be a relevant mechanism of destructive inflammation in rheumatoid joints.

2.3.1 MAST CELL PROTEOLYTIC ENZYMES

Proteolytic enzyme involvement in cartilage degradation is suggested by the detection of neutral proteases in human synovial fluid (100) and within human cartilage (101). Since neutral proteases comprise 10 to 20% of the mast cell granular components, these cells are a potential source of tissue-destructive enzymes. Granular enzymes are released along with other mediators into the tissue upon mast cell stimulation with appropriate antigen. The mast cell neutral proteases are active at physiological pH, and have specificities similar to trypsin and chymotrypsin (96), enabling them to degrade cartilage during joint inflammatory responses.

Another mast cell granule enzyme, tryptase, has been shown to cleave serum C3 protein to generate C3a anaphylatoxin (102).

The tryptase activity is down-regulated by heparin, which is part of the enzyme-heparin complex (103) released from the mast cell granules. The inflammatory and vascular effects of C3a anaphylatoxin are thus augmented by tryptase and controlled by heparin's dual ability to down-regulate the tryptase activation of C3 and increase by 100 fold the degradation of C3a (102). The synergistic activity of mast cell histamine, PGD2, and SRSAs in increasing vascular permeability ultimately results in increased influx of plasma proteins into the joint tissues, including C3, which then serves as substrate for tryptase. The tryptase-produced C3a further increases vascular permeability by the direct induction of more histamine release (53). Therefore, a form of auto-stimulation of mast cell degranulation as a result of mast cell tryptase activity provides a mechanism for the local amplification of inflammatory reactions.

2.3.2 COLLAGENASE

Active collagenase has been identified at the cartilage/pannus junction (104,105) where extensive cartilage degradation is evident. Collagenase is capable of degrading cartilage collagen as well as insoluble collagen (106). This enzymic activity is important in the pathogenesis of RA, collagen being the major structural protein of the cartilage matrix.

The recent finding that mast cell proteases are capable of activating procollagenases (96) further implicates these cells in the tissue destruction observed in rheumatoid joints. Collagenase

is released from connective tissue fibroblasts and macrophage cells in an inactive state (107) and it requires activation by enzymes such as the mast cell neutral proteases (96). Histamine, serotonin (86), and heparin (95) have been shown to enhance the activity of rodent PMN collagenases, suggesting that the mast cell's role in collagen degradation involves both protease-induced activation and mediator enhancement of local collagenase activity.

2.4 IgE AUTOANTIBODIES IN RHEUMATOID ARTHRITIS

Changes in immunoglobulin synthesis are common in RA. There are marked increases in serum IgG and IgM (24) as well as local immunoglobulin production within the rheumatoid synovium (108). Elevations in the IgE class of immunoglobulins have also been documented in both serum (109,110) and synovial fluids (111) of some RA patients.

2.4.1 ATOPY AND RHEUMATOID ARTHRITIS

The detection of raised levels of IgE in some RA patients led to the determination of the prevalence of atopy in these individuals. RA was found to occur with a frequency of 2 in 266 (0.8%) atopic patients (112), which is similar to the 1% prevalence of RA in the general U.K. population (113). In a study of 40 RA patients with an average disease duration of 14 years, O'Driscoll also found no greater prevalence of atopy than in a control group (5 of 40 compared to 9 of 40 patients

respectively) (112). In addition, no evidence was found to indicate that allergic factors contributed to the arthritis of these patients.

Although RA patients as a group do not have a higher incidence of atopy than the general population, there is some evidence to suggest that immediate hypersensitivity reactions to food antigens can produce transient joint swelling (1). It is known that healthy intestinal mucous membranes can allow macromolecules to enter the circulation in sufficient amounts to stimulate the immune system (114). Food antigens must therefore be considered a constant source of stimulation and challenges for individuals with a history of food intolerances. There is some evidence that treatment with non-steroidal anti-inflammatory drugs can increase gut permeability for macromolecule absorption (115), possibly leading to enhanced mucosal absorption of protein antigens. This could be an important source of antigen for food-sensitive arthritic individuals undergoing such drug therapy.

A variety of food intolerances have been reported in arthritic individuals, and there are studies showing clinical improvement of symptoms when these patients are placed on diets excluding the allergenic food (116-119). These patients produce food antigen-specific IgE antibodies (116,120,121), or antigen-specific IgG4 antibodies (119), both of which are cytotropic for mast cell surfaces. In these arthritic individuals, circulating homocytotropic antibodies may gain access to the highly vascular synovium and sensitize the local mast cells. Subsequent exposure

to the corresponding antigen could trigger a local synovial hypersensitivity reaction, releasing vasoactive amines that would further increase the permeability of the blood vessels in the inflamed tissues. This would allow for the entry of larger amounts of allergen and facilitate the deposition of immune complexes that could amplify the local inflammatory reaction.

Patients who derived therapeutic benefit from the exclusion of foods to which they were sensitive, later demonstrated a flare-up of joint symptoms within hours of reintroduction of these foods into the diet. Circulating immune complexes containing IgG, IgE and food allergen have been detected following oral challenge with the allergenic food antigen (120). These complexes are evident 1/2 hour after food challenge, and coincide with allergic symptoms. In these patients, both the immune complexes and allergic symptoms may be alleviated by pretreatment with sodium chromoglycate (121) which inhibits mast cell degranulation. In some cases, the joint inflammation is also shown to coincide with increases in circulating immune complexes containing IgE (116).

Animal studies have also produced some evidence to suggest that food antigens can induce antibodies that cross-react with other antigens including auto-antigens. An antibody specific for an antigen in alfalfa seeds has been shown to cross-react with DNA, and has been associated with a syndrome in experimental primates that resembles systemic lupus erythematosus (122). Rabbits fed a diet including cows' milk produce anti-IgG along

with anti-milk protein antibodies, and eventually develop a synovitis (123). Both of these experimental results are suggestive of a role for a food-allergic mechanism in the development of arthritis.

Thus allergy to food antigens appears to be a contributing factor to the pathogenesis of synovitis in some RA patients. Since food sensitivity in many cases may be difficult to diagnose, its total contribution to human arthritic inflammation might be difficult to accurately estimate.

2.4.2 IgE RHEUMATOID FACTORS

IgE rheumatoid factors (IgE-RF) were first discovered by Broder and colleagues in 1969, when he isolated immune complexes from sera of RA patients (124). The higher prevalence of IgE-RF in RA patients with systemic manifestations compared to patients with joint-restricted RA has been confirmed by several investigators (125-129). These studies further demonstrate a higher prevalence of IgE-RF in RA patients with Felty's Syndrome, which is considered to be a variant of seropositive RA with systemic complications, including leukopenia, splenomegaly, and lymphadenopathy. In addition, changes in IgE-RF levels tend to parallel arthritis activity more closely than changes in serum IgG-RF levels (130).

IgE-RF has also been detected in rheumatoid synovial fluids, along with some evidence of local synthesis in the joint (131). Furthermore, local production of IgE-RF has been proposed in the

case of an RA patient with pleural effusion (129) since the fluid was found to contain a higher concentration of IgE-RF than was present in the serum.

Serum IgE-RFs have also been associated with polyarthralgias in 42% of 26 juvenile rheumatoid arthritis patients (131), and in 9% of 32 asthmatic individuals (125). Since IgE-RFs do not occur in control or non-atopic individuals, it has been proposed that this rheumatoid factor may be directly involved in joint inflammation. Any RA patient with circulating IgE-RFs has the potential for sensitizing tissue mast cells in the synovial membrane and other highly vascularized tissues. Subsequent contact with serum IgG aggregates would trigger local inflammatory reactions at these sites, leading to extraarticular as well as joint symptoms. The possibility of this mechanism in the pathogenesis of extraarticular lesions is substantiated by the localization of IgE deposits in perivascular regions of the skin of some RA patients (132).

2.4.3 IgE ANTINUCLEAR ANTIBODIES

Permin and coworkers have observed that peripheral blood leukocytes isolated from patients with clinically severe RA released histamine on challenge with RNA (133), and 6 of 8 responded to DNA, aggregated IgG (133), and isolated leukocyte nuclei (134). However, none of the leukocytes of RA patients with inactive disease or osteoarthritis (134) responded to challenges with these antigens. In another study (135)

peripheral blood leukocytes from RA patients did not release histamine following in vitro challenge with nuclear antigens, but these latter patients were all judged to be in clinical remission.

There is therefore some suggestion of a correlation between activity or severity of RA and IgE anti-nuclear antibodies. Since the histamine release response was observed in challenges with RNA only in the most severe disease cases, one might also suggest that the presence of RNA-specific IgE antibodies might be of value in identifying the severest forms of RA.

IgE with granulocyte-specific anti-nuclear antibody (IgE GS-ANA) specificity are also detected in 60% of 20 RA patients with neutropenia compared to only 16% of 64 RA patients without this symptom (136). This type of anti-nuclear antibody has not been detected in osteoarthritic or normal individuals (134), and is believed to promote the neutropenic condition of some RA patients, especially those with Felty's Syndrome. In addition, IgE organ-nonspecific anti-nuclear antibodies have been detected in 5% of 20 RA patients (137), and might contribute to the neutropenia and other systemic manifestations of RA.

RA patients with IgE antibodies of either anti-nuclear or RF specificities do not show a significant elevation in total serum IgE levels (133,134), and no correlation can be found between these IgE autoantibodies and total serum IgE (131,133). Furthermore, the IgE-ANA titers (136), but not the IgE-RF concentrations (130), appear to parallel those of the

autoantibodies of the other four immunoglobulin classes. This suggests that at least the IgE-ANA antibodies detected in RA patients are a result of an immune response to host antigens that involves all classes of immunoglobulin (136). For the most part, RA patients with IgE-ANA (134) or IgE-RF (131) exhibit extraarticular manifestations, or other forms of systemic rheumatic diseases (137). Elevations of auto-antibodies of the IgE class thus appear to be good predictors of more severe forms of RA (5).

The detection of autoantibodies of the IgE class in certain rheumatic patients provides a possible induction mechanism for autoallergic reactions between IgE rheumatoid factors or antinuclear antibodies and the corresponding nuclear or immunoglobulin antigens. These antigens might be located or generated at the site of tissue destruction, or gain entry into the tissue via dilated blood vessels in the inflamed rheumatoid synovium.

2.4.4 IMMUNE COMPLEXES CONTAINING IgE

Circulating immune complexes are a common occurence in RA. Immune complexes are held responsible for some of the tissue destruction occurring in the inflamed joints, as well as extraarticular lesions (28). Immune complexes containing IgE (IgE-ICs, have also been detected in RA patients, primarily in those diagnosed as having Felty's syndrome (128). While only 4 out of 20 RA patients with joint-restricted disease exhibit IgE-

ICs (128), 11 of 20 patients with Felty's Syndrome possess IgE-ICs in their serum. However, no direct correlation has been found between the IgE-ICs and the total serum IgE concentration.

The circulating immune complexes may be processed by PMNs and appear as large cytoplasmic inclusions (126). IgE-IC cytoplasmic inclusions are predominantly found in PMNs of patients with Felty's Syndrome compared to patients with RA alone. Since IgE-ICs are capable of inducing mast cell degranulation (124), these immune complexes may further exacerbate the complex-mediated tissue destruction by invoking the mast cells' proinflammatory mediators.

2.5 ANTI-IgE ANTIBODIES

There have also been descriptions of rheumatoid factors with specificity for determinants on the Fc portion of IgE. Three different naturally occurring anti-IgE antibodies have been described in humans. Two of these are of the IgM isotype (138,139), while one is of the IgG isotype (140). The three differ in specificity (141), and none of the anti-IgE's were capable of directly inducing histamine release from basophils in vitro. These studies also could not establish a positive correlation between serum IgE concentrations and the presence of anti-IgE antibody. Nevertheless, this anti-IgE auto-antibody is approximately 6 times more prevalent in patients with allergic histories (140), a population with higher IgE levels compared to the general public.

It has been established that the amount of anti-IgE incorporated into ICs depends upon the relative concentrations of serum IgE and anti-IgE (139): in conditions of high anti-IgE and low IgE concentrations, only 50% of the anti-IgE is complexed, whereas with moderate anti-IgE and high IgE levels, almost all of the anti-IgE occurs in its complexed state. This implies that in individuals with elevated serum IgE levels, most of the anti-IgE antibody would be incorporated into IC structures, and therefore undetectable unless these complexes are dispersed.

Since this auto-antibody is found predominantly in conditions that elevate serum IgE, such as in patients with allergies (140), or parasitic infections (141), it is postulated to play a role in the clearance of IgE-containing ICs. At the same time, its potential for contributing to the formation of ICs could result in the pathogenic tissue deposition of ICs.

Although there is as yet no evidence for the occurrence of this anti-IgE in RA patients, considering the prevalence of serum and synovial fluid IgE-ICs, the co-existence of an anti-IgE antibody could elevate the quantity of immune complexes and thereby contribute to the existing synovial and extraarticular lesions in these patients. Such a rheumatoid factor specificity should therefore be sought in RA patients, especially those with concomitant elevations of IgE levels, and extraarticular manifestations.

2.6 GOAL OF RESEARCH

This project was aimed at developing an animal model of passive synovial anaphylaxis reactions, to examine the possible role of homocytotropic (allergic) antibodies in provoking inflammatory arthritis. Prior to this study, there was no documentation of such a model.

CHAPTER 3--MATERIALS AND METHODS

3.1 HOMOCYTOTROPIC ANTIBODY PRODUCTION

IgE production in the rat is regulated in a multitude of ways, including the participation of both genetic and soluble factors. The Brown Norway rat strain was chosen for the production of high titer antigen-specific IgE serum because this strain naturally produces high levels of IgE (142).

The experimental rats were housed in the animal quarters of the Department of Microbiology and Immunology at McGill University and were maintained on a normal diet of rat chow and water ad libitum. All manipulations were carried out on animals anesthetized with an intramuscular injection of a mixture of ketamine (Rogarsetic) and xylazine (Rompum) in a combined dosage of 8.6 mg xylazine/kg and 57 mg ketamine/kg body weight.

Both male and female 6 to 8 week old Brown Norway rats were purchased from Harlan Sprague Dawley or Charles River, and were immunized following the schedule established by Dr. H. Bazin (142) for the production of high titer antigen-specific IgE. Test rats were injected intra-peritoneally with $1x10^{10}$ Bordetella Pertussis cells (Department of Public Health, Boston) and 5 µg antigen using a 27 guage needle. Animals were boosted with the same concentration of antigen alone 21 days after immunization. The antigens and concentrations used to immunize Brown Norway rats for the production of specific IgE antiserum are listed in Table 4.1.

Control blood was collected from Brown Norway rats by retro-orbital puncture with a glass pipette or a capillary tube. After immunization, samples were obtained by cardiac puncture with a 22 guage needle (Becton-Dickinson). In either case the blood was transferred to sterile vacutainer tubes (Becton-Dickinson) and allowed to clot for 2 hours at room temperature. The tubes were then centrifuged at 3000 rpm for 15 minutes and the serum separated into aliquots of 500 µl in plastic ependorf tubes. These aliquots were stored at -20° C for use in subsequent assays.

3.2 ELISA PROCEDURE

An enzyme-linked immunosorbent assay was developed for the quantitation of total serum IgE, and antigen-specific IgE and IgG in post-immune serum samples. Optimal working dilutions for the antisera and test sera were established using a checkerboard titration system (144). Using this technique, it was determined that the greatest differences in optical density (OD) between the control and high titer serum values were obtained with dilutions of 1:100 for the primary goat anti-rat IgE antibody, and 1:500 for the enzyme-conjugated rabbit anti-goat antibody. For IgE measurements, the best serum dilution was found to be 1:64, and a dilution of 1:128 was determined as optimal for IgG quantitation. These serum dilutions were used in all subsequent assays.

Quantitation of serum IgE levels were attempted by using a purified monoclonal IgE preparation (IR2 provided by Dr. Bazin)

to construct a standard curve. The OD readings for serially diluted preparations of the purified immunoglobulin were plotted against the logarithm of the dilution. However, since a wide range of dilutions of the standard immunoglobulin protein did not produce significant changes in OD readings (Figure 3.1), the pure IgE was considered to have undergone sufficient denaturation so as to no longer be recognized by the specific antisera system in use. IgE is highly labile, and it is not uncommon for serum to loose its skin-sensitizing activity due to the denaturation of IgE following long term storage (145). Consequently, the IgE quantitation results were expressed as ratios of the immune:preimmune ODs for the serum samples collected from each test animal.

In contrast to the attempts to use purified IgE as a standard, serially diluted rat serum produced a good standard curve. This indicates the specificity of the antisera for rat IgE, and that the assay conditions in use were appropriate. The specificity of the antisera for serum IgE was further shown by the fact that preincubation of serum containing high IgE concentrations with the antiserum, prior to use of the latter in the ELISA, effectively reduced the sample OD readings to control levels (Figure 3.2).

3.2.1 TOTAL SERUM IgE MEASUREMENTS

Total serum IgE was measured using a double antibody sandwich ELISA technique. Polystyrene microtiter plates

(Immulon) were coated over night at 4° C with 1:500 dilution in phosphate buffered saline (PBS) of monoclonal mouse antibody specific for the rat epsilon heavy-chain (prepared and provided by Dr. H. Bazin (146)). The coated plates were washed 3 times with PBS, pH 7.2, to remove any unbound antibody. The non-specific protein binding sites were blocked by incubating for 1 hour at 37° C with a solution of 1% bovine serum albumin (protease-free, Miles Scientific) in PBS. The plates were again washed 3 times with PBS and then test sera diluted 1:64 in PBS was allowed to bind for 3 hours at 37° C. The plates were washed 3 times with PBS containing 0.1% Tween-20, which is a non-ionic detergent used to minimize non-specific inter-protein interactions. This was followed by a 1 hour incubation at 37°C with goat antibody against the rat epsilon heavy chain (147), diluted 1:100 in PBS/Tween buffer. The plates were then washed 3 times with PBS/Tween buffer and incubated for 1 hour with 1:500 diluted alkaline phosphatase-conjugated rabbit-anti-goat antiserum (goat IgG species-specific, BioRad). Finally, the enzyme substrate p-nitrophenylphosphate (1mg/ml in a 10% diethanolamine buffer, BioRad), was incubated for 1 hour at 37°C and optical density readings were obtained at 410 nm using a manual ELISA spectrophotometer (Dynatech).

3.2.2 ANTIGEN-SPECIFIC IgE MEASUREMENTS

This procedure is identical to that outlined above for total IgE, except for the initial preparation of the microtiter plates.

Polystyrene plates were coated over night with 50 µl of the immunizing antigen, diluted to a concentration of 100 µg/ml in PBS, rather than coating with anti-IgE antibody. All subsequent steps and antisera used were the same as those used in the assays for total IgE.

3.2.3 ANTIGEN-SPECIFIC IgG MEASUREMENTS

Antigen specific IgG was assayed using the ELISA method described above for antigen specific IgE, except that the primary antibody used was a goat-anti-rat-IgG (Cooper Biomedical) diluted 1:100 in PBS/Tween buffer.

The ELISA results are expressed as a ratio of the optical density immune:preimmune values, calculated using the following equation:

$$\frac{\text{OD of immune serum - OD of nonspecific well}}{\text{OD of preimmune serum - OD of nonspecific well}}$$

The student T-test was used to analyze the relative antibody responses to the different antigens.

3.3 PASSIVE SYNOVIAL ANAPHYLAXIS REACTION (PSA)

3.3.1 SENSITIZATION OF LEWIS RAT JOINTS

Male Lewis rats 300 to 350 grams purchased from Charles River were used throughout for the inflammatory response in PSA because of the strain's susceptibility to adjuvant and collagen type II arthritis (148), the two most studied animal models of

arthritis. The Lewis rats were anesthetized with an intramuscular injection of ketamine/xylazine. Using a 25 guage needle 5/8" (Becton-Dickinson), 50 µl of serum containing homocytotropic antibody activity was injected into the joint space of the flexed knees, entering just below the patella. The control knees received 50 µl of pre-immune BN rat serum, and the test knees received post-immune sera that had been assayed for antigen-specific IgE and IgG, and were known to contain homocytotropic antibody activity.

3.3.2 ANTIGEN CHALLENGE

After a minimal delay of 24-48 hours, sufficient to allow unfixed proteins to be cleared from the synovium (162), the rats were injected intravenously (via the dorsal vein of the penis) with the antigen corresponding to the joint-sensitizing homocytotropic antibody. It was determined that the optimal correlation between inflammation and injected antigen specific-IgE concentrations occurred when animals were challenged 24 hours after sensitization (see Results). Consequently, all challenges were routinely performed 24 hours after sensitization.

By challenging rats with a range of antigen concentrations, it was determined that 1 mg was the optimal dose for ovalbumin in producing detectable joint inflammation 1 hour after intravenous antigen challenge. The Bordetella Pertussis, when studied as antigen, gave the best challenge results if the cells were sonicated and administered at a concentration of 10^5 cells/ml.

The other immunizing antigens were not used in these challenge experiments because of a lack of antigen-specific IgE response in the immunized animals as determined by ELISA (see Results).

3.3.3 ASSESSMENT OF JOINT INFLAMMATION

The local joint hypersensitivity reaction could be visibly detected by injecting Evans Blue dye intravenously - similar to the standard passive cutaneous anaphylaxis reaction. However, more quantitative techniques were necessary to evaluate tissue inflammatory responses. Changes in joint size were measured directly using a vernier scale caliper (Fisher) capable of accurate measurements to 0.1 mm. The leg was fully extended and the calipers were placed under the patella to measure the full joint diameter; the measurement was taken at the point where free movement of the calipers was restricted by the tissue.

The results are expressed as percent changes in joint size, and were calculated according to the following formula:

$$\frac{\text{post-challenge joint size} - \text{pre-challenge joint size}}{\text{pre-challenge joint size}} \times 100.$$

A second method of assessing joint inflammation utilized an isotopic joint scanning technique. 1 mCi of ^{99}Technetium methylene diphosphonate (Osteolite kit, New England Nuclear) was injected intravenously 1 hour after antigen challenge and the joints were scanned 2 hours later. The preparation of the ^{99}Tc

and scans were carried out in the Department of Nuclear Medicine at the Royal Victoria Hospital using standard scanning equipment. Each knee was scanned by placing the probe directly against the extended knee and counting for 3 minutes. The average CPM for scans over each joint was then calculated.

3.4 PHARMACOLOGICAL MODIFICATION OF PSA

Competitive inhibitors of histamine receptors were injected into both joints at various concentrations to establish the concentration necessary to produce inhibition of inflammation on challenging with antigen. Either promethazine-HCl (Poulenc), specific for histamine H1 receptors, or cimetidine-HCl (Smith Kline and French), specific for histamine H2 receptors, was injected into the joint space of both control and immune serum-sensitized joints. Half an hour prior to antigen challenge, 50 µl of the inhibitor solutions diluted in sterile PBS to final concentrations ranging from 15 mg to 0.01 mg, were injected into each joint. This delay presumably allowed for some drug-receptor interaction before the actual antigen challenge. All pre-challenge joint measurements were taken 1 hour after the inhibitor was injected into the joints.

3.5 INDUCTION OF ADJUVANT ARTHRITIS

Adjuvant arthritis was induced in Lewis rats following an established procedure (149). Male Lewis rats were injected subcutaneously in the tail with 100 µl of Freunds Complete

Adjuvant (FCA, Gibco) emulsified with PBS, followed by a second identical injection 7 days later. Periodic measurments of knee joints were used to determine the progression of the arthritic inflammation, by calculating the percent changes in the pre-challenge joint sizes, using the following formula:

$$\frac{\text{current pre-challenge joint size} - \text{Day 1 pre-challenge joint size}}{\text{Day 1 pre-challenge joint size}} \times 100.$$

Persistent joint swelling for at least 2 days was the criteria used to diagnose arthritic animals.

3.6 INDUCTION OF COLLAGEN-II ARTHRITIS

Collagen-II arthritis was induced following an established procedure (150). Male Lewis rats were injected with 10 µg of rat collagen type II (Calbiochem) emulsified in Freunds Incomplete Adjuvant (Gibco) and 1 week later boosted with the same mixture. Arthritis development was assessed by pre-challenge knee joint measurements following the same diagnostic criteria used in the previous model.

In other experiments, a PSA reaction was provoked in one of the knee joints during the induction period, or just after the development of adjuvant or collagen-II arthritis. This involved the calculation of the PSA challenge-induced increases in joint size, as well as the percent increase in pre-challenge joint size due to the arthritic stimulus alone.

3.7 HISTOLOGICAL STUDIES

3.7.1 TISSUE PREPARATION

Following joint sensitization and antigen challenge, synovial tissue was excised from Lewis rats sacrificed with a lethal injection of anesthetic (ketamine and xylazine). Tissue was trimmed and then processed by an autotechnicon tissue processor according to the standard procedure employed in the Pathology Department, Royal Victoria Hospital (151). Tissue was fixed for 1 hour in 10% buffered formalin, which is a 1:10 dilution in PBS of a stock solution of 37 to 40% formaldehyde (Fisher). Autotechnicon tissue processing involved clearing in xylene and chloroform, dehydration in 95 to 100% ethanol, and impregnation with paraffin wax (Paraplast, Fisher) under vacuum. The processed tissue was embedded in Paraplast, and sections 6 μ thick were cut using a Spencer 820 microtome. Tissue sections were floated on water/gelatin, and then dried onto glass slides for 1 hour at 65° C and over night at 37° C. The tissue section slides were deparaffinized in three changes each of xylene and 70 to 100% graded ethanol, then specifically stained for mast cells.

3.7.2 TISSUE STAINING FOR MAST CELLS

Mast cells were stained with alcian blue and safranin-O according to the method of the Pathology Department, Royal Victoria Hospital (151). Deparaffinized tissue section slides were incubated in 3% acetic acid for 10 minutes, then stained with 5 mg/ml alcian blue (Allied Chemicals) in 0.3% acetic acid

for 45 minutes. The slides were washed in water for 5 minutes, then counterstained with 1 mg/ml safranin-O (Baker Chemical Co.) in 1% acetic acid for 15 minutes. The stained tissue was dehydrated in 3 changes of absolute ethanol for 1 to 2 minutes each, followed by 2 changes of xylene for 5 to 10 minutes each, and then mounted with permount (Fisher) and a glass coverslip.

Avidin, a protein found in egg white, is known to bind with high affinity and specificity to mast cell granules (152). This has therefore been utilized as an additional staining method for synovial mast cells (152). The process required incubating the tissues section slides with a 1:200 dilution of avidin conjugated to Fluorescein Isothiocyanate (FITC, ICN Biologicals), in PBS for 1 hour in a humid chamber at 23° C. The slides were then washed 3 times for 10 minutes in PBS pH 7.2, and covered with 90% glycerol (Fisher) in PBS and a glass coverslip. The stained slides were examined and photographed using an incident light fluorescence microscope (Zeiss).

Tissue sections were examined for both mast cell numbers and evidence of mast cell degranulation. The histological observations were later correlated with the antigen-induced joint inflammatory responses measured just prior to tissue extraction.

3.8 STATISTICAL ANALYSIS

All data are expressed as mean values +/- standard errors (SEM). Significance of differences between groups was assessed by the student T-test.

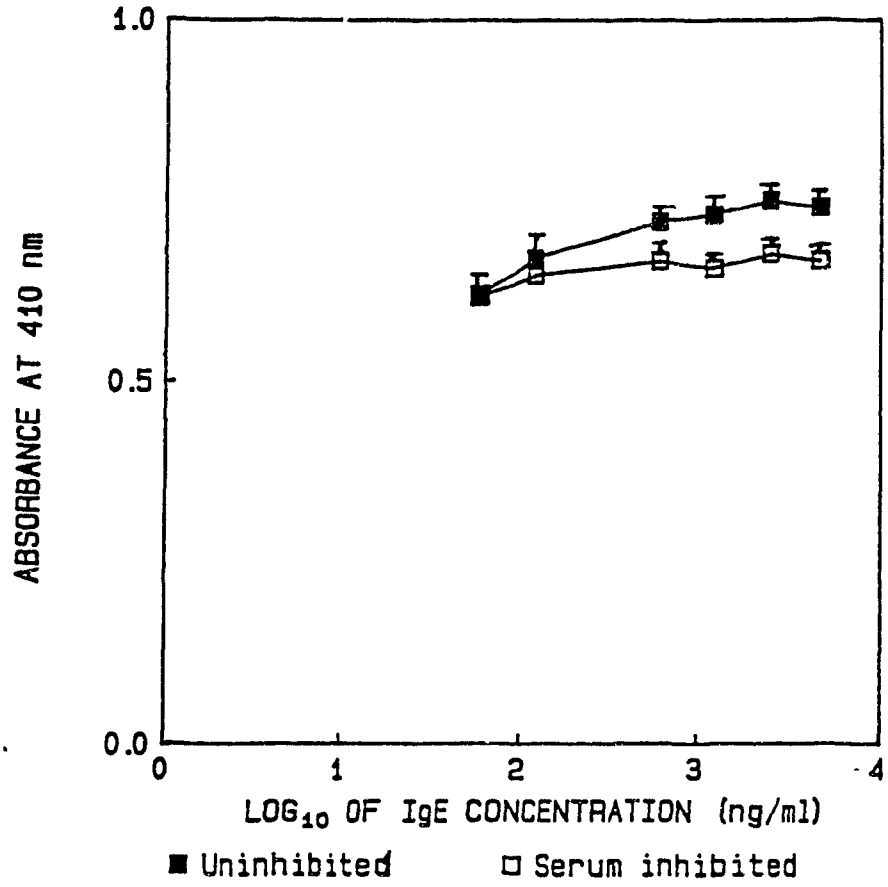

Figure 3.1 Standard Curve for Monoclonal IgE. Serial dilutions of the monoclonal IgE were assayed for total IgE by ELISA. Preincubation of the anti-IgE antiserum used in the ELISA with serum containing high IgE concentrations significantly reduced the sample OD readings, but neither of the curves showed significant changes in the optical density over a wide range of dilutions ($p > 0.05$). Values are means of 3 determinations +/- SEM.

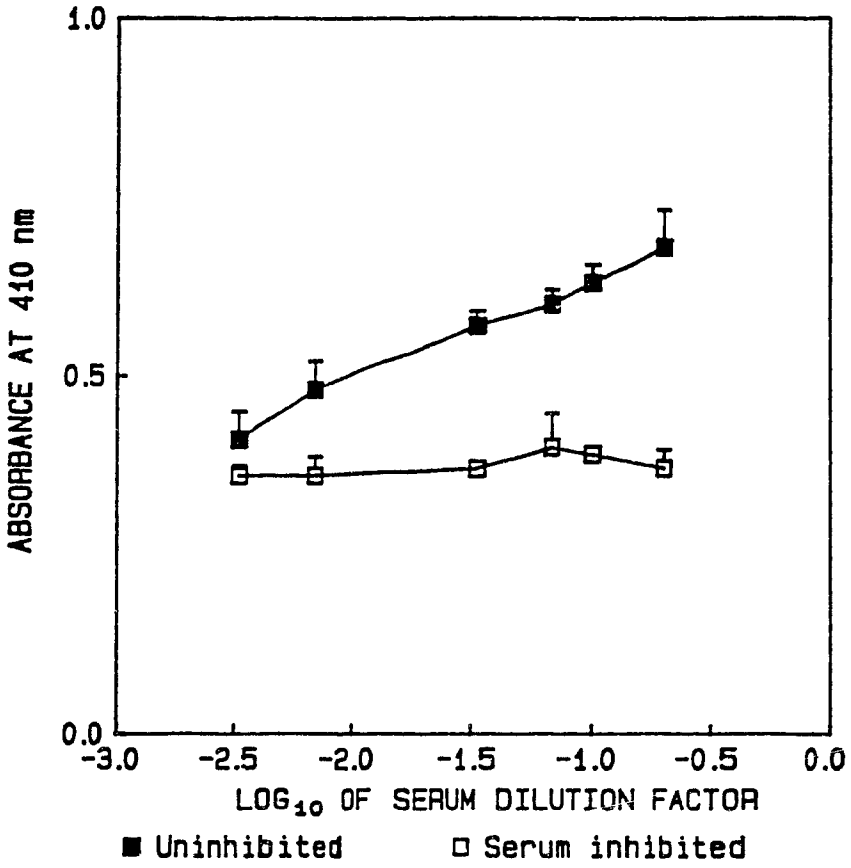

Figure 3.2 Standard Curve for Rat Serum IgE. Serial dilutions of BN rat serum were assayed for total IgE by ELISA. The specificity of the reaction is shown by the ability of a preincubation of the anti-IgE antiserum used in the ELISA with serum containing high levels of IgE, to significantly lower the sample OD readings. Values are means of 3 determinations +/- SEM.

CHAPTER 4--RESULTS

To develop the PSA model, it was necessary to prepare high concentrations of antigen-specific immunoglobulin-E. Using the immunization schedule established by Dr. H. Bazin (142), Brown Norway (BN) rats were immunized with various antigens of interest to RA. The sera obtained was assayed by an ELISA technique developed to measure rat serum IgE levels.

4.1 IgE RESPONSE TO IMMUNIZING ANTIGENS

There were notable, and as yet unexplainable, differences in the antibody responses to the various immunizing antigens (See Table 4.1). Because some antigens did not induce good IgE responses at the immunizing antigen doses used, it was deemed necessary to also measure the antigen-specific IgG levels to determine if the inoculations had been successful. As can be seen (Figure 4.1), rats immunized with pertussis alone produced significant IgG and IgE responses. The IgG response peaked and levelled off after day 28. On the other hand, the total IgE response peaked earlier, reaching levels up to 9 times the control by day 14 then falling to levels only 1.5 times the pre-immune. Pertussis specific IgE increased steadily until day 35, then decreased by day 56 to a level 7.5 times the control.

BN Rats immunized with ovalbumin and pertussis (Figure 4.2), induced a pertussis-specific IgG response that rose to plateau by day 35. By day 14 the total IgE antibody ratios were 4.5 times the pre-immune values, before decreasing steadily to levels 2.5

times the pre-immune. The ovalbumin-specific IgG ratios increased 4 to 7 fold following immunization, and remained relatively unchanged throughout the post-immune period studied. Pertussis-specific IgE levels were also significant (5 to 6 fold higher than pre-immune), while ovalbumin-specific IgE levelled off by day 35 with antibody ratios 10 times the pre-immune.

Following immunization with human IgG (Figure 4.3), experimental rats demonstrated a pertussis-specific IgG response that increased to levels 12 times the control. The pertussis specific IgE response decreased from its highest level at day 14 (6 fold higher than pre-immune) to level off at a value half of maximal. The human IgG-specific IgG antibody response did not reach levels of significance ($p>0.10$), although 3 of the 19 animals showed a moderate response to the antigen. Nevertheless, the human IgG-specific IgE antibody response reached values 2.5 fold higher than pre-immune levels, although this response was detected only in blood samples collected on day 14 ($p<0.001$).

One group of rats immunized with pertussis and human collagen-II (Figure 4.4) showed a 50 fold increase in pertussis-specific IgG and a 20 fold increase in pertussis-specific IgE ($p<0.001$), considerably higher than the pertussis-specific responses observed in any other immune group. Following immunization with rat collagen-II (Figure 4.5), experimental rats demonstrated anti-pertussis responses in both the IgG and IgE classes of immunoglobulin, with 20 and 3 fold increases respectively ($p<0.001$). However, in neither of these animal

groups was a significant IgG or IgE response detected against the collagen-II antigens ($p>0.10$).

Another population of rats was immunized with muramyl dipeptide (MDP) (Figure 4.6). The pertussis-specific IgG levels increased 40 fold and IgE increased 12 fold following immunization ($p<0.001$), but no response to MDP was observed in either of these classes of immunoglobulins ($p>0.10$). While MDP-specific IgG was detected in 3 out of 13 MDP-immune rats, this response was not significant, possibly due to the small molecular weight of the immunizing peptide. Furthermore, none of these animals produced MDP-specific IgE (Fig. 4.6).

4.2 THE PSA REACTION

4.2.1 TIME COURSE OF THE PSA REACTION

The anti-ovalbumin specific antisera contained the highest IgE titers, and these were subsequently used for sensitization of rat synovial tissues preceeding PSA reaction induction. Rat knee joints were sensitized with serum containing known levels of IgE. Following intravenous challenge with 1 mg ovalbumin antigen, there was significant swelling of the sensitized as compared to the unsensitized knee joints (Figure 4.7). The percent change in knee diameter peaked 2 hours after the antigen challenge, and the joint gradually returned to its pre-challenge diameter after 24 hours. The joint swelling observed 1 hour following antigen challenge was, on the average, 82% of the maximum response observed. In order to minimize the time of anesthesia, and for

comparative purposes, all subsequent joint measurements were made 1 hour after antigen challenge.

4.2.2 CORRELATION BETWEEN IgE AND INFLAMMATION

Rat knee joints were sensitized with control or ovalbumin-specific IgE-containing serum. After a minimal delay of 24 hours, the animals were challenged intravenously with 1 mg of ovalbumin, and the changes in joint size were recorded 1 hour following the challenge (Figure 4.8). A direct correlation was found between the levels of antigen-specific IgE present in the PSA-sensitizing serum and the magnitude of the change in joint size upon challenging with antigen. The best correlation was obtained for PSA-challenges performed 24 hours after sensitization ($r=0.81$, $p<0.0001$) and the dose response correlation tended to decrease with the increased delay between sensitization and antigen challenge (see Table 4.2). Ovalbumin-specific IgG antibody ratios were also found to correlate directly with the magnitude of the PSA-induced inflammation ($r=0.75$, $p<0.001$ for $n=29$).

A similar correlation was obtained between the pertussis-specific IgE and the PSA inflammatory response (Figure 4.9), and the best correlation was observed for antigen challenges conducted 24 hours after sensitization ($r=0.74$, $p=0.004$). See Table 4.3. Pertussis-specific IgG antibody ratios, however, did not correlate with the magnitude of the PSA inflammation ($r=0.17$, $p=0.27$ for $n=42$).

4.2.3 HISTOLOGICAL EVIDENCE OF THE PSA REACTION

In order to determine that the measurable tissue edema was indeed due to mast cell degranulation, histological studies were carried out on the synovial tissues, both before and after antigen challenge. Control synovial tissue contained numerous and densely granulated mast cells in the perivascular regions (Figure 4.10), with a great variability in total mast cell numbers observed between the different tissue samples.

Lewis rats that had been sensitized for a joint PSA response were sacrificed 30 minutes after the challenge and the synovial tissue stained and examined for mast cells. The mast cells in these tissues appeared less densely granulated when compared to cells in control tissues, and were occasionally observed releasing granules into the surrounding tissue (Figure 4.11). Histological studies on synovial tissue extracted following joint PSA reactions showed a direct relationship between the antigen-specific IgE levels used in the sensitization, and the degree of mast cell degranulation ($p=0.00016$). However, the total number of mast cells did not directly reflect the injected IgE antibody levels ($p=0.283$). These results are listed in Table 4.4.

FITC-conjugated avidin was used as a second staining technique for mast cells since it has been shown to bind directly to mast cell granules as a result of ionic interactions between the avidin and the heparin within the granules (152). Staining with FITC-avidin was found to be ideal for the rapid screening of tissue for the presence of mast cells, and a good method for

judging the density of mast cell granulation (Figure 4.12). A more diffuse pattern of staining was observed for synovial mast cells in the PSA-reactive joints compared to control joints.

4.2.4 RADIOISOTOPIC JOINT SCANS

Isotopic joint scanning was used to help establish that the measurable swelling of the PSA joint was a result of a synovial hypersensitivity reaction induced by intravenous antigen challenge. Since the ^{99}Technetium methylene diphosphonate (^{99}Tc) compound binds ionized Ca, areas of abnormal osteogenesis or increased skeletal blood perfusion during joint inflammatory responses, become the preferential sites for radioisotope accumulation. It was necessary to delay the ^{99}Tc injection 1 hour after antigen challenge in order to allow the synovial hypersensitivity reaction to progress before the radioisotope was injected. (No difference in radioisotope accumulation was detected between the PSA and control joints when the antigen and ^{99}Tc were injected simultaneously.) The joints sensitized with ovalbumin-specific IgE had on the average a 2 fold greater accumulation of ^{99}Tc than did the contralateral joints injected with control serum. One such joint scan conducted on an experimental PSA rat is shown in Figure 4.13. It was not possible however, to show a direct correlation between the ^{99}Tc counts in the knee joint and the amount of IgE used to sensitize the synovium (Table 4.5).

4.2.5 PHARMACOLOGICAL MODIFICATION OF PSA

Cimetidine pretreatment of the knees prior to antigen challenge caused an inhibition of the PSA-induced joint swelling with drug dosages ranging between 0.01 and 1 mg ($p<0.001$). However, higher drug dosages (ranging between 5 and 15 mg of cimetidine) actually caused enhancement of the PSA joint swelling. Control joints injected with similar concentrations of cimetidine did not become inflamed, thus it would appear that the exacerbation of the PSA response is not due to any possible toxic effects of cimetidine. No significant inhibition or enhancement of inflammation was observed when joints were pretreated with promethazine in the dosage range used (Table 4.6).

4.2.6 DURATION OF THE SENSITIZATION

Following the sensitization of rat knee joints, the animals were challenged with antigen at different intervals for a period of up to 36 days. At each challenge time, the magnitude of the joint inflammation was recorded. The greatest change in joint size was observed when animals were challenged 24 hours after sensitization (Figure 4.14). While the PSA-inducible joint swelling decreased with time, at the end of the 36 day period the reaction was still 60% of the maximal day 1 response.

4.3 EFFECT OF PSA ON COLLAGEN-II ARTHRITIS

The effect of a local PSA reaction on collagen-II induced joint inflammation was examined with respect to the latency for

the development of synovitis, and the severity of the inflammation induced with the collagen model. Experimental rats were simultaneously treated with a subcutaneous injection of arthritogenic rat collagen-II and an intraarticular injection of ovalbumin-specific IgE for the PSA sensitization. The joint diameters were measured prior to and following each challenge with the ovalbumin antigen, and measurements of the unsensitized knees were used as controls in comparison with values for the sensitized knees. As an additional control, the percent increases in pre-challenge joint sizes were used to indicate the degree of inflammation due to the arthritogenic collagen-II.

Superimposing the PSA reaction on a developing collagen-induced synovitis (Figure 4.15) indicates a greater increase in the pre-challenge joint sizes of the IgE-sensitized knees beginning approximately 7 days after the first PSA-induced reaction ($p<0.001$). This implies that the measurable inflammation due to the arthritogenic collagen-II (quantified as increases in pre-challenge joint sizes) is augmented by repeated joint PSA reactions. By the end of the 28 day study period, the PSA-induced inflammatory response is itself increased to levels significantly higher than the Day-1 PSA response ($p=0.047$). One week after the arthritogenic collagen injection, the pre-challenge joint sizes of both the control and IgE-sensitized joints are significantly greater than the initial measurements. Thus while the PSA reaction increases the severity of the ongoing arthritis, it does not appear to affect the latency time required

for a detectible inflammation.

The effect of the PSA reaction on pre-existing synovitis was examined in test rats allowed to develop collagen-II arthritis 2 weeks prior to joint sensitization with IgE. The progression of arthritis in these animals was again assessed in terms of changes in pre-challenge joint sizes and the articulation of affected joints. While the pre-challenge joint sizes increased during disease progression, no significant difference was detected at each time point between the control and IgE-sensitized joints ($p>0.05$). This implies that the superimposed PSA reaction did not measurably affect the severity or the latency time of the developing collagen-II arthritis throughout the full 28 day duration of the experiment. It may be noteworthy that the increases in successive PSA responses reached significance by day 28 ($p<0.001$), approximately 2 weeks following the initial sensitization with specific IgE (Figure 4.16).

4.4 EFFECT OF PSA ON ADJUVANT ARTHRITIS

Similar results were obtained with the adjuvant model of arthritis. Lewis rats were simultaneously treated with a subcutaneous injection of Freunds Complete Adjuvant, and an intraarticular injection of ovalbumin-specific IgE. The pre-challenge joint size was periodically monitored to provide an indication of arthritis onset. Beginning 14 days after the first PSA-induced reaction, the IgE-sensitized joints exhibited greater pre-challenge inflammatory changes in joint sizes compared to the

unsensitized control joints ($p<0.01$) (Figure 4.17). This implies that the PSA reaction can contribute to a developing adjuvant-induced synovitis. After repeated antigen challenges, the PSA response itself was significantly increased by day 28 ($p=.017$). This suggests a reciprocal potentiating effect of an adjuvant-induced joint inflammation on the PSA reaction.

When adjuvant arthritis was allowed to develop for 2 weeks prior to joint sensitization, no notable differences in the pre-challenge joint sizes were observed between the control and IgE-sensitized joints ($p>0.05$) (Figure 4.18). It would appear that the PSA reaction, if initiated 2 weeks after the arthritogenic adjuvant injection, does not measurably exacerbate the pre-existing inflammation. As in the collagen-II model, the PSA response itself was significantly increased compared to the initial PSA response, attaining significance 2 weeks after the IgE sensitization ($p<0.01$).

Examination of the synovial tissue of both adjuvant and collagen-II induced arthritis animals revealed intact mast cells in the control joints (Figure 4.19), while the PSA-sensitized tissue showed mast cells in the process of degranulation following antigen challenge (Figure 4.20).

The sensitized adjuvant and collagen-II arthritis rats demonstrated an additional swelling of the ankles ipsilateral to the PSA knee (Figure 4.21). Since these joints were not directly sensitized with IgE, one can speculate on the possible leakage of of IgE from the knee joint space into the circulation. While it

is doubtful that the injected IgE is recirculated in sufficient quantities to produce significant tissue-binding, actual ankle sensitization is implied by the observation of mast cell degranulation in some tissue samples following PSA reactions. Why the ankle on the PSA side might be favored for sensitization is not known. It is possible that swelling of the PSA knee affects lymphatic flow from the ankles, and thus indirectly account for this phenomenon.

Table 4.1

Summary of IgG and IgE Antibody Responses to Various Immunizing Antigens.

Antigen	Amount (μg)	Adjuvant-specific IgG	Adjuvant-specific IgE	Antigen-specific IgG	Antigen-specific IgE
pertussis	10^{10} cells	+[c]	+[c]	NA	NA
ovalbumin	5	+[a]	+[a]	+[b]	+[a]
human IgG	16.5	+[c]	+[c]	-	+[c] Day 14
human collagen II	5	+[c]	+[c]	-	-
rat collagen II	33	+[c]	+[c]	-	-
muramyl dipeptide	55	+[c]	+[c]	-	-

All immunizing solutions of antigen and adjuvant were made up to a total volume of 1 ml in sterile PBS.

N.A.= not applicable
[a] $p<0.05$
[b] $p<0.01$
[c] $p<0.001$

Responses are judged to be negative if $p>0.10$ for the comparison of immune:preimmune antibody ratios.

Table 4.2

Correlation Between Ovalbumin-specific IgE Ratios and PSA Responses at Different Challenge Times.

Time of challenge *	Intercept	Slope	T	p	r^2	r
24 hr	4.3	0.53	7.7	<0.0001	0.66	0.81
48 hr	5.0	0.45	6.0	<0.0001	0.54	0.73
72 hr	4.8	0.39	5.7	<0.0001	0.54	0.73
96 hr	4.4	0.35	3.3	0.006	0.46	0.68

n=33 Lewis rats immunized th pertussis and ovalbumin.

* Rats were challenged with 1 mg ovalbumin at the indicated times following sensitization, and change in joint size was recorded 1 hour after antigen challenge.

Ovalbumin-specific IgG antibody ratios were similarly found to correlate directly with the magnitude of the PSA-induced inflammation ($r=0.75$, $p<0.001$ for $n=29$).

Table 4.3

Correlation Between Pertussis-specific IgE Ratios and PSA Responses at Different Challenge Times.

Time of challenge **	Intercept	Slope	T	p	r^2	r
24	6.8	0.40	3.6	0.004	0.54	0.74
48	5.9	0.36	2.1	0.059	0.29	0.54
72	7.3	0.22	1.7	0.11	0.21	0.46
96	4.8	0.53	2.6	0.049	0.57	0.76

n=13 rats immunized with pertussis.

* Rats were challenged with 10^5 sonicated pertussis cells at the indicated times following sensitization, and the change in joint size was recorded 1 hour after antigen challenge.

Pertussis-specific IgG antibody ratios did not correlate with the magnitude of the PSA inflammation (r=0.17, p=0.27 for n=42).

Table 4.4

Correlation Between Ovalbumin-specific IgE Ratios and PSA-induced Mast Cell Degranulation.

IgE ratios (Immune/Control)	* Relative Mast cell Degranulation	Total Mast Cells/Field **
1.0 (Control)	0	4
2.7	1	3
7.2	1.5	4
11.0	2	2
11.8	2	4
13.2	2	5
13.5	2	4
14.3	2	3
15.5	4	2
17.0	3	3
19.5	4	2

Rats were challenged with antigen 24 hours after joint sensitization with serum containing the indicated IgE activity, the synovial tissue was extracted 30 minutes after challenge and stained with Alcian Blue and Safranin-O. Results are means of 2 experiments with the same sera.

* The density of the mast cell granules were scored from 0-4 for decreasing granule density.

There is a direct correlation between the IgE antibody ratios and the relative mast cell degranulation ($r=0.90$, $p=0.00016$).

** The average of mast cells counted in a total of 3 microscope fields at 45 X magnification.

There is no correlation between the IgE antibody ratios and the total number of mast cells ($r=-0.36$, $p=0.283$).

Table 4.5

^{99}Technetium Accummulation in the Joints Following PSA Reactions.

IgE Ratios immune/control	^{99}Tc Counts Ratios * sensitized/unsensitized	SEM
11	1.96	0.17
17	2.09	0.21
19.5	1.98	0.05

Rats were sensitized with ovalbumin-specific IgE and challenged with 1 mg ovalbumin 1 hour before the injection of ^{99}Technetium methylene diphosphonate.

* The ^{99}Tc count ratios are the means of 2 experiments using the same 3 sera.

Table 4.6

Effect of Intraarticular Antihistamines on the PSA Reaction.

Drug Dosage (mg)		Percent change in sensitized joint size With cimetidine	With promethazine
0.00		11.88 +/- 2.67	12.46 +/- 3.13
0.01	*	1.74 +/- 0.41	11.88 +/- 2.88
0.05	*	2.03 +/- 0.63	11.30 +/- 1.88
0.10	*	1.74 +/- 0.41	11.59 +/- 2.33
0.50	*	1.77 +/- 0.23	11.88 +/- 2.26
1.00	*	5.80 +/- 1.44	12.17 +/- 2.69
5.00		11.88 +/- 2.67	10.72 +/- 1.85
15.00	**	25.51 +/- 2.06	9.56 +/- 2.56

PSA reactions were induced in joints sensitized with equal concentrations of antigen-specific IgE, by challenging with 1 mg ovalbumin. Data points are the mean values for 3 experiments +/- SEM.

* Significant inhibition ($p<0.001$).
** Significant enhancement of inflammation ($p=0.015$).

Other values are not significantly different from unmodified PSA results ($p>0.05$).

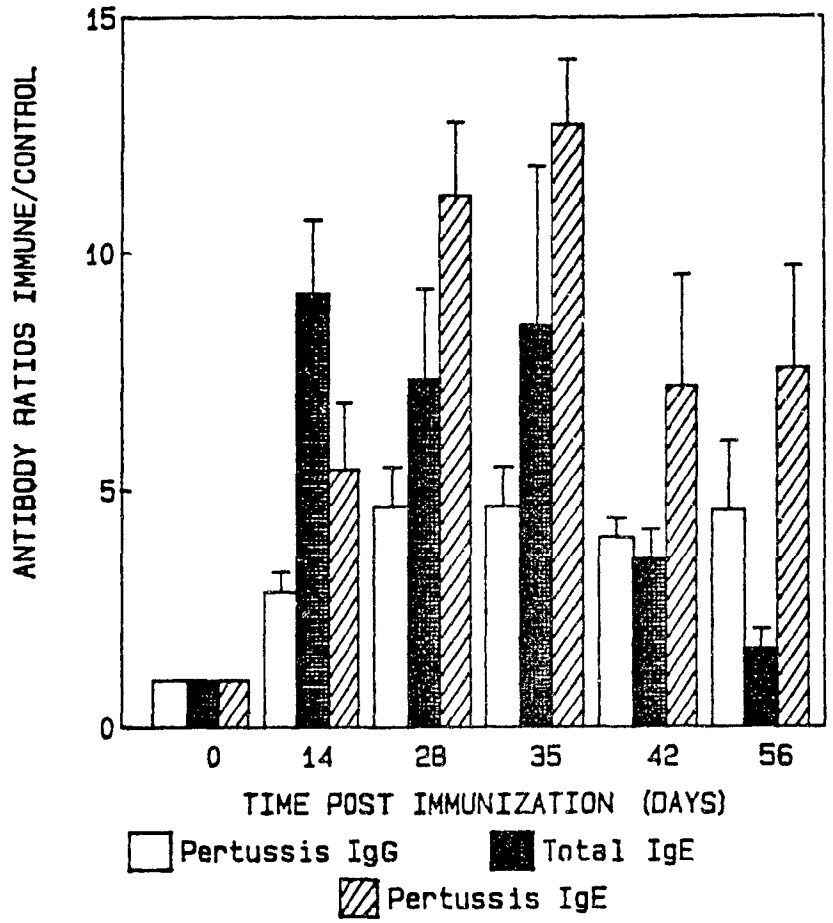

Figure 4.1. The IgG and IgE Antibody Responses in BN Rats Immunized with Pertussis (Department of Public Health, Boston)(*n*=12). Relative antibody levels are expressed as ratios of immune/preimmune +/- SEM. All post-immune antibody ratios are significantly higher than the preimmune levels ($p<0.001$).

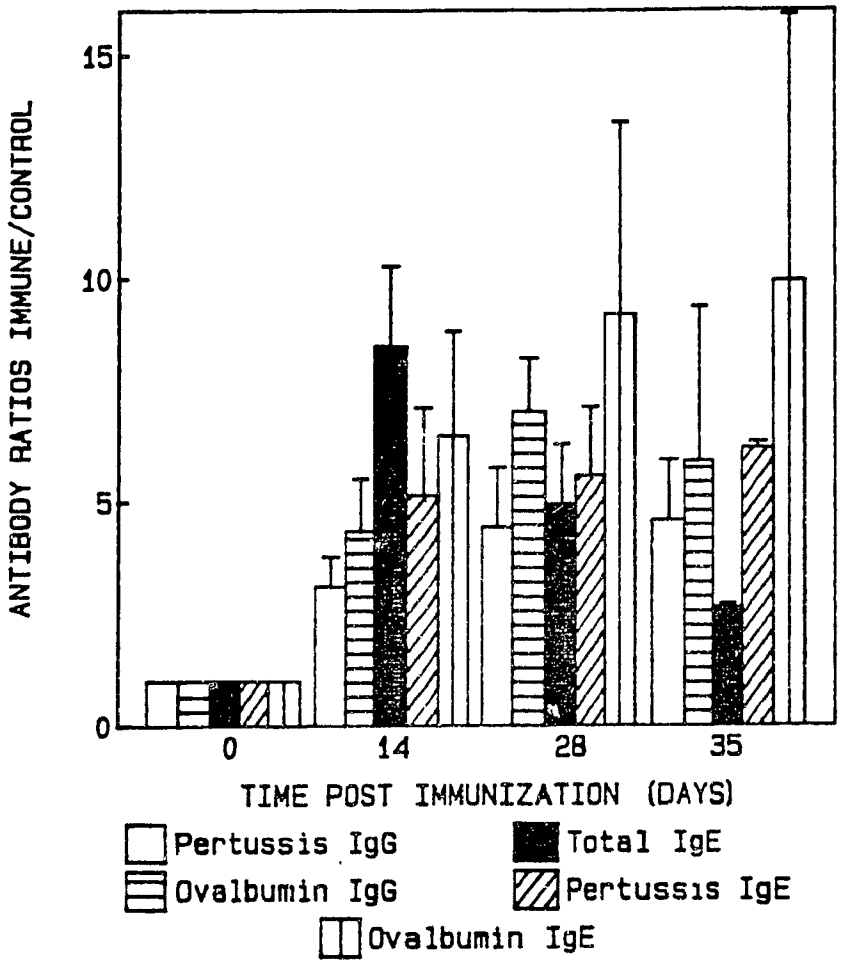

Figure 4.2. The IgG and IgE Antibody Responses in BN Rats Immunized with Pertussis and Ovalbumin (Pentex)(n=9). Relative antibody levels are expressed as a ratio of immune/preimmune +/- SEM. All post-immune antibody ratios are significantly higher than the preimmune:

ovalbumin IgG	$p<0.01$
total IgE	$p<0.0001$
pertussis IgE	$p<0.05$
ovalbumin IgE	$p<0.05$

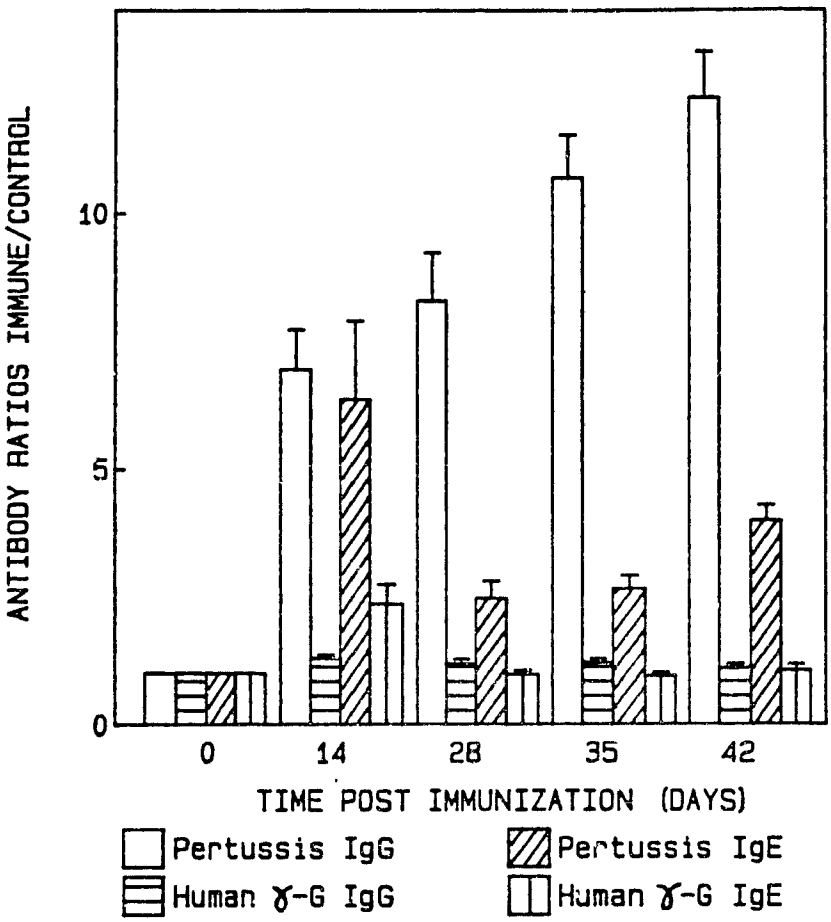

Figure 4.3. The IgG and IgE Antibody Responses in BN Rats Immunized with Pertussis and Human IgG (Sigma)(n=19). Relative antibody levels are expressed as a ratio of immune/preimmune +/- SEM. The IgE response to human IgG is significantly higher than pre-immune levels only on day 14 post-immunization ($p<0.001$). Both the IgG and IgE responses to pertussis are significantly higher post-immunization ($p<0.001$). For all other responses, $p>0.10$.

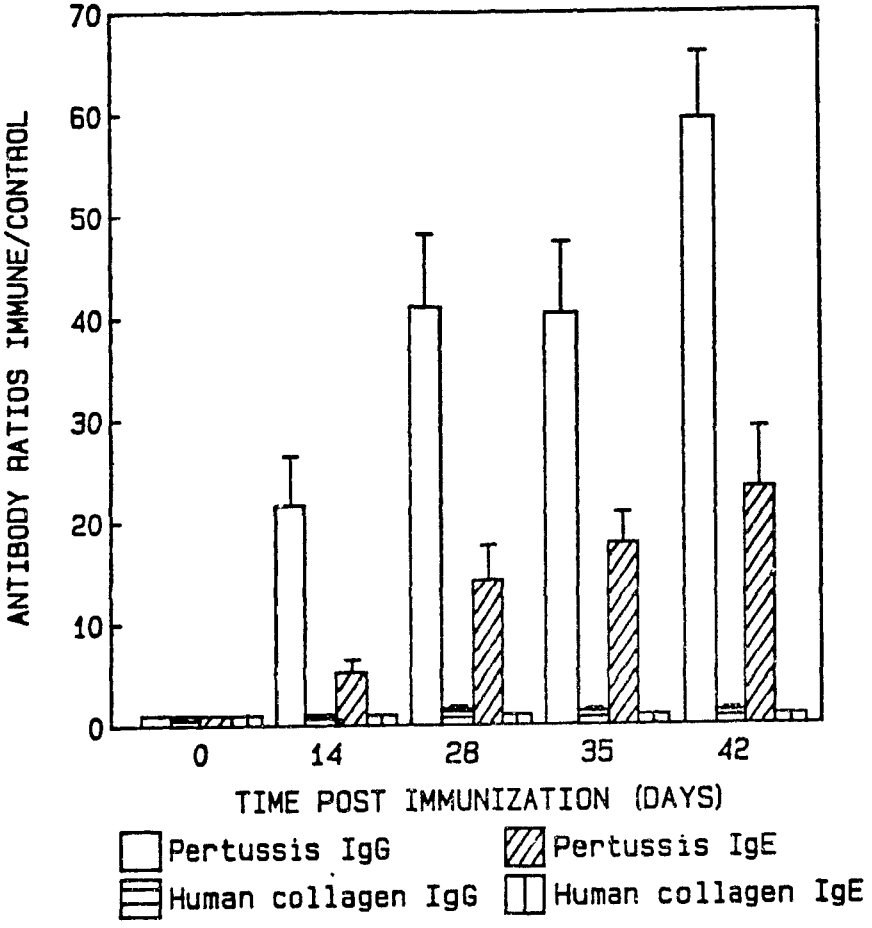

Figure 4.4. The IgG and IgE Antibody Responses in BN Rats Immunized with Pertussis and Human Collagen-II (Calbiochem)(n=7). Relative antibody levels are expressed as a ratio of immune/preimmune +/- SEM. Both IgG and IgE responses to pertussis are significantly elevated following immunization ($p<0.001$). Responses to human collagen-II are not significantly higher when compared to pre-immune antibody levels ($p>0.10$).

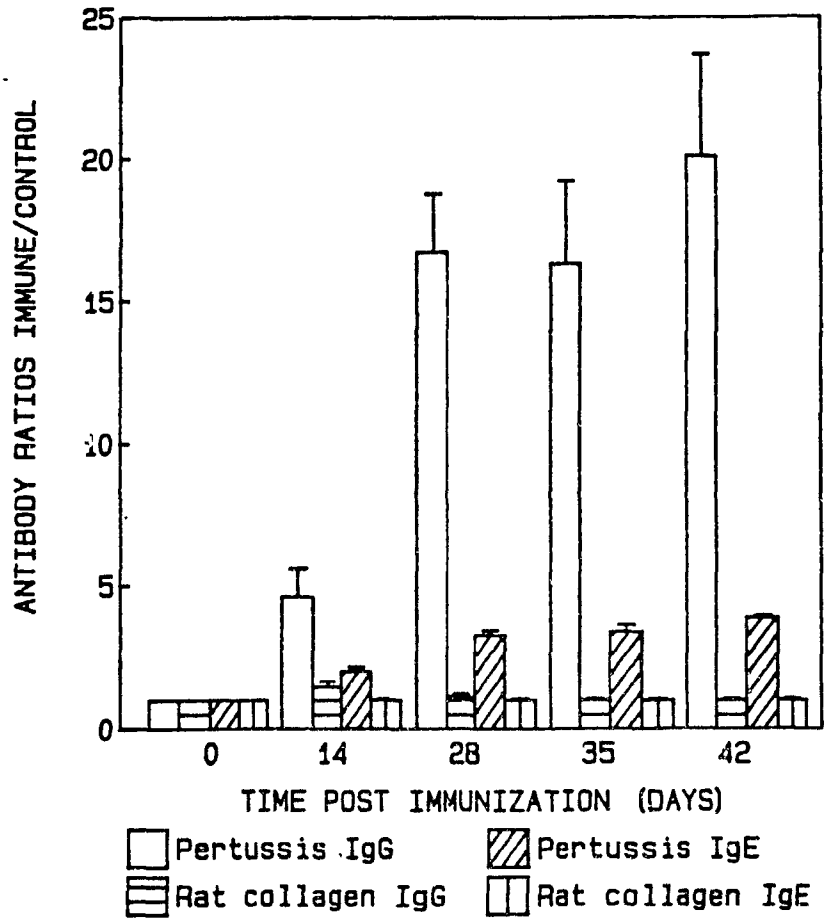

Figure 4.5. The IgG and IgE Antibody Responses in BN Rats Immunized with Pertussis and Rat Collagen-II (Calbiochem)(n=6). Relative antibody levels are expressed as a ratio of immune/preimmune +/- SEM. Both IgG and IgE responses to pertussis are significantly elevated following immunization ($p<0.001$). Responses to rat collagen-II are not significantly higher than pre-immune antibody levels ($p>0.10$).

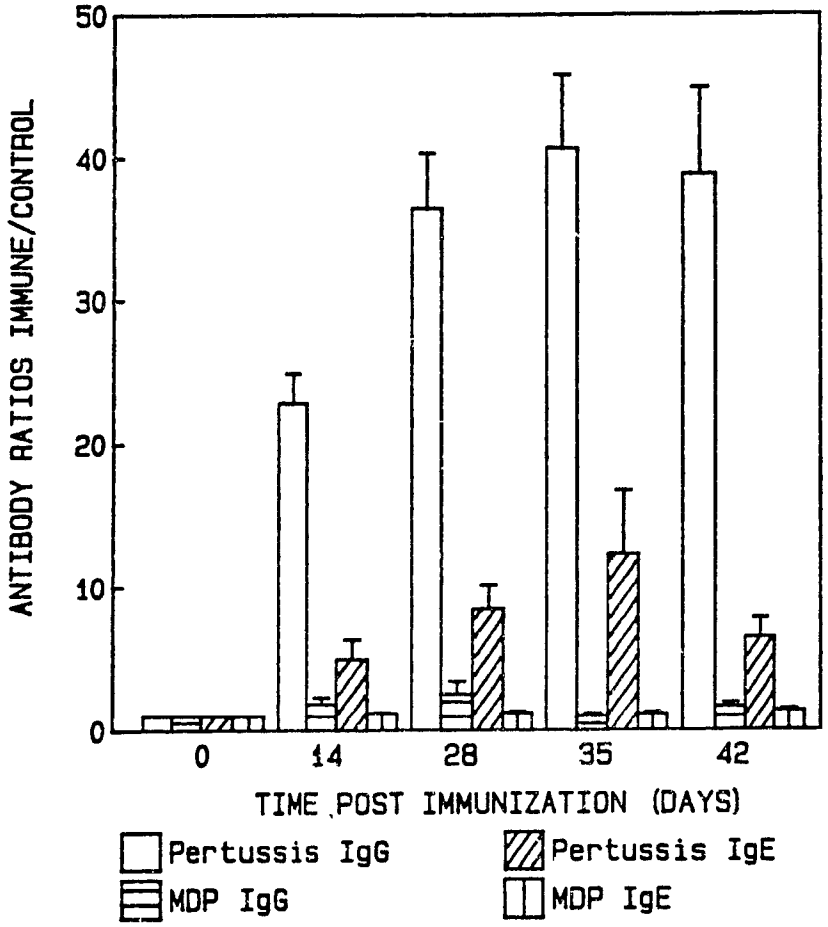

Figure 4.6. The IgG and IgE Antibody Responses in BN Rats Immunized with Pertussis and Muramyl Dipeptide (MDP, Calbiochem)(n=13). Relative antibody levels are expressed as a ratio of immune/preimmune +/- SEM. Both IgG and IgE responses to pertussis are significantly higher following immunization ($p<0.001$). Responses to MDP are not significant when compared to pre-immune antibody levels ($p>0.10$).

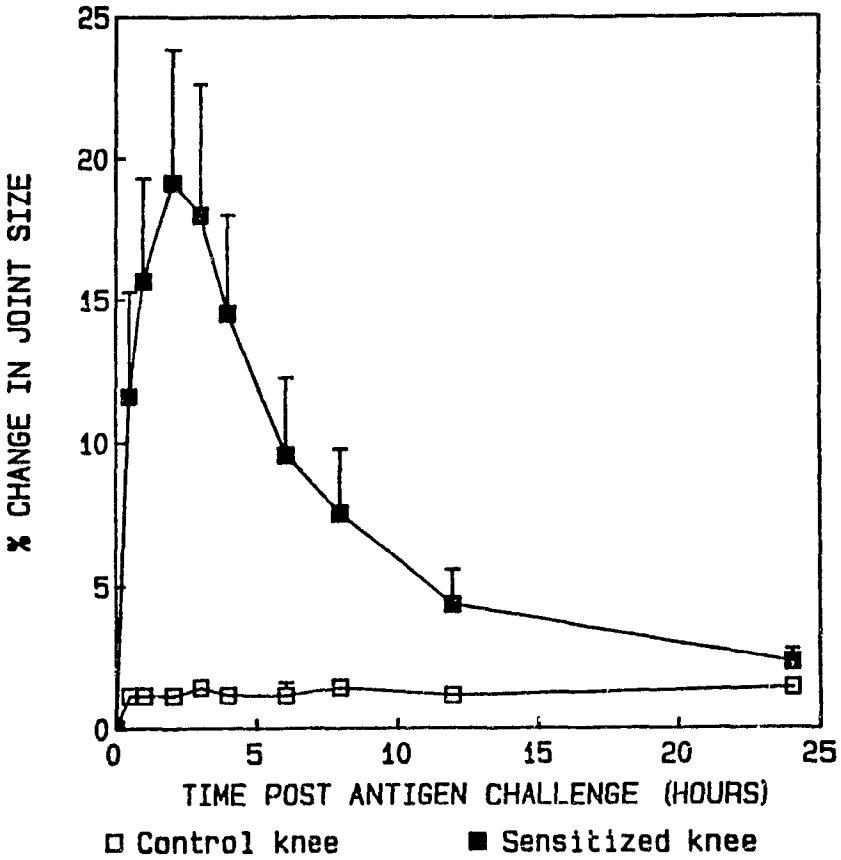

Figure 4.7. Time Course of PSA Reaction. Lewis rats (n=6) were sensitized with ovalbumin-immune serum, then challenged with ovalbumin 24 hours post-sensitization. The percent changes in joint sizes were measured at the indicated intervals following the antigen challenge. The error bars represent the SEM. Inflammation peaks at 2 hours, and returns to control levels 24 hours after the challenge.

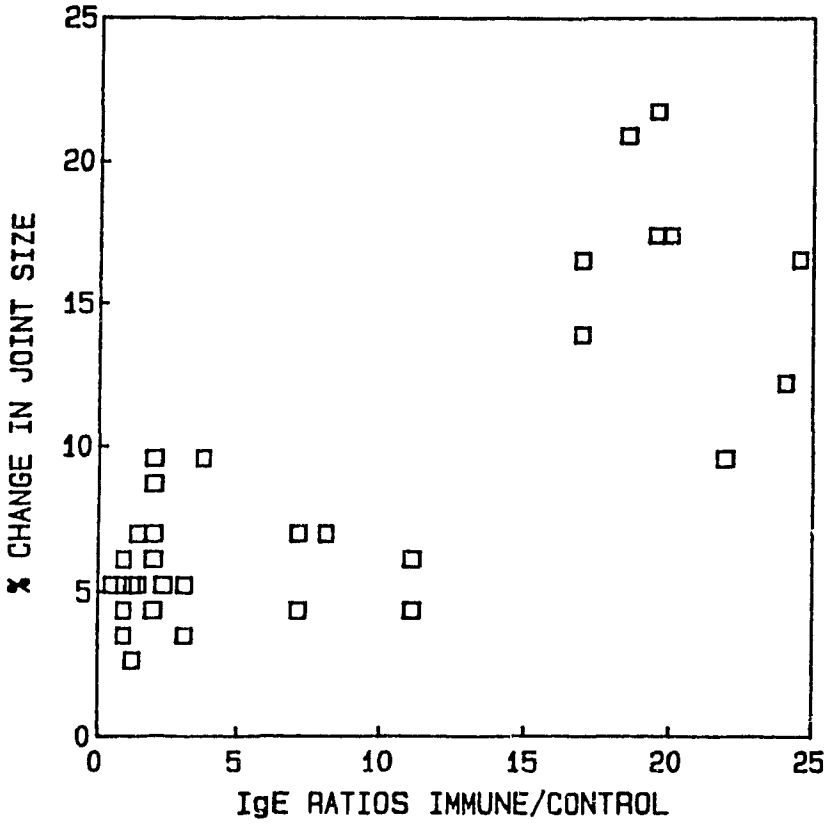

Figure 4.8. Correlation Between Ovalbumin-specific IgE and PSA-induced Joint Swelling. Rat knee joints (n=33) were sensitized with serum containing known levels of ovalbumin-specific IgE, and challenged I.V. 24 hours later with 1 mg ovalbumin. The change in joint size was recorded 1 hour after the antigen challenge. There is a significant correlation between IgE levels and the inducible swelling produced on challenging with antigen ($r=0.81$, $p<0.0001$). See Table 4.2.

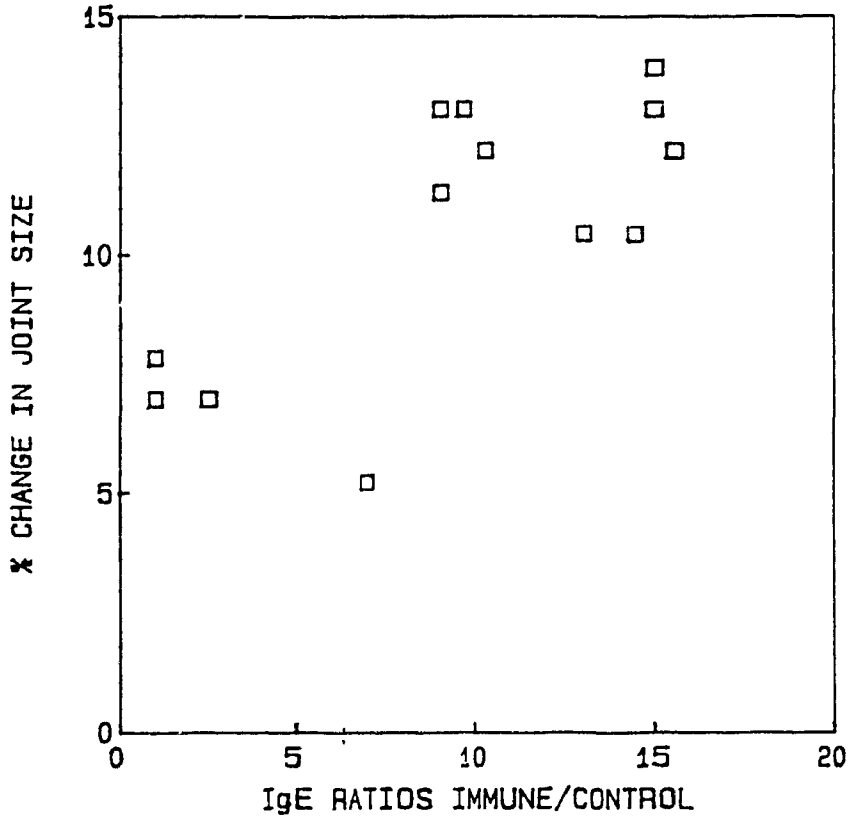

Figure 4.9. Correlation Between Pertussis-specific IgE and PSA-induced Joint Swelling. Rat knee joints (n=13) were sensitized with serum containing known levels of pertussis-specific IgE, and challenged I.V. 24 hours later with 10^5 sonicated pertussis cells. The change in joint size was recorded 1 hour following antigen challenge. There is a significant correlation between IgE levels and the inducible swelling produced on challenging with antigen ($r=0.74$, $p=0.004$). See Table 4.3.

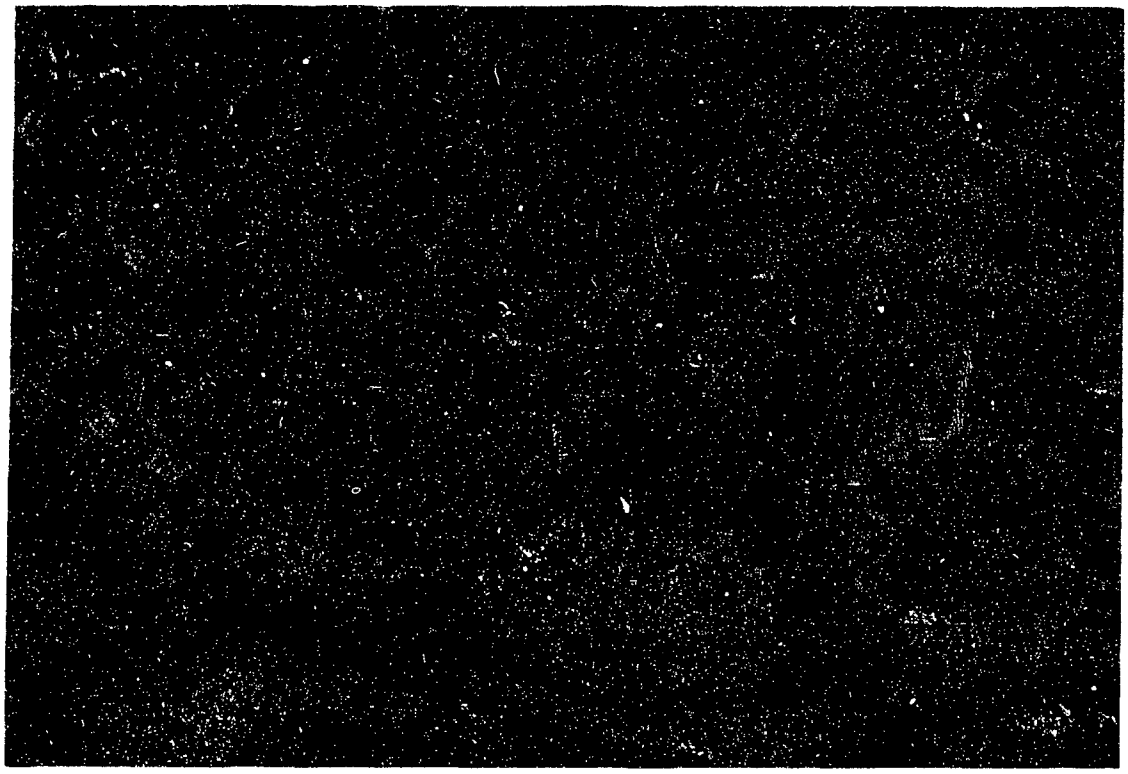

Figure 4.10. Alcian Blue/Safranin-O Staining of Mast Cells in Normal Synovium (magnification 400 X). The synovial tissue was extracted from the unsensitized knee joint of a Lewis rat, 30 minutes after an intravenous challenge with 1 mg of ovalbumin. Note the heavily granulated mast cells distributed in close proximity to the synovial blood vessels.

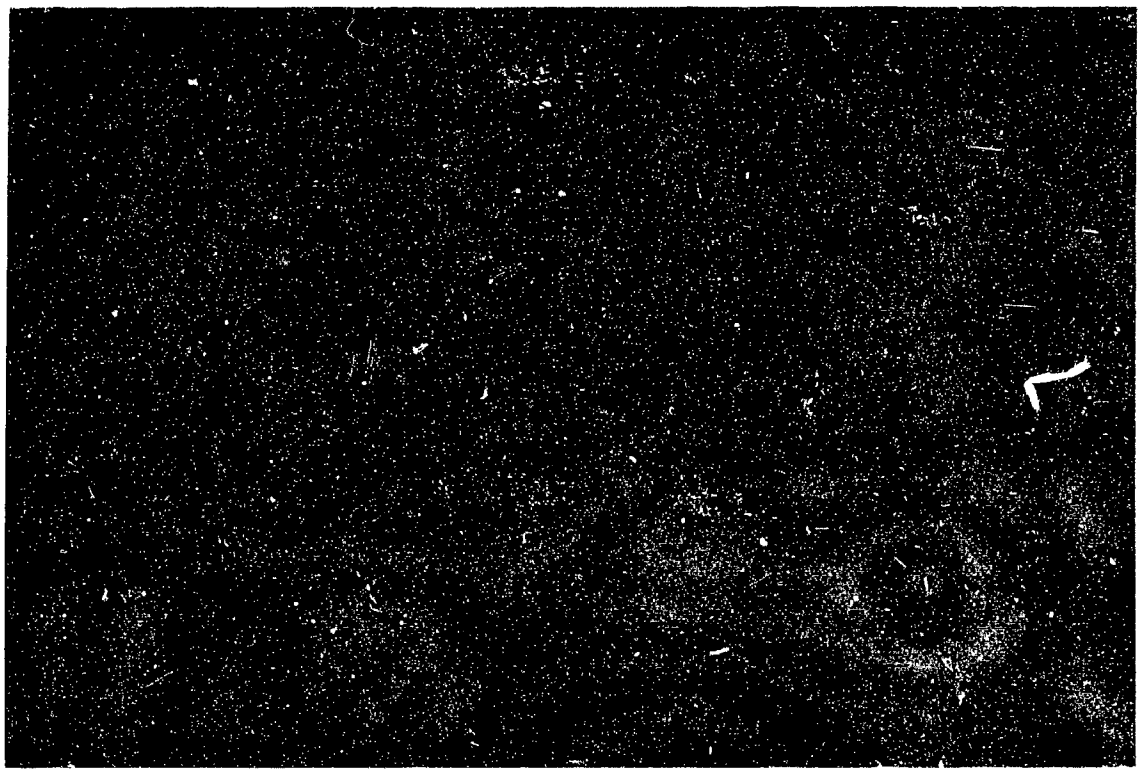

Figure 4.11. Alcian Blue/Safranin-O Staining of Mast Cells in PSA-reactive Synovium (magnification 400 X). A Lewis rat knee joint was sensitized with serum containing ovalbumin-specific IgE, then challenged intravenously after 24 hours with 1 mg of ovalbumin. The synovial tissue was extracted 30 minutes after antigen challenge, and stained for mast cells with alcian blue and safranin-O. Some of the mast cells are in the process of degranulation. See Table 4.4.

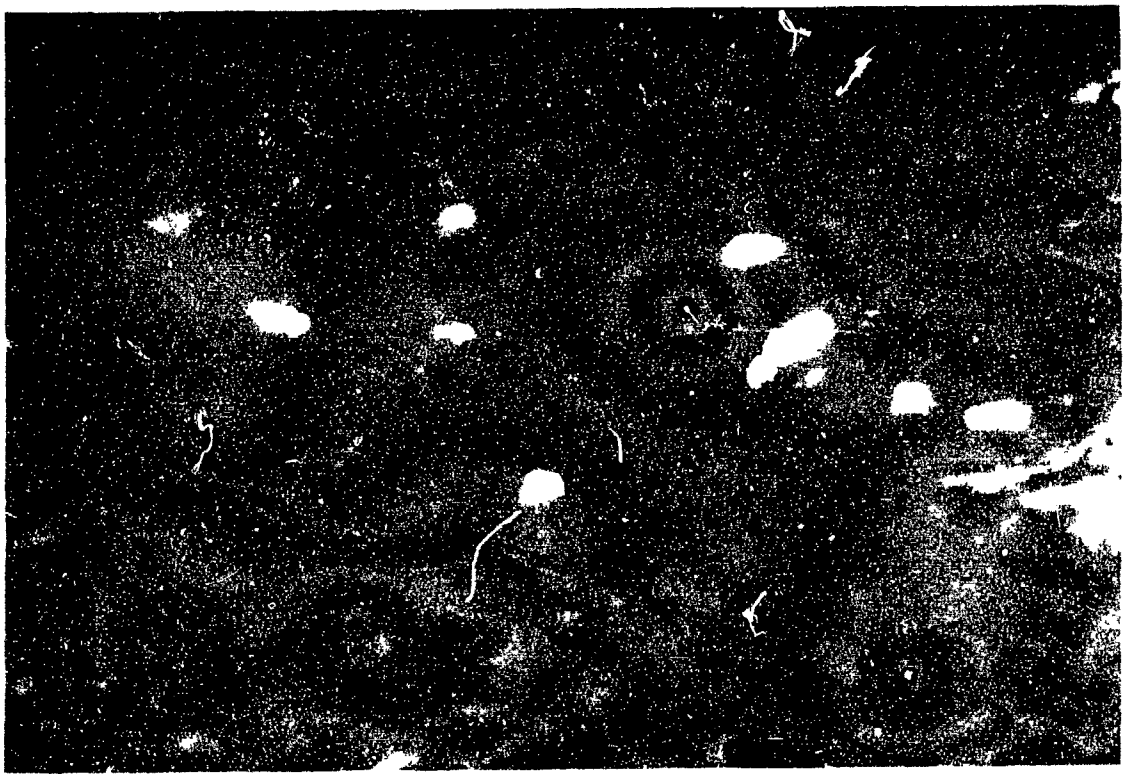

Figure 4.12. FITC-avidin Staining of Synovial Mast Cells in Normal Synovium (magnification 450 X). Normal synovial tissue was extracted from the knee joint of a Lewis rat. Note the brightly stained mast cells against the green background. This is a useful stain for the rapid tissue screening for mast cells.

Figure 4.13. ^{99}Technetium Scanning of Rat Knee Joints. A Lewis rat was sensitized with an injection of control serum into the right knee, and serum known to contain ovalbumin-specific IgE activity, into the left knee. The rat was challenged I.V. with 1 mg ovalbumin, and injected 1 hour later with 1 mCi of ^{99}Tc methylene diphosphonate. The joint scan was performed 2 hours after the radioisotope injection. There was a 2 fold higher accumulation of isotope in the sensitized (left) as opposed to the control knee (right). See Table 4.5.

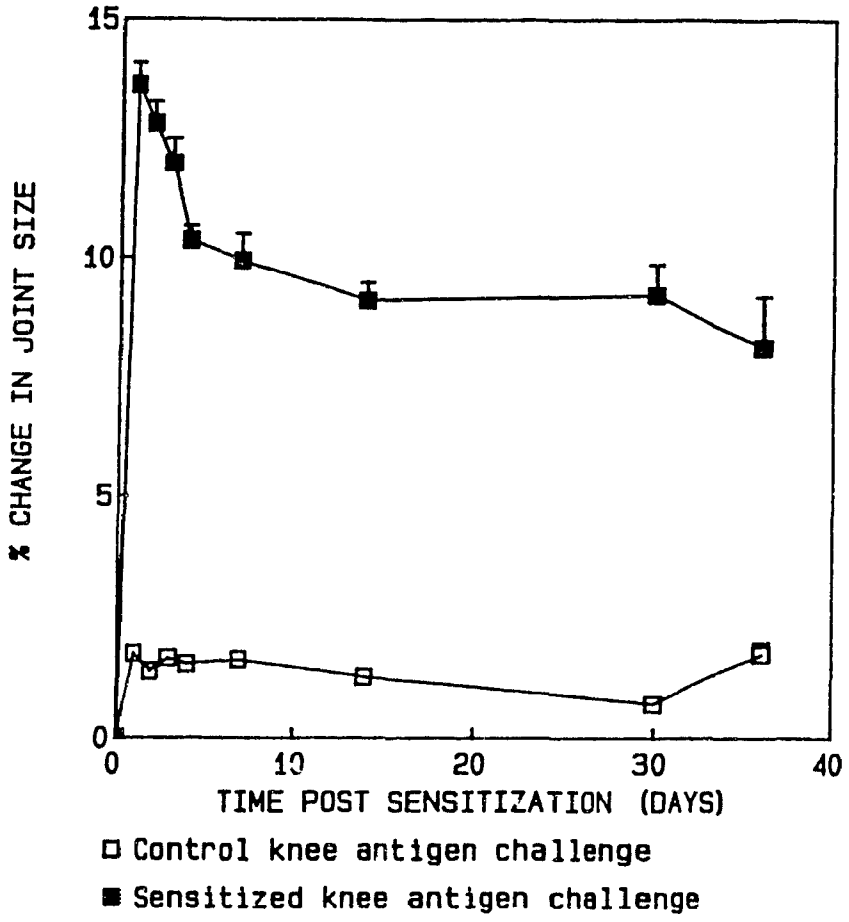

Figure 4.14. Duration of Sensitization. Lewis rats (n=9) were sensitized with ovalbumin-immune serum on day 0 and challenged on the indicated days after the sensitization. The antigen-induced inflammation is expressed as a percent change in joint size +/- SEM. The sensitized joints still respond to antigen challenge 36 days following sensitization.

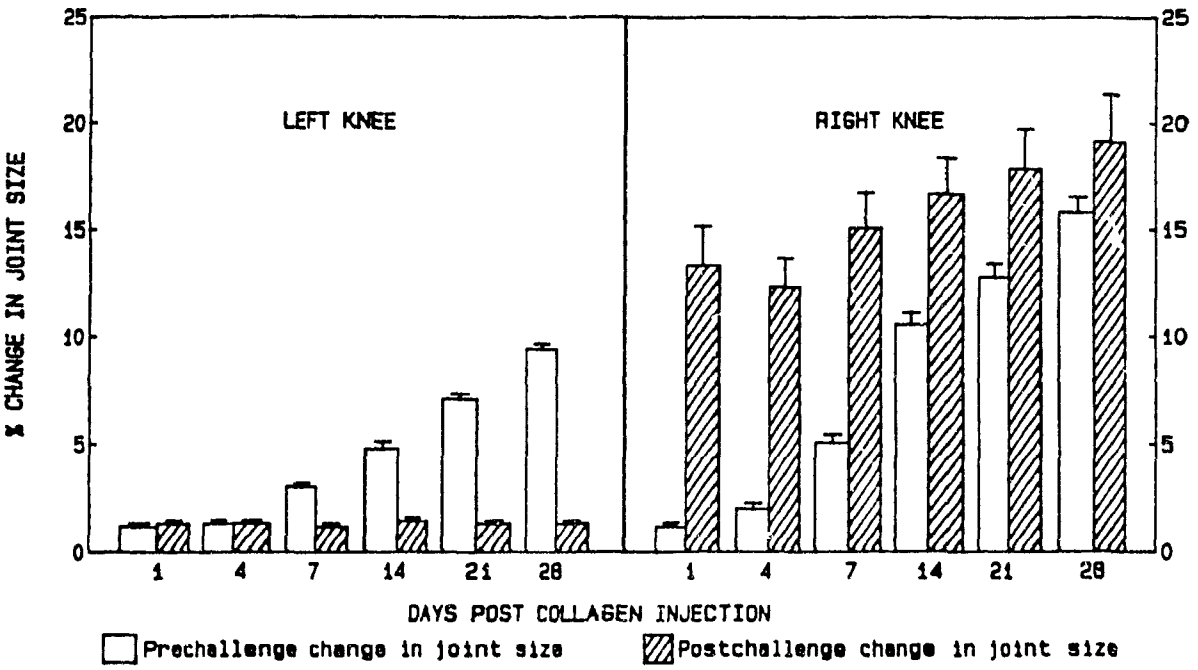

Figure 4.15. The Effect of Simultaneous Collagen-II Arthritis and PSA Reactions on Joint Swelling. Lewis rats (n=6) were injected subcutaneously on day 0 with rat collagen-II. At the same time, the right knees were sensitized with serum containing ovalbumin-specific IgE. The sensitized right knee demonstrated both an increase in pre-challenge joint size and a flare-up upon antigen challenge. The increase in pre-challenge joint size of the sensitized knee is significantly higher than the unsensitized knee beginning on day 7 ($p<0.001$). The error bars represent the SEM.

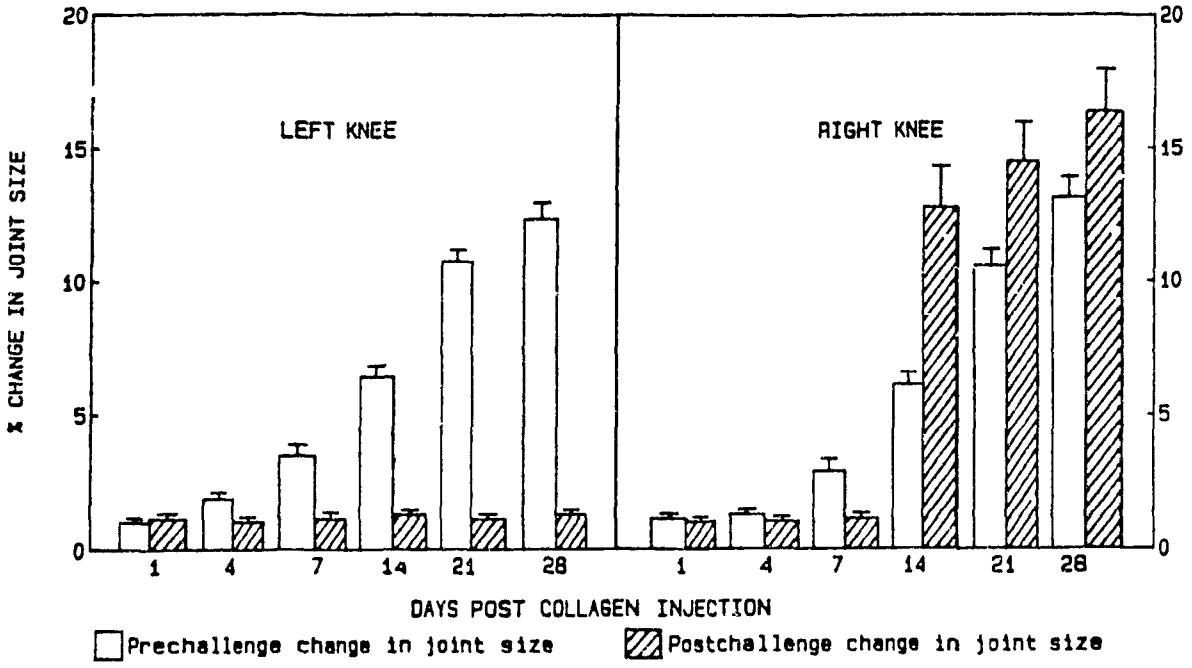

Figure 4.16. Effect of PSA Reactions on Pre-existing Collagen-II Arthritis. Lewis rats (n=6) were injected subcutaneously on day 0 with rat collagen-II. The right knees were sensitized with 50 µl of serum containing ovalbumin-specific IgE on day 13 after the injection of collagen-II. There is no statistical difference in the pre-challenge increase in joint sizes between the sensitized and the unsensitized joints ($p>0.10$). The error bars represent the SEM.

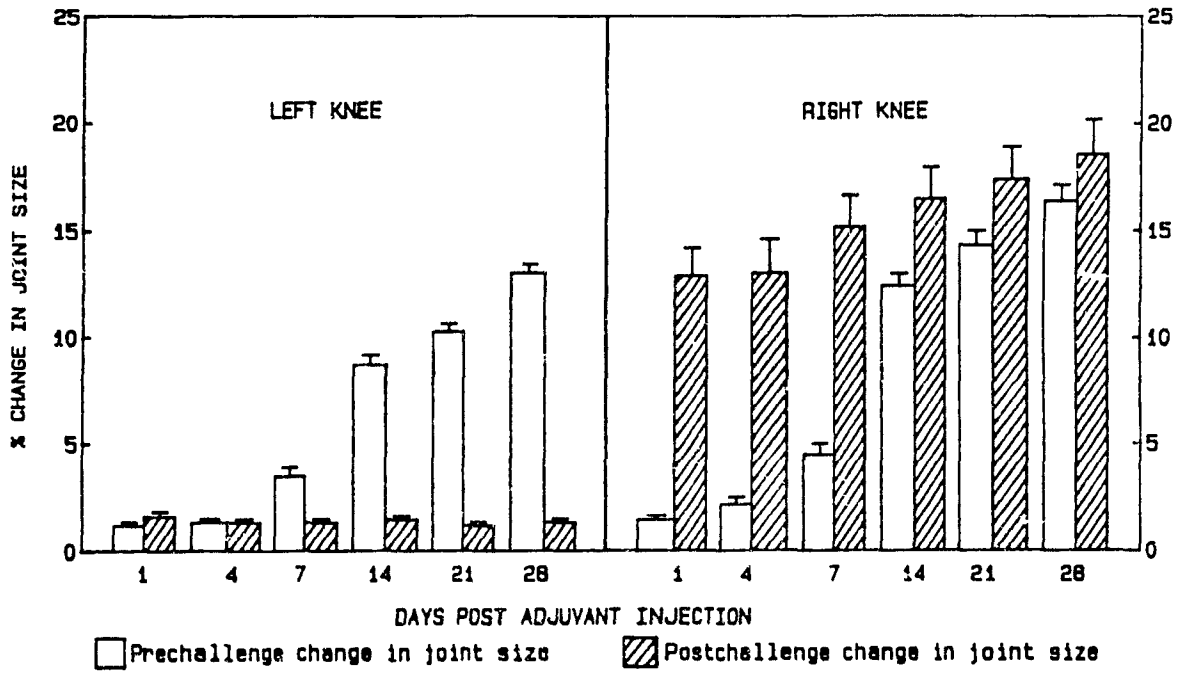

Figure 4.17. Effect of Simultaneous Adjuvant Arthritis and PSA Reactions on Joint Swelling. Lewis rats (n=6) were injected subcutaneously with 100 µl of FCA on day 0. At the same time, the right knees were sensitized with 50 µl of serum containing ovalbumin-specific IgE. The sensitized right knee demonstrated both an increase in the pre-challenge joint size and a flare-up upon antigen challenge. The increase in pre-challenge joint size of the sensitized knee is significantly higher than the unsensitized knee beginning on day 14 ($p<0.001$). The error bars represent the SEM.

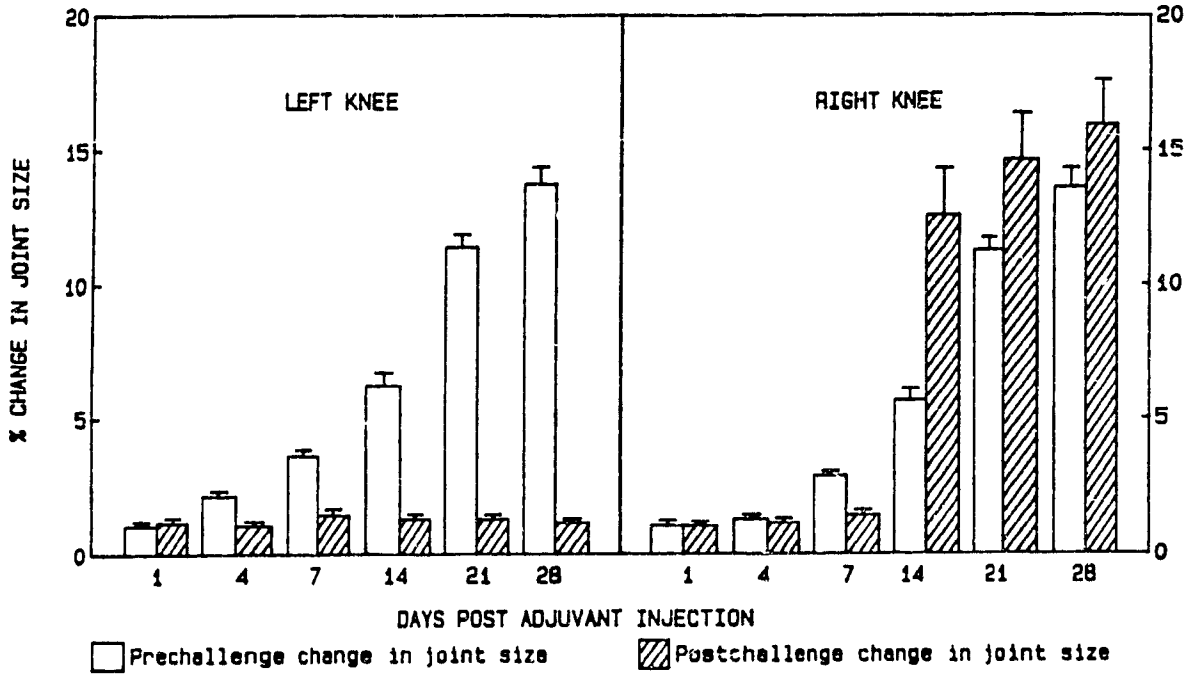

Figure 4.18. Effect of PSA Reactions on Pre-existing Adjuvant Arthritis. Lewis rats (n=6) were injected subcutaneously on day 0 with 100 µl FCA. The right knee joints were sensitized with 50 µl of serum containing ovalbumin-specific IgE on day 13 after the injection of FCA. There was no statistical difference in the pre-challenge increase in joint sizes between the sensitized and the unsensitized joints ($p>0.10$). The error bars represent the SEM.

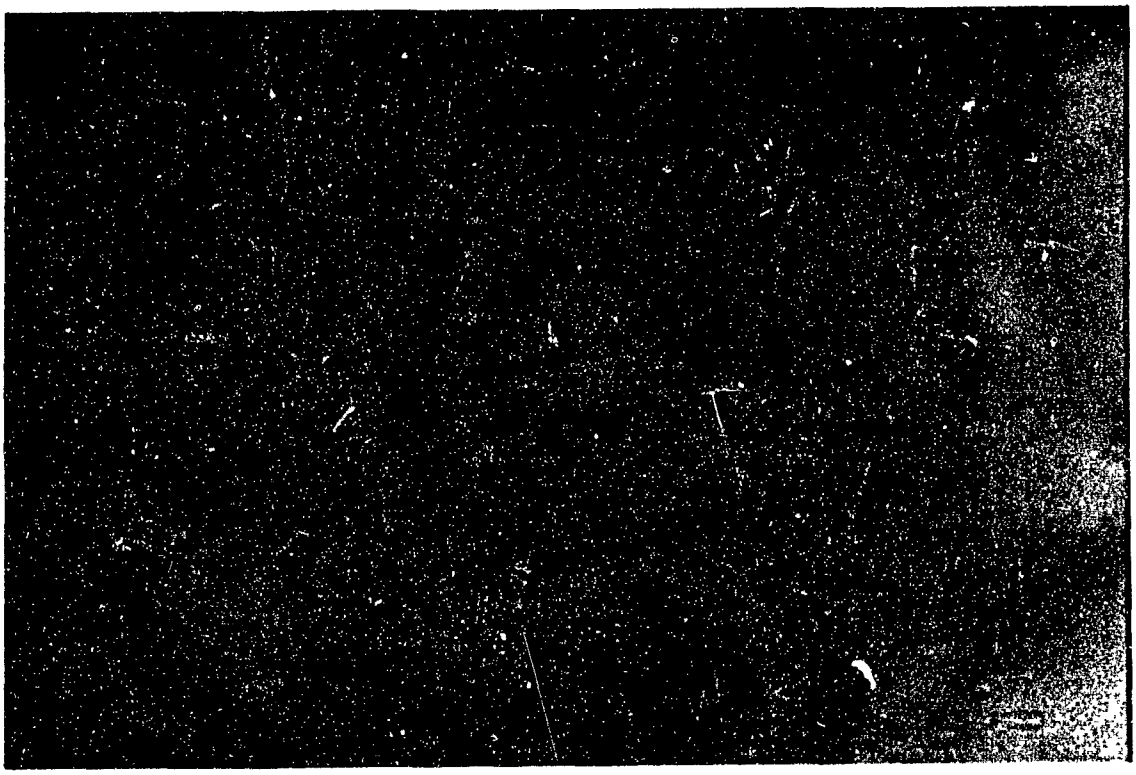

Figure 4.19. Alcian Blue/Safranin-O Staining of Synovial Tissue from a Rat with Collagen-II Arthritis. A Lewis rat was injected subcutaneously with rat collagen-II and the synovial tissue was extracted from an unsensitized knee joint 14 days later when signs of joint swelling were visible. Note the intact mast cells.

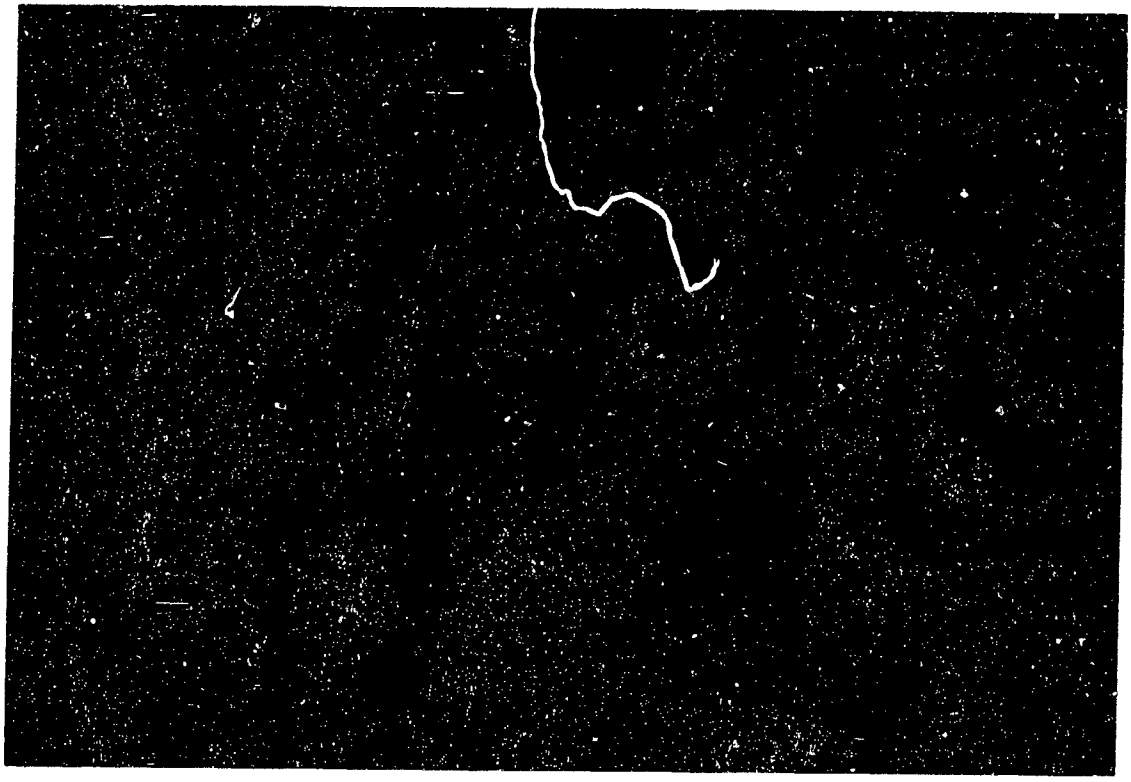

Figure 4.20. Alcian Blue/Safranin-O Staining of Synovial Tissue from a Rat with Collagen-II Arthritis, Following a PSA Reaction. A Lewis rat was injected subcutaneously with rat collagen-II. After 13 days, when there were signs of joint swelling, the rat was sensitized in one knee joint with serum containing ovalbumin-specific IgE. The rat was challenged I.V. with 1 mg of ovalbumin 24 hours after sensitization. The synovial tissue was extracted 30 minutes after antigen challenge, and stained for mast cells with alcian blue and safranin-O. The mast cells are visibly in the process of degranulation.

Figure 4.21. Ankle Swelling Response Following PSA-induction in the Knee Joint. This Lewis rat was simultaneously injected subcutaneously with FCA and intraarticularly with serum containing ovalbumin-specific IgE. Two weeks later the animal was challenged with 1 mg ovalbumin. The ankle shows a 13% increase in joint size and metacarpal joints on the same side of the sensitization are also inflamed. The sensitized knee joint shows a 5% increase in joint size following challenge.

CHAPTER 5--DISCUSSION

A number of recent reports have suggested the importance of mast cells as potential regulators of inflammatory reactions (6,7,37). Mast cell granular contents are known to have immunomodulating effects (57), and are directly proinflammatory (61,62). Mast cell mediators may also initiate and promote some of the pathologic changes seen in the rheumatoid joint, such as bone resorption (75), hyperplastic growth of the synovial layer during pannus formation (94), and synovial infiltration and activation of inflammatory cells (61,62). RA patients frequently demonstrate elevations of serum (109,110) and synovial fluid (111) IgE concentrations. This has led to the proposal that local mast cell activation might also contribute to the perpetuation of synovial inflammation, either through an allergic-type IgE-mediated reaction, or as a result of anaphylatoxins (53) or lymphokines (54) produced during an on-going immune response. A rat model of passive synovial anaphylaxis (PSA) was designed to examine the potential role of the IgE/mast cell system in the induction or exacerbation of synovitis.

Specific IgE antibody synthesis is extensively regulated by genetic factors and further modulated by lymphokines (142). Within inbred rat populations, the age and strain of the rat, in addition to the type of antigen and adjuvant used in the immunization, are important determinants of the antigen specific IgE production (142). These variables were taken into

consideration to maximize the yield of antigen-specific IgE in test rats. The Brown Norway (BN) strain of rat was selected for the production of high titer IgE antiserum because this strain naturally produces higher levels of IgE (142), and it has been determined that strains of rats with higher IgE levels produce greater IgE responses on immunization with specific antigen (143).

The failure of some animals to generate antigen-specific IgE against the immunizing antigen made antigen-specific IgG measurements necessary to ascertain that the inoculation had been successful. Significant IgG and IgE titers were elicited following immunization with pertussis antigen and ovalbumin. In both groups, antigen-specific IgE concentrations continued to increase during the post-immunization period. At the same time, the concentration of total serum IgE actually decreased. The decrease in total IgE levels is unexpected and currently unexplained.

In view of the prevalence of rheumatoid factor activity in RA serum, including IgE RFs, an attempt was made to induce an IgE response against human IgG antigen. However, only a transient IgE response to human IgG was observed, insufficient to induce a PSA reaction. The difficulty in inducing anti-human IgG responses in rats might be due to some of the antigenic epitopes being recognized as auto-antigenic. This is supported by previous findings showing that there is sufficient homology between the two antibodies for the rat IgG to be immunologically

identified by antisera to the human immunoglobulin (153). Thus an anti-human IgG response, once initiated, might be suppressed by the rat immune system.

In the mouse, genetic factors are important regulators of the immune response to low antigen concentrations (154). Since low antigen dosages were administered in these experiments to favor the production of predominantly IgE immunoglobulins (142), one might anticipate a similar genetic determination of immune responsiveness. Mouse strains that are high responders to ovalbumin possess the $H\text{-}2^b$ or $H\text{-}2^q$ genotype, whereas these genotypes are simultaneously associated with non-responsiveness to bovine-IgG (153). Since ovalbumin elicited a strong antibody response in the BN rat, this suggests that the BN strain might posses a major histocompatibility complex gene analogous to the mouse $H\text{-}2^b$ or $H\text{-}2^q$ genotype, and might additionally be a genetic non-responder to IgG antigen. However, this genetic typing information for the BN strain is as yet unavailable.

Since various IgE autoantibodies have been described in human RA, attempts were made to induce the PSA reaction using antigens such as those used in other experimental models of arthritis: i.e. MDP, which is capable of inducing an adjuvant form of joint inflammation (155), and collagen-II which is considered an antigen relevant to both animal and human disease. However, it has not yet been possible to induce specific IgE following immunization with these antigens. Despite its known adjuvant activities, MDP did not itself elicit any antibody

response in the immunized BN rats, possibly due to the low molecular weight of this peptide. This is in contrast to the significant anti-pertussis adjuvant response observed. The lack of an immune response to the human and rat collagen-II antigens might also reflect the possible genetic non-responsiveness of the BN rat strain for these antigens. Other investigators have indeed demonstrated that the immune response to collagen-II is genetically regulated in both rats (148) and humans (156).

The sera of rats with the highest titers of antigen-specific IgE were the most useful in the subsequent induction of PSA joint inflammatory responses. The PSA reaction involves injecting IgE-containing serum into the knee joint of a test animal. The homocytotropic immunoglobulins become attached to the synovial tissue mast cells via the Fc portion of the heavy chains. Subsequent challenge with corresponding antigen induces a local hypersensitivity reaction, resulting in a measurable swelling of the sensitized joint.

The magnitude of the PSA-induced joint swelling was found to correlate directly with the amount of ovalbumin- and pertussis-specific IgE, as well as the ovalbumin-specific IgG levels in the sensitizing serum. While the IgG2a component of the IgG antibody fraction has not yet been established, it is a second class of rat homocytotropic antibody that may also contribute to the PSA-induced joint inflammation. The magnitude of the observed swelling was maximal 24 hours after sensitization, and decreased with an increased delay between the sensitization and challenge.

This decreased response over time might be due to some turnover of mast cell bound homocytotropic antibody. Nevertheless, the inflammation could still be induced on challenging with ovalbumin 36 days after the initial sensitization of the joint, by which point the response had decreased to 60% of maximum. This long duration of the sensitization is of great interest. It becomes possible for a joint, once sensitized, to respond to subsequent exposures to the corresponding allergen (or even cross-reactive antigen) for up to several months after the initial sensitization. This might be relevant to the repeated exacerbations of inflammation frequently observed in the human disease.

Our thesis is that the mast cell mediators are largely responsible for the microvascular changes which produce the measurable accumulation of fluid in the joint tissues. This was supported by the histological observation of mast cell degranulation in the joints undergoing an active PSA response, as well as the evidence of the joint edema using direct caliper measurments, and radioisotopic scans of the PSA knee joints. Although several attempts were made, the failure to procure sufficient synovial fluid, even from significantly inflamed joints, prevented the measurement of histamine in the PSA joint synovial fluid. This may be due to the predominant tissue edema component of the PSA inflammation, and extremely small volumes of synovial fluid available in the joints.

Pharmacological blocking studies of the PSA response further

demonstrate that the joint swelling is a result of the PSA-induced local hypersensitivity reaction. Pretreatment with the H2 receptor blocker, cimetidine, prevented the PSA response at low doses (0.01-0.5 mg). This implies that histamine H2 receptor activation may be the predominant regulator of synovial microvascular permeability, since promethazine, a H1 blocker did not affect the response. This is in agreement with the identification of histamine H2 receptors on canine synovial blood vessels (157).

In vitro studies have shown that exogenous histamine can activate basophil surface H2 receptors and thereby inhibit further histamine release (58). Consequently, treatment of synovial mast cells with H2 blockers might effectively eliminate the negative feedback mechanism that would normally prevent excessive histamine release. Such a disruption of regulated histamine release during a PSA reaction might also contribute to the local vascular effects of histamine and account for the enhanced joint swelling produced by high cimetidine dosages. However, since mast cell activation releases a complex array of mediators, one must also consider the possible vascular effects of the other mast cell products released during the PSA reaction, which would not be affected by antihistamine drugs.

Adjuvant and collagen-II induced arthritis are the most extensively studied experimental models of arthritis, and they share many of the same clinical and histopathological features of the human disease (158). Despite their many similarities, the

pattern of immune responses associated with the development of collagen-II and adjuvant arthritis models differs. Collagen-induced arthritis has been shown to correlate with the levels of collagen-specific IgG produced following immunization (159), and the disease can be transferred by either lymphoid cells (150) or by collagen-immune serum (160). On the other hand, adjuvant arthritis in the rat is associated with the immuno-enhancing properties of adjuvants (including Freunds Complete Adjuvant, intact bacterial cell walls, or purified bacterial peptidoglycan such as MDP (155)). Delayed hypersensitivity is believed to play the major role in the pathogenesis of adjuvant arthritis (149).

Superimposing the PSA reaction on pre-existing joint inflammation demonstrated a similar effectiveness of the PSA reaction to exacerbate an on-going collagen-II or adjuvant-induced arthritis. This implies that local hypersensitivity reactions can augment inflammation of differing immunologic origins. Despite the fact that collagen-II arthritis is predominantly auto-antibody mediated, while adjuvant arthritis has a mainly cellular mechanism, the immunoenhancing effects of mast cell mediators appear to contribute to the existing immune response, resulting in more pronounced inflammation in both animal models.

For the 28 day period of these studies, the PSA reaction did not shorten the latency time for the development of a measurable joint inflammation in either the collagen or adjuvant arthritis models. Repeated PSA reactions in a knee joint significantly

increased the magnitude of the swelling produced by the primary models of arthritis. Compared to unsensitized control joints, the arthritis-induced swelling became significantly more pronounced in the joints also undergoing PSA reactions, and significance was attained approximately 1 to 2 weeks after the administration of collagen-II or FCA, respectively. This implies that the PSA reaction can exacerbate the arthritic inflammation, predisposing the joint for a more aggressive synovitis. However, the PSA reaction did not measurably affect the severity of arthritic joint inflammation when it was first provoked 2 weeks after the arthritis induction.

By the end of the 28 day study period, the PSA inflammatory response itself was significantly greater than the initial Day-1 response in both arthritis models. This escalation of the PSA response in actively inflamed joints may be due to leakage of plasma proteins into the synovial tissue via intracellular endothelial gaps (161) enhanced by the local inflammation. Increases in local vascular permeability would increase the supply of intravenous antigen to the joints during PSA reactions, and might account for the progressive increase of the PSA response.

The occasional swelling of ankles ipsilateral to the sensitized knee joints was an unexpected observation since the ankles had not been directly sensitized. It is possible for some leakage of homocytotropic antibody to have occurred from the joint space into the circulation, and subsequently into the ankle

synovial tissue. This indirect sensitization is possible since material injected into the joint space is primarily cleared via the the effluent venous channels to the femoral vein, and less so through the lymphatics (157). This would allow some of the recirculating IgE and/or IgG2a to bind at other joints, including the ankles. Actual ankle sensitization is also implied by the observed degranulation of mast cells in some tissue samples. However, why the ankle on the PSA side would be favored is not known. It is also possible that swelling of the PSA knee affects lymphatic flow from the ankles, and might indirectly account for this phenomenon.

In conclusion, the rat PSA model does support the initial proposal that local hypersensitivity reactions can cause arthritic-like swelling of IgE-sensitized joints, and can exacerbate or promote arthritic inflammations of other immunologic origins. Future work on this rat PSA model should include the testing of the effectiveness of other pharmacological blocking agents to inhibit the PSA resporse. Additional antigen challenges should be conducted to determine if the PSA response can be elicited in appropriately sensitized animals using cross-reactive antigens. The joint-sensitizing serum must also be assayed to determine the concentration of antigen-specific IgG2a, the other rat homocytotropic antibody, which may contribute to the PSA-inflammation. Immunohistochemical detection of the mast cell mediators released into the synovial fluid or tissue during PSA reactions might also be attempted.

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