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An Experimental Study on Immune Complex Induced Athritis in Rabbits

— Reference to Macrophages and M-type Cells of the Synovium —

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This study evaluates the pathogenesis of rheumatoid arthritis by producing immune complex induced arthritis with an intra-articular injection of BSA in immunized rabbits, and the effect of systemic administration of cyclophosphamide and local administration of anti-macrophage serum.

The reduction of inflammatory reaction by cyclophosphamide administration appears to be caused mainly by selective depletion of the neutrophils, and partly by immune suppression. It appears that the rabbit abdominal macrophage has the common morphologic, functional and antigenic patterns with the M-type synovial lining cells. There is another possibility that the cross-reacting antigens between macrophage and the M-type cell of the synovial lining may exist. It is concluded that in this experimental immune complex arthritis, the site of localization of immune complexes seems to be the synovial, M-type cell, and the tissue injury of synovium is largely mediated not only by neutrophils and complement, but also by macrophages.

Key Word: Antimacrophage serum, Cyclophosphamide, Immune complex induced arthritis, Rheumatoid arthritis, Synovial cell.

The term "rheumatoid arthritis," introduced by Sir Alfred Garrod in 1858, describes a systemic disease of unknown cause that is characterized by symmetrical inflammatory polyarthropathy, female predominance, spontaneous remission and exacerbation (Salter, 1975; Turek, 1977; Brashear & Rakey, 1978; Beary et al., 1981; Kelley et al., 1981; Christian, 1982).

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Compelling evidence implicates genetic and immunologic processes in the initiation and perpetuation of the inflammatory events characteristic of rheumatoid arthritis. The susceptibility to rheumatoid arthritis is an inherited trait, determined by gene products of the major histocompatibility system. Such genes probably control the humoral and cell-mediated immune mechanisms thought to participate in the pathogenesis of rheumatoid arthritis (Paget & Gibofsky, 1979; Kelley, 1981). Antigen-stimulated T and B lymphocyte and macrophage interactions are taking place with

resulting release of lymphokines from T-cells and immunoglobulins from plasma cells. So it postulates that the main event is an antigen-antibody reaction in the joints. Such immune complexes activate the cascade of complement-generating factors that promote chemotactic migration of polymorphonuclear leukocyte. The latter phagocytose the complexes and release lysosomal enzymes causing articular damage (Cooper, 1968; Moskowitz, 1968; Persellin, 1968).

Bennett (1978) introduced four general mechanisms by which infectious agents can initiate synovitis: 1) multiplication of the agent within a joint space, 2) antigens derived from an infectious agent within the joint space and initiated immune response, 3) infectious agents or their derived antigens at a distant site, and 4) "arthrogenic toxins" produced by infectious agents. Bennett (1981) declared that the study of rheumatoid arthritis should be based on such topics as knowledge of the link to genetically controlled host response factors, immune complexes and induction of the inflammatory process and organisms implicated in the initiation of the rheumatoid process.

Recent discoveries of the relationship of histocompatibility markers to the epidemiology of certain disease have indicated strong genetic relationships of these cell surface molecules to apparent disease susceptibility. Sasazuki et al., (1977) insisted that the associated molecule is the major histocompatibility complex located on the human 6th chromosome. It is specifically the glycoprotein molecule (Terhorst et al., 1977). Such associations are quite striking between HLA-B27 and ankylosing spondylitis, as well as with certain related diseases including Reiter's syndrome, reactive arthritides and some cases of juvenile rheumatoid arthritis, and HLA-Dw4 with rheumatoid arthritis (Bennett, 1981).

Although much evidence can be brought foward to indicate that known microbial infection may be followed by polyarthritis and multisystemic rheumatic disease in both animals and men, it is difficult to clearly analyze such a course of events when dealing with human rheumatoid disease (Bennett, 1981). Several microorganisms are found in experimentally induced rheumatoid arthritis: clostridium perfringens in fecal material (Mansson & Olhangen, 1974); diphtheroid organism in synovium (Person et al., 1971); mycoplasma in synovium (Barden & Decker, 1971; Ross, 1973; Decker & Barden, 1976; Taylor-Robinson & Taylor, 1976); virus or virus like particles (Grayzel & Beck, 1970; Baringer, 1971; Person et al., 1971; Schumacker, 1975; Gajdusek, 1977); viral induced enzyme (Spruance et al., 1975); newly synthesized RNA & DNA (Person et al., 1976); antiviral antibody (Chandler et al., 1971); and cytotoxic antibody to viral products (Person et al., 1976).

Dumonde and Glynn (1962) described an experimental animal model of rheumatoid arthritis, which was induced by intra-articular injection of autologous and heterologous fibrin in previously immunized rabbits. The induction of a similar chronic synovitis after intra-articular injection of soluble proteins such as egg albumin and bovine serum albumin in immunized rabbits was described (Glynn, 1968; Webb et al., 1971; Cooke et al., 1972; Cooke & Jasin, 1972; DeShazo et al., 1972; Stastny et al., 1973). Some authors (Steffen & Timpl, 1963; Michaeli & Fudenberg, 1974; Andriopoulos et al., 1975; Cracchiolo et al., 1975; Andriopoulos et al., 1976) reported the presence of an antibody to collagen in rheumatoid arthritis patients. Type I and III collagens are found in the skin and parenchyma of several organs, whereas type II exists in cartilage (Seraffini-Fracassini & Smith, 1974). Thus an immune response to the cartilage type of collagen could explain the predilection of rheumatoid arthritis to involve

diarthrodial joints. Injection of homologous and heterologous type II collagen induces inflammatory arthritis in immunized animals (Trentham et al., 1977; Trentham et al., 1978; Stuart et al., 1979; Rogers et al., 1980; Trentham et al., 1980; Trentham, 1981; Caulfield et al., 1982).

Immune complexes are important in the pathogenesis of rheumatoid arthritis due to the following points: 1) presence of immunoglobulin & complement in the collagenous tissue of the joint (Cooke et al., 1975), 2) experimental arthritis induced by fibrin and antigens (Dumonde & Glynn, 1962), and 3) presence of C3 byproducts in the synovial fluid of rheumatoid arthritis patients (Zvaifler, 1969). The mechanism of tissue damage by immune complexes is still unclear, but in glomerulonephritis, the correlation with polymorphonuclear leukocytes (Dixon et al., 1961; Germuth et al., 1972), the correlation with complements and deposition of immune complexes (Cochrane, 1971; Germuth & Rodriguex, 1973), and the correlation with platelets (McCuskey & Vassali, 1971) are suggested.

There is general agreement that anti-macrophage serum is cytotoxic to macrophages and can decrease the phagocytic activity of peritoneal macrophages (Panijel & Cayeux, 1968; Unanue, 1968; Jennings & Hughes, 1969). Some investigators find immuno-suppressive effects after the receipt of anti-macrophage serum (Panijel & Cayeux, 1968; Kim et al., 1975), but others report no immuno-suppressive effects (Unanue, 1968; Loewi et al., 1969).

Depletion of circulating leukocytes reduces the vascular damage of an Arthus reaction and the necrotizing arteritis of serum sickness (Kniker & Cochrane, 1965). The presence of polymorphonuclear leukocytes is related to the glomerular damage of induced nephritis (Unanue & Dixon, 1965; Cochrane, 1971; Germuth & Choi et al., 1972), and the depletion of circulating polymorphonuclear leukocytes reduces the glomerular damage (Henson, 1972). Recently there have been several interesting reports that macrophages and monocytes are essential in human and animal experimental glomerulonephritis (Atkins et al., 1976; Holdsworth et al., 1978; Schiffer & Michael, 1978; Schreiner et al., 1978).

This study evaluates the pathogenesis of rheumatoid arthritis by producing immune Complex induced arthritis with an intra-articular injection of BSA in immunized rabbits, and the effect of systemic administration of cyclophosphamide and local administration of anti-macrophage serum.

MATERIALS AND METHODS

A total of 45 rabbits were used, each weighing about 1.8 – 2.5 Kg, and were divided into 4 groups as follows:

Group I :	Control group	
A.	Non-immunized control . . .	5
B.	Immunized control	5
Group II :	Experimental arthritis group	15
Group III :	Cyclophosphamide treated group	15
Group IV :	Anti-macrophage serum treated group	5

Immunization: Bovine serum albumin dissolved in saline (BSA 10 mg/mlL was mixed with equal parts of complete Freund adjuvant (Difco Laboratory, with 1 mg/ml of dried tubercle bacilli) to produce a water-in-oil emulsion. The immunization was conducted through intradermal injection of 2 ml of this emulsion in 7 or 8 sites of the neck, back, and buttocks. A booster immunization was performed 1 week after the first immunization.

Knee joint injection: The skin around both

knee joints was shaved and cleaned with 75% alcohol. With the knee partially flexed, the joint was entered either by a medial approach into the suprapatellar pouch or through the patellar ligament. Then 5 mg of BSA in 1.0 ml of saline was injected into both knee joints. The interval between immunization and intra-articular injection was 17 days.

Skin test for the existence of delayed-type hypersensitivity: An intradermal injection of 0.1 ml BSA solution (as described above) was done. The skin test was regarded as positive if at 24 hours after injection there was an easily palpable area of industration at least 5 mm in diameter at the injection site. A positive skin test was taken to indicate the existence of delayed-type hypersensitivity to BSA.

Macrophage suspension: The peritoneal macrophage suspension was made by the method of Dyminski and Argyris (1969).

Anti-macrophage serum: The preparation of anti-macrophage serum was done by the method of Marsman et al. (1970).

Specimens taken from synovium were fixed in 10% neutral formaline, embedded in paraffin, and hematoxylin-eosin staining was done. For ultrastructural examination, tissues from the synovium were fixed in 3% glutaraldehyde and 1% osmium tetraoxide with the ordinary processing, and the examination was made using a Hitachi H-500 electron microscope.

Frozen sections were made at a thickness of 3 microns stained with fluoresceintagged antisera to rabbit immunoglobulin (IgG, IgA, IgM), C_3, fibrin and bovine serum albumin, and examined with the Leitz Dialoux fluorescent microscope.

RESULTS

A) Gross findings

In the control group, the synovial membranes showed slight congestion and the inner surfaces were glistening, but there was no swelling or fibrosis. In group II the synovium showed marked swelling and congestion, and much mucinous inflammatory exudate was contained in the intra-articular space on the 3rd day. On the 7th day the synovium showed irregular thickening. On the 14th day the synovium showed less swelling and congestion and more irregular thickening. In group III edema and congestion were slight. In group IV edema and congestion were marked in the early stage and the amount of inflammatory exudate was less than in group II.

B) Rheumatoid factor

The rheumatoid factor was positive in two out of ten in the control group, twelve out of fifteen in group II, six out of fifteen in group III, and four out of five in group IV (Table 1).

Table 1. Rheumatoid factor

Group	Positive	Percentage	Total
I	2	20	10
II	12	80	15
III	6	40	15
IV	4	80	5

$X^2 = 9.04$ $P < 0.05$

C) Light microscopic findings

In the control group the synovium showed one cell layer of synovial cells and infiltrations of a few inflammatory cells. In group II the synovial cell proliferated to 8 layers by the 4th week, and the synovium showed necrotizing inflammation and neutrophilic and eosinophilic infiltration on the 3rd day. In the 4th and 8th week the infiltrated inflammatory cells were predominantly lymphocytes. In group III there was no synovial proliferation and the inflamma-

Table 2. Light microscopic findings

Group	Sacrifice	Layers of synovial cell	Inflammatory cells				Necrosis	Location
			N	E	M	L		
I		1	−	−	−	−	−	
	3D	0 − 1	+++	+++	+	−	++	
	1W	1 − 4	+++	+++	+	+	+	Surface &
II	2W	2 − 4	+	++	+	+++	−	Deeper
	4W	4 − 8	+	++	−	+++	−	
	6W	1 − 4	−	++	−	+++	−	
	3D	0 − 1	+	++	+	−	−	
	1W	1 − 2	+	+	+	+	−	
III	2W	1 − 2	+	+	−	+	−	Surface
	4W	1	−	+	−	+	−	
	6W	1	−	+	−	+	−	
	3D	0 − 1	++	+	+	−	+	
	1W	1 − 2	++	+	+	+		
IV	2W	1 − 2	+	+	−	+	−	Sub-synovial
	4W	1	+	+	−	++	−	
	6W	1	−	+	−	++	−	

N : Neutrophil E : Eosinophil M : Macrophage L : Lymphocyte

tory cell infiltration was less severe than in group II. In group IV there was no synovial proliferation and the inflammatory cells were neutrophils and eosinophils in the early stage and lymphocytes in the late stage (Table 2).

D) Electron microscopic findings

The synovial cells were mainly of 2 types, M-type (macrophage-like) and F-type (fibroblast-like), and another cell type, I-type (intermediate). The M-type cells had active phagocytic activity and large numbers of lysosomes, well-developed rough endoplasmic reticulum, and Golgi apparatus. The synovial cells didn't have junctional apparatus such as tight junction and basement membrane. In group II the synovial cell proliferation was marked by the 4th week, but less severe in group III and IV. In group IV the M-type cells were less prominent than in group II and III.

E) Immunofluorscent findings

In the control group there was no specific deposit. Group II showed immunoglobulin (Ig G, IgA, IgM on the synovial surface in the early stage and a small amount of BSA, C_3 and fibrin. In group III the depositions were minimal and there were no depositions in group IV (Table 3).

DISCUSSION

The evidence implicates genetically controlled immunologic processes in the initiation

Table 3. Immunofluorescent findings

Group	Sacrifice	BSA	IgG, IgA, IgM	C_3	Fibrin
I		−	−	−	−
	3D	− ~ ±	+ −	± ~ +	± ~ +
	1W	± ~ +	+	− −	± ~ +
II	2W	± ~ +	+ −	± ~ +	± ~ +
	4W	± ~ +	± ~ +	+	+
	6W	− ~ ±	± ~ +	−	−
	3D	− ~ ±	− ~ ±	−	~ ±
	1W	− ~ ±	−	−	− ~ ±
III	2W	− ~ ±	− ~ ±	− ~ ±	−
	4W	−	−	−	−
	6W	−	−	−	−
	3D	− ~ ±	− ~ ±	−	− ~ ±
	1W	−	− ~ ±	−	−
IV	2W	−		−	
	4W	−			
	6W	−	−	−	−

and perpetuation of the inflammatory events characteristic of rheumatoid arthritis. Presently, it is believed that rheumatoid arthritis arises through immunopathologic responses to an as yet unidentified antigen, possibly related to an atypical microbial infection in a genetically susceptible host. The antigen-induced synovitis was initially described as an inflammatory response of rabbits previously immunized to homologous and heterologous fibrin (Dumonde and Glynn, 1962). Subsequently, the induction of a similar chronic synovitis after intra-articular injection of soluble proteins such as egg albumin and bovine serum albumin in immunized rabbits was described (Glynn, 1968; webb et al., 1971, Cooke et al., 1972; Cooke & Jasin, 1972; DeShazo et al., 1972; Stastny et al., 1973). Type I and III collagens are found in the skin and parenchyma of several organs, whereas type II exists in cartilage (Seraffini-Fracassini & Smith, 1974). Thus, an immune response to the cartilage type of collagen could explain the predilection of rheumatoid arthritis to involve diarthrodial joints.

Injection of homologous and heterologous type II collagen induces inflammatory arthritis in immunized animals (Trentham et al., 1977; Trentham et al., 1978; Stuart et al., 1979; Rogers et al., 1980; Trentham et al., 1980; Trentham, 1981; Caulfield et al., 1982). In vivo experimental animal models of rheumatoid arthritis have contributed significantly to pathogenetic concepts of rheumatoid arthritis. The experimental arthritis has many features in common with rheumatoid arthritis. Serum immunoglobulin levels are usually elevated, and rheumatoid factors are present in the serum and synovial fluid. Approximately 20% of synovial fluid immuno-

globulin is synthesized by the synovial membrane, the predominant class being Ig G (Paget & Gibofsky (1979). Webb et al., (1971) stated that almost all of a foreign protein injected into a normal knee joint is rapidly cleared in a few days. If an animal is given foreign protein into a knee joint and a delayed type hypersensitivity is produced later, then a chronic proliferative synovitis can also develop. This suggests that munute amounts of foreign protein can persist in an antigenic form in normal rabbit synovial membrane. The persistance of this small amount of antigen may account in part for the chronicity of this from of form of experimental synovitis.

This experiment evaluates the pathogenesis of rheumatoid arthritis by producing immune complex induced arthritis with an intra-articular injection of bovine serum albumin in immunized rabbits, the effect of systemic administration of cyclophosphamide and local administration of anti-macrophage serum, and also the changes of the synovial membrane as noted by the light microscope, electron micrsocope and immunofluorescent microscope.

In group II the rheumatoid factor was detected in 80%, but in the cyclophosphamide treated group (group III) it was detected in 40%. Group III was the same as group II. It was difficult to decide whether the anti-macrophage serum treatment could decrease the positivity of the rheumatoid factor because the number of rabbits in group IV was small.

Approximately 80% of rabbits were seropositive for rheumatoid factor. The presence of rheumatoid factor is not specific for any disease, and the actual role it plays in the production of rheumatoid arthritis has not been defined. Seropositivity is present in other connective tissue diseases such as systemic lupus erythematosus and scleroderma and in a variety of other illnesses such as tuberculosis, sarcoidosis, and bacterial endocarditis (Paget & Gibofsky, 1979).

In group II the synovial cell proliferation was marked in the later stages and the infiltration of polymorphonuclear leukocytes and necrosis was marked in the earlier stages. The infiltrated inflammatory cells were predominantly lymphocytes in the later stages. The depositions of immune complexes were located on the synovial surface and decreased in the later stages. In group III there was little synovial cell proliferation and little inflammatory cell infiltration in the superficial synovial tissue. There were less depositions of immune complexes and fibrins than in group II. In group IV there was no synovial proliferation and no immune depositions.

Cyclophosphamide has immunosuppressive and bone marrow suppressive action with resulting leukocyte depletion. The cyclophosphamide administration decreased the inflammatory infiltration of polymorphonuclear leukocytes and decreased the immune and fibrin depositions. These results suggest that depletion of leukocytes inhibit the tissue injury mediated by polymorphonuclear leukocytes as did other reports (Henson, 1972; Naish et al., 1975). Patients with severe rheumatoid arthritis and unresponsive to conventional therapy received oral cyclophosphamide. Thirty eight continued on the drug for six months or longer. Of the thirty eight patients, twenty nine (75%) improved (Fosdick et al., 1968).

By electron microscopy, the synovial cells are mainly of two types, M-type (macrophage type) and F-type (fibroblast type), and another cell type, I-type (intermediate). The M-type cells have active phagocytic activity and large numbers of lysosomes, well-developed rough endoplasmic reticulum, and Golgi apparatus. The F-type cells have secretory actions and rough endoplasmic reticulums (Muirden, 1966). In this experiment there were more marked proliferations of M-type cells than F-type cells as in other reports (Muirden, 1966; Norton & Ziff, 1966; Grimley, 1967). These results suggest that the M-type synovial cells have similar structures, functions, and anti-

genecity to the macrophages. Muirden (1966) showed that ferritin, when introduced into the articular space, was engorged by the synovial cell and this suggested phagocytic activity in the synovial cell.

Interest has recently been revived concerning the properties of antimacrophage serum. There is general agreement that antimacrophage serum is cytotoxic to macrophages and can decrease the phagocytic activity of peritoneal macrophages (Panijel & Cayeux, 1968; Unanue, 1968; Jennings & Hughes, 1969). The erythophagocytosis by mouse peritoneal macrophages consists of an attachment phase and an ingestion phase. The phagocytosis inhibitory effect of anti-macrophage serum would be explained by a blackade at the attachment site (Isa, 1971). Some investigators reported that anti-macrophage serum cross-reacts with lymphocytes (Bodmer et al., 1966; Marsman et al., 1970; Kim et al., 1975), but others reported no cross-reaction (Unanue, 1968; Jennings & Hughes, 1969). It is interesting to note the cross-reaction between macrophages and erythrocytes (Jennings & Hughes, 1969). The systemic use of anti-macrophage serum suppresses antibody production (Panijel & Cayeux, 1968; Argyris & Plotkin, 1969; Isa, 1971) and slows the rejection of autologous skin grafts (Dyminski & Argyris, 1969). There are several interesting reports that macrophages play an important pathogenetic role in tissue injury of human and experimental glomerulonephritis (Atkins et al., 1976; Holdsworth et al, 1978; Schiffer & Michael, 1978; Schreiner et al., 1978; Thomson et al., 1979; Holdsworth et al., 1980). In this experiment the local treatment of anti-macrophage serum in experimentally induced arthritis inhibited the synovial cell proliferation, the inflammatory cell infiltration, and immune and fibrin deposition. Rosenthal (1980) reported that the macrophage had 4 main actions: 1) proliferation of the T-cell, 2) a central role in the genetically controlled immunologic process, 3) possession of a plasma membrane receptor bearing an Fc receptor to enhance phagocytic activity, and 4) biologically active materials to initiate an immune response. Therefore, it was suggested that the peritoneal macrophage had similar structure, function and antigenecity to the M-type synovial cells and cross-reacted with the M-type cells.

CONCLUSION

This study evaluates the pathogenesis of rheumatoid arthritis by producing immune complex induced arthritis by intra-articular injection of BSA in immunized rabbits and the effect of systemic administration of cyclophosphamide and local administration of antimacrophage serum.

The results are summarized as follows:

1. The changes in the rabbits resembled rheumatoid arthritis in man and initiated prominent proliferation of synovial lining cells. Neutrophilic infiltration in the synovial tissue was present in the early stage, but later lymphocytic and plasma cell infiltrations were dominant.
2. The systemic administration of cyclophosphamide significantly inhibited the inflammatory reaction of the immune complex induced arthritis, and the local administration of anti-macrophage serum also markedly reduced the inflammatory changes.
3. By electron microscopy, the proliferation of the M-type cells was prominent in the synovial lining, which was not particularly influenced by the cyclophosphamide administration, but was inhibited prominently by the anti-macrophage serum administration.
4. By immunofluorescent microscopy, the deposits of BSA, immunoglobulins, C_3,

and fibrin were clearly demonstrated mainly in the synovial lining. These deposits were somewhat reduced by the cyclophosphamide administration, but were greatly inhibited by the anti-macrophage serum administration.

In summary, the reduction of inflammatory reaction by cyclo-phosphamide administration appears to be caused mainly by selective depletion of neutrophils, and partly by immune suppression. It appears that the rabbit abdominal macrophage has the common morphologic, functional and antigenic patterns with the M-type synovial lining cells. There is another possibility that cross-reacting antigens between macrophage and the M-type cell of the synovial lining may exist. It is concluded that in this experimental immune complex arthritis, the site of localization of the immune complexes seems to be the synovial, M-type cell, and the tissue injury of synovium is largely mediated not only by neutrophils and complement, but also by macrophages.