Ann. rheum. Dis. (1972), **31**, 186

Elution of antiglobulins and antinuclear antibody from rheumatoid synovial membrane

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Immune factors are currently believed to be important in the pathogenesis of rheumatoid synovitis. Several findings have suggested that immune mechanisms are operative within the rheumatoid joint; these include the detection of antiglobulins and low levels of complement in the synovial fluid (Hannestad and Mellbye, 1967; Ruddy and Austen, 1970), the detection of immunoglobulins and complement within synovial leucocytes (Hurd, LoSpalluto, and Ziff, 1970), and the presence of immune complexes consisting of antiglobulins and IgG in synovial fluid (Winchester, Agnello, and Kunkel, 1970). Furthermore, the synovial membrane in rheumatoid arthritis has also been shown to contain certain immune factors. Rheumatoid factor, immunoglobulins, and complement components have been detected in rheumatoid synovial membrane using immunofluorescent techniques (Bonomo, Tursi, Trizio, Gillardi, and Dammacco, 1970; Kinsella, Baum, and Ziff, 1970). These findings have led to the concept that the deposition of immune complexes within the synovial membrane may initiate or perpetuate the inflammatory process (Zvaifler, 1970).

Elution procedures, using either high molarity or acidic buffers, are known to free immune complexes which are bound to certain tissues. Similar methods have been employed to elute and study the immune factors from the glomeruli of immune-induced nephritis (McGiven and Ironside, 1968; Krishnan and Kaplan, 1967; Koffler, Schur, and Kunkel, 1967). We undertook the present study in an attempt to elute and study immune factors present in rheumatoid synovial membrane.

Material and methods

SYNOVIAL SPECIMENS

Specimens were obtained from 24 patients who had undergone synovectomy of the knee. Eighteen patients had a diagnosis of classical or definite rheumatoid arthritis (RA). Other patients included with two a diagnosis of juvenile rheumatoid arthritis (JRA), two with a chronic unremitting knee effusion secondary to knee trauma (traumatic arthritis (TA)), one with degenerative joint disease (DJD), and one with a chronic monoarticular arthritis (MA) of unknown aetiology. All specimens removed at the time of operation were placed in sterile saline and dissected to remove gross amounts of fat and bony debris. A representative section was placed in formalin for routine pathological examination. The remainder was stored at −80°C.

ELUTION PROCEDURE

The specimens were thawed and maintained at 4°C. throughout the elution sequence. Sodium azide was used to prevent bacterial growth. The synovial membrane was again dissected to remove all fat and other debris such as remnants of menisci, and washed in three changes of phosphate buffered saline (PBS), 0·15 M, pH 7·4, with constant stirring to remove all blood. The tissue was minced and then mechanically homogenized by using first a commercial blender (Waring Products Division, New Hartford, Connecticut), and then a polytron (Willens Polytron, Luzern, Switzerland). The homogenized membrane was washed six to ten times in ten volumes of PBS, 0·15 M, pH 7·4. Washes were concentrated by negative pressure dialysis to a total volume of 5 ml. and analysed for rheumatoid factors and antinuclear antibody activity as described below. When these tests were unequivocally negative, the homogenized membrane was eluted with ten volumes of 2 M NaCl, pH 7·0, for 10 hours with constant stirring. The eluates were dialysed against PBS, 0·15 M, pH 7·4, and concentrated to a final volume of 5 ml. by negative pressure dialysis.

ANALYSIS OF SYNOVIAL ELUTIONS

All specimens were placed in agar gel double diffusion against antisera monospecific for human IgG, IgA, IgM, beta-1-C, fibrinogen, and albumin (Hyland Laboratories, Los Angeles, California). Total protein was determined by the technique of Lowry, Rosebrough, Farr, and Randall (1951). IgG, IgA, and IgM were quantitated utilizing radial immunodiffusion plates (Kallestad Laboratories, Inc., Minneapolis, Minnesota). The antiglobulin activity of the heat-inactivated eluates (56°C. for 30 minutes) was

Accepted for publication September 20, 1971
Supported in part by USPHS Grant GM 15759 and by a grant from the Orthopedic Research and Education Foundation
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measured with latex particles coated with aggregated human IgG and sheep erythrocytes sensitized with rabbit gamma globulin. Heterophil antibody titres were performed simultaneously in microtitre plates using a 2 per cent. solution of unsensitized sheep erythrocytes. Antinuclear antibodies of the IgG, IgA, and IgM class were detected using a two-layer immunofluorescent technique (Barnett, Bienenstock, and Bloch, 1966). Human peripheral blood smears were incubated with 0·1 ml. eluate for 45 minutes. Fluorescein isothicyanate conjugated rabbit anti-human IgG and IgA were diluted 1:30 with PBS and the IgM diluted 1:10 with PBS before use in the test. All slides were incubated with conjugate for 45 minutes and read using a Zeiss fluorescent microscope. Immunoelectrophoresis was carried out using agar-coated slides, and rabbit antisera specific for whole normal human sera and for human IgG, IgA, and IgM. Aliquots of all elutions were also placed in 0·4 per cent. agarose and diffused against native and single-stranded calf thymus DNA, 500 μg./ml. (Worthington Biochemical Corp., Freehold, New Jersey).

Aliquots of each patient's whole sera and whole synovial fluid were also analysed by immunoelectrophoresis, quantitated for total protein and immunoglobulin content, and measured for antiglobulin and antinuclear antibody activities.

IgG SUBCLASSES AND LIGHT CHAINS IN SYNOVIAL MEMBRANE ELUATES

After the above analyses, synovial membrane eluates and matching whole synovial fluids containing IgG at a concentration of 1 mg./ml. were analysed semi-quantitatively (Tojo, Friou, and Spiegelberg, 1970) for the four IgG subclasses as well as for kappa and lambda light chain types (Kindly performed by Dr. Hans Spiegelberg, Scripps Institute, LaJolla, California).

Results

All three major immunoglobulin classes were detected in elutions of 22 or 24 synovial membranes. Two elutions showed complete abscence of any immunoglobulins; one was from a rheumatoid synovial membrane, and the other from the synovium of the patient with degenerative joint disease. IgG was most frequently detected, being present in 22 eluates in concentrations varying from 0·05 to 2·0 mg/ml. IgA was detected in thirteen eluates in concentrations of 0·03 to 0·39 mg./ml. IgM was present in thirteen eluates in concentrations of 0·02 to 0·8 mg./ml. Three elutions from rheumatoid synovial membrane contained beta-1-C detectable by agar gel diffusion. The synovial eluate from one of the traumatic arthritis patients also contained detectable beta-1-C; but, the eluant from this patient contained no antibody activity. Fibrinogen was not detected in any synovial membrane elutions. The total protein content of the synovial elutions ranged from 0·42 to 26·4 mg./ml.

Elutions from thirteen of the twenty rheumatoid synovial membranes (12 RA and 1 JRA) contained antibody activity. Antiglobulin activity only was detected in six eluates, ANA only was demonstrated in four synovial eluates, and both ANA and antiglobulin activities were found in three eluates. Quantities of immunoglobulins and total protein of the thirteen elutions in which antibody activity could be detected are recorded in Table I. The sixth wash of all synovial tissue was uniformly negative for detectable antibody activity and showed low levels of total protein, with a range of 0·026 to 0·069 mg./ml.

Antiglobulin activity was detected in the eluates from eight of eighteen rheumatoid synovial membranes, and in one of the JRA synovial membranes (Table II). Latex agglutination titres ranged between 1:80 and 1:40,960, whereas the sensitized sheep cell agglutination titres were between 1:8 and 1:128. In one patient (No. 5), antiglobulin activity was detected only in the synovial eluate, and not in the serum or in

Table I *Immunoglobulin levels in eluates from thirteen rheumatoid synovial membranes in which antibody activity was detected*

Diagnosis	*Patients*	*Synovial eluates (mg./ml.)*				*Synovial fluids (mg./ml.)*		
		γG	*IgA*	*γM*	*Total protein*	*γG*	*γA*	*γM*
RA	1	1·05	0·16	0·082	1·60	13·0	4·40	1·45
	2	0·07	0·03	0·027	0·69	13·5	4·80	3·50
	3	0·05	—	0·01	1·00	7·3	0·91	0·80
	4	0·75	—	0·03	ND	10·5	2·60	0·80
	5	0·01	—	0·01	5·80	4·3	0·24	1·50
	6	0·125	0·09	0·053	0·42	8·0	1·90	3·50
	7	0·56	0·11	0·07	1·04	7·2	ND	0·84
	8	0·078	—	0·01	1·00	4·3	0·31	0·80
	9	1·50	0·25	0·06	18·00	8·6	1·20	0·80
	10	0·47	0·07	0·052	26·40	10·0	ND	0·58
	11	0·48	0·04	0·053	13·40	ND	ND	ND
	12	0·70	0·39	0·06	1·83	7·8	2·0	0·84
JRA	1	2·00	0·29	0·18	1·88	7·3	1·3	0·66

— = undetectable; ND, not done; RA, rheumatoid arthritis; JRA juvenile rheumatoid arthritis.

Table II *Antiglobulin titres* in synovial eluates which demonstrated antibody activity*

Diagnosis	*Patients*	*Synovial eluates*	*Synovial fluids*	*Serum*
RA	1	—	—	—/8
	2	—	2,560/16	5,120/32
	3	—	2,560/128	160/—
	4	—	—	—
	5	320/0	—	—
	6	40,960/128	10,240/128	10,240/128
	7	320/8	—	80/8
	8	80/32	1,280/128	640/128
	9	1,280/16	1,280/128	160/—
	10	2,560/32	40,960/128	320/8
	11	160/8	—	640/8
	12	40,960/128	640/8	1,280/64
JRA	1	20,480/128	40,960/128	160/8

* Latex agglutination titre/sensitized sheep cell agglutination titre expressed as the reciprocal of titres
— = undetectable

the synovial fluid. In two others (Nos. 7 and 11), antiglobulin activity was present in the eluate and serum but absent in the whole synovial fluid. The titres of antiglobulins in the eluates were generally higher than the titres in the respective sera. There was no correlation between the antiglobulin titres detectable in the eluates and those in the whole synovial fluid.

Antinuclear antibodies of the IgG and IgM class were present in seven of eighteen rheumatoid synovial elutions, and were not detected in elutions from any of the other synovial membranes (Table III). IgG type ANA was present in six and IgM type ANA in four of the rheumatoid elutions. Antinuclear antibody titres of the IgM class ranged from 1:16 to 1:64. In two instances, the ANA was detectable only in the undiluted eluate. Antinuclear antibodies were present in four synovial elutions, but could not be detected in the patients' synovial fluid or sera (RA patients Nos. 1, 4, 10, and 12). In one other patient, the ANA was present in the serum and synovial eluate but was not detectable in the patient's synovial fluid (RA patient No. 11). IgA class ANA was not present in any of the elutions or whole synovial fluids, but was found in one patient's sera. There was no correlation between the presence or titre of antiglobulins and antinuclear antibodies. Some eluates with high titre rheumatoid factor had no detectable ANA. The converse was also observed. For example, RA patient No. 2 had high titres of ANA (type IgG and IgM) but no detectable antiglobulins in the eluate.

No paraproteins were detected in the sera, synovial fluids, or eluates. Albumin was detectable in sixteen of the eluates. None of the synovial membrane elutions showed any reaction when diffused against calf thymus DNA. Sufficient quantities of synovial eluates remained to determine the IgG subclass and light chain content of eight paired eluates and whole synovial fluids (7 RA patients and 1 JRA patient). IgG_1 was present in all specimens. IgG_2 was present in all but two RA eluates and in all the whole synovial fluids. IgG_3 was present in two rheumatoid synovial eluates but could not be detected in the patient's corresponding whole synovial fluid. This subclass was

Table III *Antinuclear antibody* in synovial eluates which demonstrated antibody activity*

Rheumatoid arthritis patients	*Synovial eluates*			*Synovial fluids*			*Serum*		
	γG	*γA*	*γM*	*γG*	*γA*	*γM*	*γG*	*γA*	*γM*
1	16	—	1	—	—	—	—	—	—
2	16	—	16	—	—	256	64	4	256
3	—	—	64	—	—	64	—	—	—
4	1	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—
6	—	—	—	16	—	64	—	—	—
7	—	—	—	—	—	—	—	—	—
8	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—	—
10	16	—	—	—	—	—	—	—	—
11	16	—	16	—	—	—	1	1	1
12	256	—	—	—	—	—	—	—	—

* Expressed as reciprocal of titre — = undetectable

also present in both the eluate and whole synovial fluid of two other RA patients and in only the whole synovial fluid of one JRA patient. IgG_4 was not detected. In all the eluates and in all but one of the matched whole synovial fluids, kappa light chains appeared to be present in greater amount than lambda chains.

Elutions from the seven rheumatoid synovial membranes (6 RA and 1 JRA) which showed no antibody activity contained low levels of IgG; but IgM was detected in only one eluate. Five of these patients had neither antiglobulins or ANA in their sera or synovial fluids. One had antiglobulin present in the serum and synovial fluid, and one had ANA present in both serum and synovial fluid, and antiglobulins in serum only. Elutions of the synovial membranes from the five remaining non-rheumatoid patients contained no detectable antibody activity despite the presence of eluted protein and immunoglobulins. One of the two patients with traumatic arthritis had a significant quantity of protein (87·3 mg./ml.) including all three immunoglobulin classes, and beta-1-C was present in the synovial membrane eluate; however, antinuclear antibody or antiglobulin activity was not detected.

Discussion

The results of this study indicate that rheumatoid synovial membrane frequently contains immunoglobulins possessing antiglobulin and antinuclear antibody activities. Furthermore, these antibodies appeared to be firmly fixed within the synovial membrane since they were eluted only after the tissue was treated with a buffer of high molarity.

Certain synovial eluates appeared to contain antibody activity selectively, in that the matched whole synovial fluid or serum from these patients showed no evidence of this same antibody activity. However, in most eluates which contained demonstrable antiglobulins or antinuclear antibody, similar activities were found in the patients' synovial fluid and/or serum. These observations suggest that certain antibodies may either be sequestered by the synovial membrane or be synthesized by the synovium. Alternatively, failure to detect antibody activity in the serum or synovial fluid despite its presence within the synovium may be a consequence of circulating inhibitors.

Antinuclear antibody was detected in the synovial eluates but not in the synovial fluids or sera of four patients, suggesting that these antibodies may have been synthesized or selectively sequestered within the synovial membrane. However, rheumatoid synovial fluid may contain DNA or DNA-like substances which could interfere with the detection of antinuclear antibody *in vitro* (Barnett, 1968). The presence of such an inhibitor in synovial fluid and its absence in the eluates may account for our findings. A similar mechanism may also explain why Munthe and Natvig (1970) were able to detect nuclear antigen but not antinuclear antibody in their eluants from rheumatoid synovial membrane. In a subsequent report, these authors (Munthe and Natvig, 1971) studied synovial eluates from 97 patients with RA and found only weak ANA activity in two. In our specimens, seven of eighteen rheumatoid synovial eluates contained ANA, one with a titre of 1:256. The reason for these somewhat divergent results is not apparent. Perhpas the 10-hour elution time we employed as compared to the 1-hour elution time of Munthe and Natvig (1971) resulted in greater amounts of protein being eluted. A second explanation is that ANA may have been complexed to antigen and that the longer exposure to high molarity buffer may have caused dissociation of these complexes.

In contrast to the antinuclear antibody, detection of antiglobulins in the synovial eluates usually correlated with the presence of antiglobulin activity in the synovial fluid. The more frequently observed elution sequence (detectable antiglobulin in synovial fluid, its absence in the washes of homogenized synovium, and its subsequent elution from synovium in high titre) suggests a nonspecific sequestration of antiglobulins by the synovial membrane. However, in three of the eluates containing antiglobulins, this activity was not found in the matching whole synovial fluid. Moreover, in three other eluates, the antiglobulin titres were greater than or equal to that of the matched whole synovial fluid, despite the fact that all eluates contained significantly lower concentrations of immunoglobulins than were present in the whole synovial fluid. These findings could imply either a selective sequestration of a high avidity antiglobulin by the synovium (Bluestone, Cracchiolo, Goldberg, and Pearson, 1970) or perhaps synovial synthesis of antiglobulins. An alternative and more likely explanation is that the failure to detect antiglobulin in synovial fluid despite its presence in the synovial eluates is due to the presence of some factor, such as IgG, in the synovial fluid which could inhibit the detection of antiglobulins *in vitro* (Hannestad, 1967). Indeed, the studies of Munthe and Natvig (1971) clearly demonstrated the presence of IgG complexes in eluates from rheumatoid synovial membrane, and other studies (Winchester and others, 1970) showed similar complexes in rheumatoid synovial fluid. Since these IgG complexes bind IgM antiglobulin, they may conceal antiglobulin activity (Cracchiolo, Bluestone, and Goldberg, 1970). The results of our study probably underestimate the frequency of antiglobulin in synovial fluid and eluates, since we did not search for these hidden antiglobulins. Munthe and Natvig (1971) found hidden antiglobulin in sixteen of 31 eluates from seronegative patients.

In an effort to delineate whether specific types of IgG immunoglobulins were present or had been

deposited within the synovial membrane, IgG subclasses and light chain classes were typed semi-quantitatively in eight eluates and matching synovial fluids. IgG_1 and IgG_2 subclasses were seen in almost all specimens; IgG_3 was detected in two rheumatoid eluates, but not in the whole synovial fluid of the same patients. The latter finding suggests that at least in certain individuals immunoglobulin deposition within the synovial membrane may be selective rather than random. Although IgG_1 and IgG_3, which are known to have complement-fixing activity, were present in the majority of the eluates, the beta-1-C component of complement was detected in only three rheumatoid synovial elutions. Previous studies have clearly shown that beta-1-C is often a component of the immune complexes in rheumatoid synovial membrane (Kinsella and others, 1970). Moreover, Lindström (1970) has shown the presence of beta-1-C in most of his rheumatoid synovial eluates, using the technique of agar gel immunoelectrophoresis. Our inability to detect beta-1-C in many of the synovial specimens may be due to the insensitivity of our method; alternatively, the complement components might not have been as firmly bound to the synovial membrane as the immunoglobulins, and were perhaps lost during the prolonged washing procedures which we employed before elution. Both kappa and lambda light chains were present in the eight eluates and whole synovial fluids which we tested. Kappa chains appeared to predominate in all eight eluates. In twenty eluates from rheumatoid synovial membranes (Lindström, 1970), quantitative determinations indicated that kappa light chains predominated in five eluates, and in seven eluates the same amount of each light chain was detected. However, in eight eluates, high levels of lambda type light chains were found.

The observations presented here, along with the reports of Lindström (1970) and Munthe and Natvig (1971), indicate that immunoglobulins with specific antibody activity are firmly bound to or contained within the synovial membrane in certain patients with rheumatoid arthritis. The presence of the antibodies, presumably in complex with their respective antigens, would be in accord with the current belief that rheumatoid synovitis is mediated *via* immune mechanisms.

Summary

Synovial membranes from eighteen patients with rheumatoid arthritis, two with juvenile rheumatoid arthritis, and four with other forms of arthritis were exposed to high molarity buffer in an effort to elute and characterize synovial immune factors. The synovial membranes were subjected to 2 M NaCl for 10 hours and the eluates were studied for the presence of antinuclear antibody and antiglobulins. Elutions from twelve rheumatoid membrane and one juvenile rheumatoid membrane contained antiglobulins and/or antinuclear antibodies. In certain patients the particular antibody activity present in the eluates was not detected in the patient's serum or whole synovial fluid. The majority of patients with antibody activity in their synovial eluates, however, also showed this activity in their synovial fluids. Antibody activity was not detected in the eluates from the four non-rheumatoid synovial membranes, although they did contain the three major classes of immunoglobulins. These findings suggest that rheumatoid synovial membranes may sequester immune factors from the synovial fluid or may perhaps synthesize immunoglobulins containing antinuclear antibody and antiglobulin activities.

The authors thank Miss Jo Ellen Cunningham for her skilled technical assistance.

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