***Corynebacterium acnes* in rheumatoid arthritis**

II. Identification of antigen in synovial fluid leucocytes

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The previous paper described the isolation of *Corynebacterium acnes* from blood, synovial fluid, and synovial tissue of patients with rheumatoid arthritis. Immunological studies showed that these patients had significantly lower titres of complement-fixing antibody to *C. acnes* than a normal control group, but that there was no difference in precipitating antibody to disrupted *C. acnes* organisms or to soluble antigens present in the culture medium.

One could postulate that in rheumatoid arthritis an immunological deficiency is present and subnormal or abnormal antibody response occurs to antigenic stimuli. Antigen excess might be predicted during exacerbations as with deoxyribonucleic acid in systemic lupus erythematosus (Tan, Schur, Carr, and Kunkel, 1966; Barnett, 1968; Schur and Sandson, 1968). This could explain the very low complement-fixing antibody titres.

To support this hypothesis, *C. acnes* antigen should be present in synovial fluid leucocytes from rheumatoid arthritis, having been phagocytosed as an immune complex with fixation of complement. These inclusion-containing leucocytes have been shown to contain complement, rheumatoid factor, IgG, and nucleoprotein, presumably as antigen-antibody complexes (Rawson, Abelson, and Hollander, 1965; Zvaifler, 1965; Barnhart, Riddle, and Bluhm, 1967; Brandt, Cathcart, and Cohen, 1968; Vaughan, Barnett, Sobel, and Jacox, 1968; Vaughan, Jacox, and Noell, 1968; Zvaifler, 1970). The low levels of complement in rheumatoid synovial fluid have been explained by its fixation in the formation of various immune complexes, including rheumatoid factor-IgG complexes (Fostiropoulos, Austen, and Bloch, 1964; Pekin and Zvaifler, 1964; Hedberg, 1967; Sonozaki and Torisu, 1970). Fibrin and its breakdown products also play a role in the process (Barnhart and others, 1967). Specific antigens of micro-organisms have not been reported. This paper will describe the identification of *C. acnes* antigen in rheumatoid synovial fluid leucocytes.

Material and methods

A number of procedures were used to extract antigens from whole washed *C. acnes* organisms. Methanol and hot phenol-water extraction procedures were found to be satisfactory, yielding several distinct precipitin lines on double gel diffusion plates. These extracts are probably carbohydrate- or lipid-containing antigens, or lipopolysaccharides. The same methods were then used to extract antigens from synovial fluid leucocytes and from synovial fluid.

Synovial fluid was obtained by routine arthrocentesis under sterile conditions. Cells were separated by centrifugation as soon as possible and washed three times with buffered saline. If the fluid was viscous, it was first treated with hyaluronidase to facilitate separation of cells.

After the third wash, cells were suspended in 10 ml. normal saline. 1 ml. was saved for protein determination by the method of Oyama and Eagle (1956). The remaining 9 ml. were divided equally for two extraction methods.

METHANOL EXTRACTION

The cell button was dried by rotary evaporation and extracted twice with 10 ml. methanol. Methanol fractions were combined and desiccated by rotary evaporation. The residue was dissolved in 1 ml. normal saline. 2 ml. of the supernatant synovial fluid of each specimen were dried by lyophilization and extracted with methanol as described above.

PHENOL-WATER EXTRACTION

The procedure used is a modification of that of Westphal and Jann (1965) described by Osborn, Rosen, Rothfield, and Horecker (1962) for the extraction of lipopolysaccharide bacterial antigen. Synovial cells were washed three times with normal saline and then extracted twice with 45 per cent phenol in water at 68°C. for 20 minutes. Each extract was cooled to 4°–10°C. and centrifuged at 2,000 r.p.m. The aqueous layers were pooled and washed with sufficient ether to remove the remaining phenol. After removal of the ether, the water phase was rotary-evaporated to dryness and the residue dissolved in 1 ml. normal saline.

2 ml. of the supernatant synovial fluid of each specimen were dried by lyophilization and extracted with phenol-water as described above.

COMPLEMENT-FIXATION AND DOUBLE GEL DIFFUSION STUDIES

Cell and synovial fluid extracts (phenol-water and methanol) were used as antigens in complement-fixation and double gel diffusion tests using the methods described in the previous paper.

The cell and synovial fluid extracts were diluted 1:4, 1:8, 1:16, and 1:32 and tested against *C. acnes* rabbit antibody by the microtitre complement-fixation method. The greatest dilution of antibody giving a 2+ or greater reaction was recorded. The extracts were used as antigens in Ouchterlony gel diffusion plates and reacted against the same antiserum for precipitating antibody.

Rheumatoid factor titres were determined on the supernatant synovial fluid specimens by the method of Singer and Plotz (1956). Total haemolytic complement (CH_{50}) levels were also determined on the synovial fluids. The method of Kent and Fife (1963) as modified by Pekin and Zvaifler (1964) was used. Normal values for sera are 125–200 units.

Results

The original group of synovial fluid leucocytes which were extracted by methanol and tested by complement-fixation against rabbit *C. acnes* antibody are shown in Table I; 81 per cent. of the rheumatoid cell extracts were positive, 60 per cent. in titres of 1:16 or greater, some being as high as 1:1024.

Table I Synovial fluid leucocytes—rheumatoid methanol extraction

Leucocytes	Complement-fixation titre*			
	0	1:2–1:8	1:16–1:64	1:128 >
No.	9	10	17	11
Per cent.	19	21	36	24

* Extracts tested against anti-*Corynebacterium acnes* serum.

Table II shows the results for nonrheumatoid synovial cells extracted with methanol. Only one

specimen of twenty (5 per cent.) was positive. The control group included patients with degenerative arthritis, gout, pseudogout, and ankylosing spondylitis.

Table II Synovial fluid leucocytes—nonrheumatoid methanol extraction

Leucocytes	Complement-fixation titre*			
	0	1:2–1:8	1:16–1:64	1:128 >
No.	19	0	1	0
Per cent.	95	0	5	0

* Extracts tested against anti-*Corynebacterium acnes* serum.

Because many of the rheumatoid effusions had higher concentrations of leucocytes than the control group, additional studies were carried out in which the protein content of the cells was determined. The supernatant synovial fluid complement levels and latex-fixation titres were determined in addition to the two procedures to extract *C. acnes* antigen.

Table III shows the results of the combined extraction procedures, using both complement-fixation and gel diffusion for identification of *C. acnes* antigen. In each case the extracts were used as antigens and reacted against rabbit *C. acnes* antibody. 88 per cent. of methanol rheumatoid cell extracts were positive by complement-fixation and none by the precipitin test. Only 12 per cent. of the supernatant synovial fluid methanol extracts were positive by complement-fixation. On the other hand, phenol-water extracts gave positive precipitin reactions in one-fourth of the cell extracts and in only 4 per cent. of the supernatant fluid extracts.

Nonrheumatoid cell and synovial fluid extracts are shown in Table IV (overleaf). These include diagnoses of degenerative arthritis, gout, pseudogout, ankylosing spondylitis, and an acute viral infection. The numbers are small in the second series, but all were negative for *C. acnes* antigen.

Table V (overleaf) shows the synovial fluid complement levels in the latex positive and latex negative rheumatoid cases and the nonrheumatoid controls.

Table III Synovial fluids and leucocytes—rheumatoid

Extraction	Methanol*						Phenol-water*								
	Complement-fixation				Gel diffusion		Complement-fixation				Gel diffusion				
	Pos.		Neg.		Pos.	Neg.	Pos.		Neg.		Pos.	Neg.			
	%	No.	%	No.	%	%	%	No.	%	No.	%	No.			
Cells	88	22	12	3	0	100	25	25	6	75	18	25	6	75	18
Fluid	12	3	88	22	0	100	25	17	4	83	20	4	1	96	23

* Extracts tested against anti-*Corynebacterium acnes* serum.

Table IV *Synovial fluids and leucocytes—nonrheumatoid*

Extraction	Methanol*				Phenol-water*			
	Complement-fixation		Gel diffusion		Complement-fixation		Gel diffusion	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
		% No.		% No.	% No.	% No.	% No.	% No.
Cells	0	100 7	0	100 7	0	100 9	0	100 9
Fluid	0	100 7	0	100 7	22 2	78 7	11 1	89 8

* Extracts tested against anti-*Corynebacterium acnes* serum.

Table V *Synovial fluid complement levels and rheumatoid factor*

Rheumatoid factor	C ₅₀ units		
	0-20	21-40	41 >
Positive	13	2	1
Negative	3	0	4

As in other studies, rheumatoid factor positive fluids have lower synovial fluid complement levels than factor negative fluids.

Since the total number of cells used for extraction varied a great deal, the possibility existed that *C. acnes* antigen could be ubiquitous and that its apparent presence in rheumatoid synovial leucocytes was due only to the greater number of cells present in the inflammatory exudate. Table VI compares the total protein of the cells extracted (function of total cell count) with presence of *C. acnes* antigen. Again, although the number of nonrheumatoid cells is small, it is apparent that *C. acnes* antigen may be found in rheumatoid extracts having low total protein (0.1 to 1 mg.) and that nonrheumatoid cell concentrates with greater protein content may be negative for this antigen.

The majority of rheumatoid synovial fluid cells studied contained the antigen, were latex positive and had low complement levels. Only four of the 25 rheumatoid extracts were latex negative. Three of these contained *C. acnes* antigen. There is thus no apparent correlation between the presence of antigen and rheumatoid factor.

Table VI *Cell protein and Corynebacterium acnes antigen*

Cells	Corynebacterium acnes antigen	Cell protein (mg.)		
		0.1-1.0	1.1-10	11-100
Rheumatoid	Present	2	11	10
	Absent	1	2	0
Nonrheumatoid	Present	0	0	0
	Absent	4	4	1

IDENTIFICATION OF *Corynebacterium acnes* ANTIGEN

Seven high-titred extracts were tested against the pre-immunized rabbit serum and against rabbits immunized with *Mycoplasma hominis* I and *Staphylococcus aureus* to show antigen specificity of the cell extracts (Table VII, opposite). Titres were very low in each instance except to the *C. acnes* antibody.

Cell extracts which developed precipitin lines against *C. acnes* antibody were tested for specificity by developing lines of identity with *C. acnes* antigen. The Figure (opposite) shows a phenol-water extract of synovial leucocytes from a rheumatoid patient demonstrating a line of identity with *C. acnes* antigen. No lines of identity occurred with the following organisms in the same concentration: *Escherichia coli*, *S. aureus*, *Proteus mirabilis*, *Pseudomonas vulgaris*, *Streptococcus viridans*, *St. pyogenes*, *M. hominis* I, *M. hominis* II, *M. salivarium*, and *M. hyorhinis*.

Rabbit *C. acnes* antibody was absorbed with *C. acnes* organisms and tested by gel diffusion against cell extracts which gave precipitin lines. No precipitin lines appeared, indicating complete absorption and removal of *C. acnes* antigen.

Discussion

This study has shown that an antigen(s) identical to *C. acnes* antigen is present in the synovial fluid leucocytes of rheumatoid patients. Studies have not so far shown this to be present in other types of arthritis.

That this is a specific antigen present only in *C. acnes* is suggested by the fact that it was not found in a

Table VII Complement-fixation titre

Synovial Cell Extract	Rabbit antisera to:			
	<i>C. acnes</i>	Pre-immune	<i>M. hom. I</i>	<i>Staph. aureus</i>
BK	>1:64	1:2	1:4	0
CB	>1:64	1:4	1:8	1:16
MJ	>1:64	1:4	1:2	1:8
FM	>1:64	0	0	0
RK	>1:64	0	0	0
EP	>1:64	1:2	1:2	1:2
CN	1:64	0	1:2	1:2
<i>C. acnes</i>	1:2048	0	0	0
<i>M. hom. I</i>	0	0	1:128	0

FIG. 1 Double gel diffusion, showing line of identity between synovial fluid leucocyte extract and *Corynebacterium acnes* antigen.
C.E. = phenol-water cell extract
C. acnes = disrupted bacterial antigen
Anti-C. acnes = rabbit antiserum

number of other bacteria or mycoplasma, but this does not completely eliminate the possibility of an antigen common to a number of micro-organisms. It is known that *C. acnes* is present normally in deep skin structures. It can be pathogenic and has been associated with acne vulgaris (Puhvel, Barfatani, Warnick, and Sternberg, 1964), subacute bacterial endocarditis (Wittler, Malizia, Kramer, Tuckett, Pritchard, and Baker, 1960; Reid and Greenwood, 1967; Zierdt and Wertlake, 1969), septicaemia, meningitis, and abscesses (Johnson and Kaye, 1970). In a few of these reports, antibody titres were studied and found to be elevated. Antibody titres by comple-

ment-fixation tests were very low or absent in the rheumatoid specimens studied (Bartholomew and Nelson, 1972). Conceivably, antibody to *C. acnes* is depressed because it is continually being complexed with complement and antigen. The form in which *C. acnes* antigen is present in synovial fluid leucocytes is not known. Possibilities include: bacterial form (active or inactive), L-form or a transitional phase as described by Zierdt and Wertlake (1969), or a soluble antigen. Presence of the bacteria itself or of L-form is unlikely, as this should be readily identifiable by electron microscopy in synovial tissue or synovial fluid leucocytes

and this has not been reported. L-forms or transitional forms of *C. acnes* may be present, in other tissues or in the blood stream, as a persisting source of antigen. Many of the positive cultures reported in the preceding paper (Bartholomew and Nelson, 1972) appeared first in this form. Most likely, the antigen present in the synovial fluid leucocytes is a soluble noninfective antigen which can form antigen-antibody complexes and fixes complement.

The successful extraction methods used to isolate and concentrate the antigen favour a carbohydrate or lipid-containing antigen. The hot phenol-water extraction has been used to extract lipopolysaccharide bacterial antigen including endotoxin. Preliminary studies have shown the antigen to be pronase resistant and unaffected by boiling for 10 minutes, indicating that the antigenic sites are probably not proteins. Studies are in progress to further identify the antigens.

Summary

Present theories of the pathogenesis of joint inflammation in rheumatoid arthritis favour the concept of

phagocytosis of immune complexes which have fixed complement as basic to the process. If microorganisms are responsible for a minor or major role in this disease, their specific antigens might be present in the immune complexes phagocytosed by synovial fluid leucocytes using conventional biochemical methods to extract carbohydrate or lipid-containing antigens.

This study shows that *C. acnes* antigen can be extracted in 88 per cent. of rheumatoid synovial fluid leucocytes using conventional biochemical methods to extract carbohydrate or lipid-containing antigens. Nonrheumatoid cells have been positive in only one of the twenty tested.

Specificity of this leucocyte antigen is shown by gel diffusion precipitin lines of identity with disrupted *C. acnes* organisms. While this antigen has not been found in a number of common bacteria or mycoplasma, the possibility remains that it could be present in other microorganisms.

C. acnes is a ubiquitous anaerobic diphtheroid which can be pathogenic. It is conceivable that rheumatoid patients are unable to produce effective or sufficient antibody to neutralize or eliminate this antigen and to allow formation of soluble immune complexes.

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