Corynebacterium acnes in rheumatoid arthritis

I. Isolation and antibody studies

L. E. BARTHOLOMEW* and F. R. NELSON

From the Rackham Arthritis Research Unit and Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan

Numerous studies have reported the isolation of various micro-organisms from patients with rheumatoid arthritis (Hill, 1968). Several unrelated organisms have been isolated including various bacteria (Schueller, 1906; Rosenow, 1914), Bedsonia (Schachter, 1967), and mycoplasma (Arai, Ishikawa, and Hotta, 1964; Bartholomew, 1965; Williams, 1968). The isolation of diphtheroid organisms from synovial fluid, synovial tissue and blood in rheumatoid arthritis has been reported (Cadham, 1942; Duthie, Stewart, and Dayhoff, 1967; Hill, McCormick, Greenbury, Morris, and Kenningale, 1967; Alexander, Stewart, and Duthie, 1968; Bartholomew, 1968; Stewart, Alexander, and Duthie, 1969). Exact identification of the isolated strains was not made. Complement-fixing antibody was not found in the sera of twenty rheumatoid patients (Stewart and others, 1969).

The significance of diphtheroid bacteria found in rheumatoid patients is uncertain. These organisms are ubiquitous and commonly present in deep structures of skin. Therefore the possibility that they represent contaminants introduced at the time of sampling has to be considered. Other interpretations suggest that their presence in tissue, blood, and fluid may be of no pathogenic significance, or that they could be directly related to rheumatoid arthritis aetio logically.

The theory that a common organism (antigen) could provoke and perpetuate rheumatoid arthritis is attractive. This could imply a deficiency or abnormality in the immunological mechanism of rheumatoid patients whereby antibody was ineffective or insufficient to neutralize specific antigen. Antibody directed against antigen–antibody complexes could produce autoantibodies such as rheumatoid factor. Thus, in rheumatoid arthritis, the two mechanisms of chronic infection and autoimmunity could be harmonized into a unified concept.

This study confirms the reports of isolation of diphtheroid organisms (Corynebacterium acnes) from rheumatoid patients (Duthie and others, 1967; Alexander and others, 1968; Stewart and others, 1969). It appears from this study that a number of the isolates first appear as L-forms and may revert to the typical bacterial or cell-wall phase. In addition, results of complement-fixing and precipitating antibody in rheumatoid patients and controls are presented. The following paper describes the identification of a specific bacterial antigen of C. acnes in synovial fluid leucocytes.

Material and methods

CULTURE MEDIA

The culture media used was that described for the isolation of L-forms of bacteria (Gutman, Turck, Petersdorf, and Wedgwood, 1965). It contains the following: (g/l) sucrose 100-0, phytone 20-0, NaCl 5-0, MgSO4·4H2O 2-5, yeast extract 10-0, cholesterol 0-04, dissolved in 10 ml 95 per cent. ethanol. The medium was modified by substituting 20-0 g. gelatin (Difco) for 8-0 g. Ionagar. The pH was adjusted to 7-8 with 0-5N NaOH. For blood and synovial fluid cultures, 35 ml medium was autoclaved for 1 hr at 121°C. in Type I 50-ml rubber-sealed bottles. Before use 10 per cent. sterile, heat-inactivated horse serum was added by sterile syringe.

For synovial tissue cultures, 10 ml medium was autoclaved in 20-ml screw-cap culture tubes. Ten per cent. horse serum was then added. Complete medium was incubated for one week before use at 37°C. All tubes and bottles were then inspected for turbidity. Ten per cent. of the uninoculated tubes and bottles were subcultured and aliquots were examined under ×1,000 magnification after Giemsa staining. If contamination was present the entire lot was discarded. Uninoculated control medium was included with every set of inoculated bottles or culture tubes.

Solid medium contained 1-5 per cent. agar (Nobel's special agar) and no gelatin. Horse serum was added when the autoclaved medium had cooled to 40–45°C. Bacterial
forms were subcultured in standard brain heart infusion (BHI) broth prepared in tubes and bottles. Standard blood agar plates were used to detect haemolysis and routine fermentation reactions were performed on actively growing subcultures. No antibiotics or inhibitory substances were added.

**BLOOD AND SYNOVIAL FLUID CULTURES**

Approximately 5 ml. blood or synovial fluid were added directly to the culture bottles from the syringe at the time of venepuncture or arthrocentesis. The skin was previously cleaned with pHisohex, iodine, and alcohol. The rubber caps on the culture bottles were cleaned with iodine.

**SYNOVIAL TISSUES**

Specimens obtained in the operating room were placed in sterile dry bottles at the time of synovectomy. The tissue was cut into small fragments with sterile scissors in a tissue culture cubicule provided with forced draft sterile air. Aliquots were inoculated into tubes containing culture medium.

All cultures were incubated for 8 weeks at 37°C. The screw-cap tubes and solid medium were incubated in candle jars. Type I bottles were incubated aerobically but were sealed. At weekly intervals aliquots were removed from the original cultures under sterile conditions for the following: Giemsa stain, subculture into L-form medium, BHI (liquid and solid), and blood agar.

**ANTIGEN PREPARATION AND RABBIT ANTIBODY**

Two prototype strains of *C. acnes* isolated from blood cultures were used to inject rabbits. Antigens were prepared by growing the organisms in 500 ml. BHI medium for 5 days on an oscillating platform. The bacteria were harvested by centrifugation and washed three times in buffered saline. The final concentration was adjusted to McFarland Tube 10. The original supernate was saved for soluble antigen preparations.

**Injection schedule**

1 ml. antigen was homogenized with 1 ml. complete Freund’s adjuvant and injected subcutaneously at three sites and intramuscularly at two sites. This schedule was repeated weekly for 4 wks at which time high-titred complement-fixing (1:4096) and precipitating antibody were present. Thereafter booster injections were given as necessary.

**COMPLEMENT-FIXATION TESTS**

A concentrated bacterial antigen was prepared from I 1. of a 5-day culture of each of the above strains and prepared as above. Bacterial cells were disrupted by means of a French pressure cell. This stock antigen was used for both complement-fixation and double gel diffusion tests.

For complement-fixation tests the microtitre method was employed (Sever, 1962). Antigen was titred against rabbit antibody and 1:200 dilution of the stock antigen was satisfactory for routine testing. Buffer for complement-fixation tests consisted of the following 10X stock solution per litre, which was diluted with distilled water: NaCl 87-66 gm., triethanolamine 28 ml., 1N HCl 162 ml., 0-03M CaCl, 50 ml., 0-2M MgCl, 6H2O 50 ml. The pH was adjusted to 7-4 ± 0-1 (with HCl or NaOH).

**PRECIPITATING ANTIBODY**

Double diffusion gels were performed by the Ouchterlony technique on glass microscope slides covered with 1 per cent. ionagar 2. Antigen for the centre well was an undiluted disrupted *C. acnes* suspension. Various sera and synovial fluids were used in outer wells. Specimens giving a precipitin line were checked for identity with known *C. acnes* antigen using high titre undiluted rabbit antibody. Gels were incubated in a humidifying chamber and precipitin lines were recorded as they appeared for a 5-day period. They were washed for 24 hrs in saline, followed by 3 hrs in distilled water, then air-dried and stained with Amido-schwartz.

**PARTIAL PURIFICATION OF SOLUBLE ANTIGEN IN CULTURE MEDIUM**

Corynebacteria were grown in BHI medium for 5 days. They were then centrifuged at 12,000 r.p.m. for 15 min. The supernate was saved and dialysed against distilled water for 24 hrs with three changes. The dialysed supernate was lyophilized and resuspended in normal saline (25 mg./ml.). The processed supernate was chromatographed on a Sephadex G75 column 70 cm. long and 2-5 cm. in diameter. The column was eluted with normal saline and 5-ml. fractions were collected. Fractions containing antigen were detected by double gel diffusion, testing each aliquot against rabbit antibody. Antigen appeared at the end of the void volume and in the first fifteen 5-ml. fractions.

**IDENTIFICATION OF CORYNEBACTERIUM ACNES**

Initial identification of diphtheroids was made by their characteristic morphological appearance using Gram and Giemsa stains of cultures showing turbidity. Four strains isolated from rheumatoid patients were sent to Dr. W. E. C. Moore, Professor of Microbiology, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, and were identified by him as *Propionibacterium acnes* (*C. acnes*) by means of multiple biochemical reactions and acid fermentation products separated by silicic acid and gas chromatography.

In general, the various isolates were microaerophilic or anaerobic, Gram-positive, and did not haemolyse blood agar. They fermented maltose, mannitol, and glucose, and digested gelatin. About half were producers of catalase.

High-titre rabbit antibody was developed against two of these strains. Subsequent isolates were characterized by their morphological appearance, biochemical reactions, and double gel diffusion lines of identity with prototype strains. No precipitin lines occurred with other Corynebacteria, including *C. xerosis*, *C. pseudodiphtherium*, *C. hoffmani*, and a number of diphtheroids obtained from the clinical bacteriology laboratory. Likewise, strains of *Staphylococcus aureus*, *Streptococcus pyogenes*, Enterobacteria coli, *Pseudomonas aeruginosa*, and Proteus vulgaris showed no precipitin lines; nor did four strains of mycoplasma (*M. hominis I, M. hominis II, M. pneumoniae*, and *M. hyorhinis*).

**Results**

**Isolation of Corynebacterium acnes**

The isolation rate of *C. acnes* from various sources is
shown in Table I; 13 per cent. of 195 rheumatoid blood cultures were positive, while only 2 per cent. of 46 control blood cultures were positive. This difference is statistically significant: \( P = 0.049 \) by the \( \chi^2 \) method. Synovial fluid cultures were positive in 6 per cent. of 130 rheumatoid samples and in none from 22 cases of degenerative arthritis. Rheumatoid synovial tissue obtained during surgical synovectomies were positive in 43 per cent. of fourteen cultured.

**Table I Isolation of Corynebacterium acnes**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Source</th>
<th>Positive No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid arthritis Control*</td>
<td>Blood</td>
<td>13 per cent. (25) 195†</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Synovial fluid</td>
<td>2 per cent. (1) 46†</td>
</tr>
<tr>
<td>Degenerative joint disease</td>
<td>Synovial fluid</td>
<td>6 per cent. (8) 130</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Synovial tissue</td>
<td>0</td>
</tr>
</tbody>
</table>

* Controls include normal subjects, gout, and degenerative joint disease. † \( P = 0.049 \).

**Antibody studies**

Results of antibody levels determined by complement-fixation tests of 100 specimens each of rheumatoid sera, synovial fluid, and normal sera are shown in Table II; 44 per cent. of control sera had titres of 1:8 or higher, while only 14 per cent. of rheumatoid sera had titres of 1:8 or above. The \( P \) value for this data is less than 0.0001 using the \( \chi^2 \) formula and is thus highly significant. Rheumatoid synovial fluid titres were somewhat lower than rheumatoid sera but these were not matched pairs.

**Table II Complement-fixation tests Corynebacterium acnes antigen**

<table>
<thead>
<tr>
<th>Material</th>
<th>Titre (per cent.)</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-1:4</td>
<td>1:8-1:32</td>
</tr>
<tr>
<td>Rheumatoid sera</td>
<td>86</td>
<td>13</td>
</tr>
<tr>
<td>Rheumatoid synovial fluid</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>Control sera</td>
<td>56</td>
<td>32</td>
</tr>
</tbody>
</table>

* \( P = <0.0001 \).

Precipitating antibody as determined by double gel diffusion using the same specimens are shown in Table III. The antigen present in the BHI medium was more reactive than the disrupted bacterial antigen. However, there was no significant difference between rheumatoids and controls.

**Table III Precipitating antibody gel diffusion**

<table>
<thead>
<tr>
<th>Material</th>
<th>Antigen (per cent.)</th>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacterial*</td>
<td>Medium†</td>
</tr>
<tr>
<td>Rheumatoid sera</td>
<td>10</td>
<td>47</td>
</tr>
<tr>
<td>Rheumatoid synovial fluid</td>
<td>9</td>
<td>33</td>
</tr>
<tr>
<td>Control sera</td>
<td>9</td>
<td>56</td>
</tr>
</tbody>
</table>

* Bacterial antigen—disrupted by French pressure cell. † Medium antigen—in culture medium partially purified by Sephadex G-75.

**Isolation of other organisms**

There was no difference between rheumatoid and non-rheumatoid specimens in the rate of isolation of other organisms. Approximately 5 per cent. of all cultures grew out other organisms divided about equally among staphylococci, Gram-positive bacilli, and yeasts.

**Apparent isolation of L-forms**

Approximately 40 per cent. of the rheumatoid cultures appeared to have L-forms present when Giemsa-stained preparations of cultures were viewed under \( \times1,000 \) magnification. Fig. 1 shows the appearance of these forms at 1 week. They appear as clumps of small granular or spherical forms. Fig. 2 (opposite) is from the same culture 10 days later, showing the apparent reversion to the typical bacterial forms of diphtheroids. In no instance were we able to grow the variants on solid medium in typical L-form colonies, or in various liquid media.
Discussion

Diphtheroid type bacilli have been reported by other workers in association with rheumatoid arthritis (Duthie and others, 1967; Alexander and others, 1968; Stewart and others, 1969). The isolation rates from rheumatoid specimens were not significantly different from the present study; they isolated diphtheroids from 8 per cent. of blood cultures, 10 per cent. of skin samples, and 27 per cent. of synovial fluids.

Others have isolated diphtheroids from lymph nodes of rheumatoid patients (Cadham, 1942) and from patients with lymphoreticular neoplasms (Wuerthele-Caspé, Alexander-Jackson, Anderson, Hillier, Allen, and Smith, 1950; Fleisher, 1952; Alexander-Jackson, 1954).

Corynebacterium acnes is commonly found in deep skin structures and could easily contaminate blood or synovial fluid obtained by introducing a needle through skin. However, the significantly increased isolation rate of blood cultures from rheumatoid patients would imply that other factors are involved. The highest isolation rate was from rheumatoid synovial tissue obtained during surgery. Skin contaminants would be unlikely in these specimens, as a second set of surgical instruments is used after skin incisions are made and wound edges retracted under sterile drapes.

Additionally, all of the diphtheroid type isolates were identified as belonging to a single species (C. acnes) rather than to a heterogeneous group of diphtheroids.

The possibility that the transitional or L-form growth of C. acnes may be involved is supported by finding apparent L-forms in rheumatoid cultures and their reversion to cell-wall forms. Unfortunately, L-form antigens could not be developed. The role of transitional forms and L-forms of organisms in disease is not well defined, but there is evidence that this type of growth may be important in latent or recurrent infections, as in septicemia (Wittler, Malizia, Kramer, Tuckett, Pritchard, and Baker, 1960; Mattman and Mattman, 1965), subacute bacterial endocarditis (Rosner, 1966; Zierdt and Wertlake, 1969), and urinary tract infections (Gutman and others, 1965; Conner, Coleman, Davis, and McGaughey, 1968). The isolation of the L-form of a diphtheroid-like bacteria in a very high percentage of blood cultures from rheumatoid patients has been described (Pease, 1969, 1970). Alexander and others (1968) inoculated diphtheroids in bacterial form into cultures of rheumatoid synovial cells. The bacteria became intracellular and lost their cell-wall. Conceivably intracellular infection with transitional or L-forms of organisms could provide the chronic low-grade antigenic stimulus responsible for the 'autoimmune' feature of rheumatoid arthritis.

Studies of circulating antibody by complement-fixation against this C. acnes strain showed very low titres in both rheumatoid sera and synovial fluid. However, precipitating antibody to both the whole disrupted organism and to partially purified antigens in culture media revealed little difference between rheumatoid subjects and controls. This suggests that complement-fixing antibody is either rapidly consumed or underproduced in rheumatoid arthritis.

Several studies are reported indicating abnormalities in the immune mechanism of rheumatoid patients. The frequent increase of immunoglobulins...
in rheumatoid arthritis, particularly IgG and IgM, suggests an immunological abnormality (Hong and West, 1964; Claman and Merrill, 1966). However, others demonstrated in rheumatoid patients an IgM deficiency in the immune response to Brucellin, sheep red cell stroma, and Limulus polyphemus haemocyanin (Bandilla, Pitts, and McDuffie, 1970). Rheumatoid factor titre increased in rheumatoid patients receiving antigenic stimulation, suggesting a non-specific antibody stimulation. Lower antibody titres to blood groups A and B were reported in the 7S and 19S classes in rheumatoid arthritis (Rawson, Abelson, and McCarty, 1961; Rawson and Abelson, 1964), but no difference was found in IgG antibody response to tetanus toxoid (Vaughan and Butler, 1962). IgA deficiency has been reported in 3 per cent. of cases of juvenile rheumatoid arthritis (Cassidy and Burt, 1968).

The results of these studies suggest that rheumatoid patients might not be capable of handling chronic antigenic stimuli from *C. acnes* in the usual fashion. In particular, the very low complement-fixing antibody levels in both sera and synovial fluid might indicate that antigen excess was present or that a particular defect in antibody response occurs in rheumatoid patients.

Support for this hypothesis requires that *C. acnes* antigen be identified in serum, synovial fluid, synovial fluid leucocytes, or in the synovial tissue of rheumatoid patients, and that it be absent in other forms of arthritis and in normal subjects.

Present theories of the pathogenesis of chronic inflammation in rheumatoid joints suggest that antigen–antibody complexes present in synovial fluid play a major role (Rawson, Abelson, and Hollander, 1965; Zvaifler, 1965, 1970; Barnhart, Riddle, and Bluhm, 1967; Brandt, Cathcart, and Cohen, 1968; Vaughan, Barnett, Sobel, and Jacox, 1968; Vaughan, Jacox, and Noell, 1968). These complexes which have been shown to contain rheumatoid factor, IgG, and complement are phagocytosed by leucocytes. Therefore, if a specific antigen is present, it should be identifiable in washed synovial fluid leucocytes. The following paper describes the identification of *C. acnes* antigen in synovial fluid leucocytes of rheumatoid patients.

### Summary

*Corynebacterium acnes*, an anaerobic diphtheroid bacillus, has been isolated from 13 per cent. of 195 rheumatoid blood cultures, 6 per cent. of 120 synovial fluid cultures, and 43 per cent. of fourteen synovial tissue cultures. Non-rheumatoid blood cultures were positive in 2 per cent. of 46 and none of 22 synovial fluid cultures. The enriched hypertonic culture medium employed favoured growth of L-form organisms and this type of growth was commonly noted during early cultures.

Studies of circulating antibody to this organism showed that complement-fixing antibody was absent or very low in rheumatoid serum and synovial fluid, while precipitating antibody by double gel diffusion showed no difference between rheumatoid subjects and controls.

The significance of these results is discussed in terms of possible pathogenetic and immunological mechanisms.

We wish to thank Emil Jebe, Ph.D., for his assistance in the statistical analyses. This work was supported in part by USPHS Grant AI06729 and by the Michigan Chapter, Arthritis Foundation.

### References


BRANDT, K. D., CATHCART, E. S., AND COHEN, A. S. (1968) *J. Lab. clin. Med.*, 72, 631 (Studies of immune deposits in synovial membranes and corresponding synovial fluids)


Corynebacterium acnes in rheumatoid arthritis


Fleisher, M. S. (1952) Amer. J. med. Sci., 224, 548 (Significance of diphtheroid microorganisms in blood cultures from human beings)


Hong, R., and West, C. D. (1964) Arthr. and Rheum., 7, 128 (Gamma-1 globulin levels in rheumatic fever)


—— (1970) Ibid. 29, 439 (Morphological appearances of a bacterial L-form growing in association with the erythrocytes of arthritic subjects)


——, ——, and McCarty, D. J. (1961) Arthr. and Rheum., 4, 463 (The isohemagglutinin partition in rheumatoid arthritis)


Schueller, M. (1960) Arch. med. Sci., 43, 231 (The relations of chronic villous polyarthritis to the dumb-bell shaped bacilli)


——, Jacox, R. F., and Noell, P. (1968) Arthr. and Rheum., 11, 135 (Relation of intracytoplasmic inclusions in joint fluid leukocytes to anti-γG globulins)


—— (1970) Ibid., 13, 895 (Further speculation on the pathogenesis of joint inflammation in rheumatoid arthritis)