RHEUMATOID ARTHRITIC AND NON-RHEUMATOID SYNOVIUM IN CELL CULTURE*

MORPHOLOGICAL OBSERVATIONS, ACRIDINE ORANGE, AND FLUORESCENT FRACTION II STUDIES

BY

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Cultures *in vitro* of synovial membranes and synovial cells from patients with rheumatoid arthritis have been reported to show changes not seen in control cultures. Multinucleated cells were more numerous (Stanfield and Stephens, 1961; Lackington, 1959), a greater degree of aneuploidy occurred (Castor, 1960), and epithelial transformation was observed (Castor, Prince, and Dorstewitz, 1961).

The pathogenesis of rheumatoid arthritis may be a virus infection that triggers off or produces an abnormal immune mechanism, which in turn affects synovial tissue. A virus might also directly affect synovial cells and in the established disease might exist in a latent form. Latent virus-infected tissues *in vivo*, when propagated *in vitro*, may show spontaneous degeneration and possibly the development of inclusion bodies. Rowe, Huebner, Gilmore, Parrott, and Ward (1953) found that 62 per cent. of surgical human adenoid tissue specimens grown in tissue culture grew initially as ciliated epithelium, but that after 8 to 28 days spontaneous degeneration occurred. The cytopathogenic agent could be transferred with filtrates of the culture fluid. Working along similar lines, Clarke (1958) cultured biopsy material from the knee joints of two rheumatoid arthritics and found non-specific degeneration in one case after 35 days and in the other after 56 days. The culture fluid had no specific influence on other cell cultures.

Studies of synovial cell cultures were designed to extend these observations to examine growth patterns, nuclear, and other cytological changes, as well as the development of spontaneous degeneration. Special procedures were carried out to

demonstrate the influence, if present, of any latent infectious agent, possibly a virus. The cellular presence of rheumatoid factor was also examined.

Material and Methods

Tissue cultures were prepared from synovial membranes obtained at surgery from twelve patients with rheumatoid arthritis and from ten non-rheumatoid subjects (three with osteo-arthritis, three with chronic bursitis, two with ankylosing spondylitis, and two normal persons).

Synovial membranes were washed, minced, and explanted into 2-oz. prescription bottles and into Leighton tubes containing coverglasses. Primary Maximow slide cultures were also prepared, but were more frequently used as subcultures for special studies. The nutrient medium was CMRL 1066 (67 per cent.) (Parker, Castor, and McCulloch, 1957), heat-inactivated, pooled human serum (negative for rheumatoid factor by the latex Fraction II fixation test) (17 per cent.), calf serum (8 per cent.), human cord serum (5 per cent.), and embryo extract (3 per cent.), together with penicillin and streptomycin. Cultures were fed at 4-day intervals and transferred by trypsinization, usually every 3 weeks.

These cells were maintained *in vitro* for various periods up to 230 days, and were examined at intervals by phase-contrast microscopy and after differential staining.

Acridine Orange (AO) Studies.—This fluorochrome stains deoxyribonucleic acid (DNA) yellow-green, and nucleolar and cytoplasmic ribonucleic acid (RNA) orange to red. Inclusion bodies of such materials are similarly stained. Cultures were also stained after being treated with DN-ase or RN-ase.

Fluorescein-conjugated Heat-aggregated Human Gamma Globulin Studies.—This reagent demonstrates rheumatoid factor in plasma cells in sections of rheumatoid synovium and lymph nodes from rheumatoid patients (Mellors, Nowoslawski, Korngold, and Sengson, 1961). Washed cultures were fixed with acetone, treated with the fluorescent material for 30 to 60 minutes, washed in buffered saline, and mounted in glycerol.

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TABLE
DAYS *IN VITRO* OF RHEUMATOID AND NON-RHEUMATOID SYNOVIUM SUBCULTURES

No. of Days	..	..	..	..	..	..	20	40	60	100	160	220
Non-Rheumatoid Subjects	1. Normal									+		
	2. Normal											+
	3. Osteo-arthritis								+			
	4. Osteo-arthritis									+		
	5. Osteo-arthritis										+	
	6. Ankylosing Spondylitis								+			
	7. Ankylosing Spondylitis									+		
	8. Bursitis											+
	9. Bursitis*											
	10. Bursitis									+		
Rheumatoid Arthritics	11											
	12								+			
	13*								+			
	14											
	15						+					
	16*											
	17										+	
	18							+				
	19							+				
	20								+			
	21							+				
	22*											

* = Not subcultured.

Observations

Growth Patterns

These were observed at various intervals. In the primary cultures, the synovial cells were usually narrow, fibroblast-like cells, but in later and established cultures, they were not typically spindle-shaped, but appeared as broader, bipolar cells with broad processes. Transitional and epithelioid cells, though present in later subcultures, were never dominant in numbers. Individual differences in the

growth patterns are described later.

Rheumatoid tissue proliferated early, within 10 days, but the cultures, with one exception, could not be maintained beyond 58 days. The rapid spontaneous degeneration as described for latent virus-infected adenoid tissue did not occur.

Non-rheumatoid tissue proliferated later, generally by 20 days (except for bursitis tissue, which proliferated early), but maintained their growth for longer periods, mostly over 100 days (Table).

Fig. 1.—Case 1. Normal 113 days *in vitro*. Acridine orange. $\times$ 400.

Non-Rheumatoid Cultures

Normal.—Synovium was obtained incidental to cartilage removal in one case and to bunionectomy in a second. Primary cultures grew slowly and contained the types of cells described by others (Vaubel, 1933; Murray, Stout, and Pogogeff, 1944)—round, stellate, and tulip-shaped. Abundant, uniform, fibroblast-like growth began after 21 days

and continued in one case for 113 days (Fig. 1) and in the other for 230 days. Giant or multinucleated cells were infrequent.

Osteo-arthritis.—Synovium from three patients also grew slowly. Early cultures contained round cells, spindle-shaped, and stellate fibroblast-like cells which were finely granular (Fig. 2*a, b*), while subcultures continuing for 6 to 16 weeks *in vitro* consisted

Fig. 2.—Case 3. Osteo-arthritis.

(*a*) 9 days *in vitro*. Phase contrast. $\times 200$.

(*b*) 11 days *in vitro*. Phase contrast. $\times 200$.

Fig. 3.—Case 7. Ankylosing spondylitis.

(*a*) 51 days *in vitro*. Phase contrast. $\times 600$.

(*b*) 65 days *in vitro*. Acridine orange. $\times 400$.

of smooth, fibroblast-like cells resembling those seen in cultures of normal synovium, interspersed with

broader and transitional fibroblast-like cells and occasional mononucleated giant cells.

Fig. 4.—Case 8. Bursitis. 182 days *in vitro*. Acridine orange. $\times 400$.

Ankylosing Spondylitis.—Synovium from two patients produced uniform fibroblast-like cells indistinguishable from the normal. In the later stages the cells became broader and a few giant cells appeared (Fig. 3 *a, b*).

Chronic Bursitis.—Synovium from three patients showed some changes in growth pattern which differed from the other non-rheumatoid subjects. In two cases, the primary culture consisted of smoothly granular “tulip” cells and bipolar cells, both resembling those found in cultures of normal synovium. In one of these cases, older cultures changed to coarsely granular, fibroblast-like cells of varying size, and later (17th passage) moderate numbers of giant and multinucleated cells appeared (Fig. 4). Synovium from the third case produced in early cultures a population of mixed fibroblast-like cells accompanied by epithelioid cells. In subsequent passages many binucleated cells were present. In the sixth passage the mixed growth of epithelioid, transitional, and fibroblast-like cells continued.

Fig. 5.—Case 11. Rheumatoid arthritis.

(*a*) 3 days *in vitro*. Phase contrast. $\times 300$.

(*b*) 30 days *in vitro*. Phase contrast. $\times 600$.

Rheumatoid Arthritic Cultures

Synovial tissues from twelve patients with rheumatoid arthritis produced cultures which differed characteristically in some respects from non-rheumatoid cultures. Outgrowth from primary explants was generally more rapid. Cells early and late in culture varied frequently in size, and cytoplasm was coarsely granular. Nuclear changes were prominent, with wide variations in size, nuclear budding, and multinucleation, and there also seemed to be a greater number of nucleoli.

Synovium from one patient at 3 days produced an outgrowth of spindle-shaped, round, and dense "comma" cells. By 30 days, these had been replaced by mixed fibroblast-like cells and giant cells with large nuclei and coarsely granular cytoplasm (Fig. 5 *a, b*), and at 55 days this pattern remained unchanged.

Synovium from a second rheumatoid patient after 3 days in culture contained round cells, small spindle-shaped cells, and large unidentified multinucleated cells. At 44 days, although numerous giant multinucleated cells persisted, the principal growth consisted of fibroblast-like and epithelioid cells (Fig. 6).

A third patient's synovial culture at 19 days was composed of fibroblast-like cells, many of which became markedly broader by the 22nd day. At this time, frequent multinucleated giant cells appeared. These two types of cell persisted until the 156th day of culture (Fig. 7 *a, b*).

Fig. 6.—Case 14. Rheumatoid arthritis. 44 days *in vitro*. Acridine orange. $\times 400$.

Fig. 7.—Case 17. Rheumatoid arthritis.

(a) 36 days *in vitro*. Acridine orange. $\times 400$.

(b) 156 days *in vitro*. Acridine orange. $\times 400$.

Fig. 8.—Case 17. Rheumatoid arthritis. 62 days *in vitro*.

- (a) Acridine orange. $\times 1500$.
(b) Ribonuclease followed by acridine orange. $\times 1500$.
(c) Deoxyribonuclease followed by acridine orange. $\times 1500$.

Acridine Orange Studies

No gross differences were seen in the content and distribution of DNA and RNA in any of the 22 synovial cultures, whether examined as primary or subcultures. Typical studies are seen in Fig. 8 (*a, b, c*). In many cultures from rheumatoid patients, however, there was an apparent increase in the quantity of DNA which was due to the presence of large, lobulated, and multiple nuclei. Characteristic inclusion bodies may develop in cells as a response to some viruses, and such inclusions were assiduously searched for in primary and subcultures, but were never found.

Fluorescein-conjugated Human Gamma Globulin Studies

Primary cultures of rheumatoid synovial membrane may contain macrophages and mononuclear round cells including plasma cells (Fig. 9). Primary cultures of two out of three such patients showed the cellular presence of rheumatoid factor. A typical plasma cell with Russell-like bodies is seen in Fig. 10 (*a*), and a round cell with perinuclear fluorescence and a fluorescent cell that may be fibroblast-like in Fig. 10 (*b*). Fluorescent cells were not found in subcultures of these primary cultures, nor in non-rheumatoid primary synovial cultures.

Fig. 9.—Case 22. Rheumatoid arthritis. 4 days *in vitro*. Acridine orange. $\times 100$.

Fig. 10 (*a, b*).—Case 20. Rheumatoid arthritis. 11 days *in vitro*. Fluorescent human gamma globulin. $\times 1000$.

Discussion

The examination of cell cultures of synovium from twelve rheumatoid arthritics and ten non-rheumatoid subjects revealed some differences in the growth pattern. Rheumatoid synovium primary cultures proliferated earlier but could not be maintained (subcultured) as long as the non-rheumatoid specimens. Early and later cultures of eight of twelve patients' synovium frequently contained large and giant cells that were usually multinucleated. Nuclear budding and micronuclei were not uncommon. Nucleoli were prominent and increased in number. There was normal distribution of DNA and RNA, except for the expected increase of the former in the multinucleated cells. In two of three rheumatoid primary cultures treated with fluorescent human gamma globulin, plasma and round cells containing rheumatoid factor were found. Rheumatoid factor was not detectable either in or on synovial cells, except in one instance. Mellors and others (1961) did not find fluorescence of synovial cells in their studies of rheumatoid synovium sections. Cooper (1964), in similar studies, found that fluorescent materials were sometimes present in synovial cells. Barland, Novikoff, and Hamerman (1962) have suggested that type A synovial cells as seen by electron microscopy have the properties of phagocytosis and pinocytosis. This could explain the presence of rheumatoid factor in synovial cells, the cell getting it from that present in its environment before explantation or that produced or released by the plasma cells of the culture.

Non-rheumatoid synovium cultures proliferated later (except in the case of two bursitis donors), but could be subcultured for longer periods. Occasional multinucleated cells were present in five of the ten patients, two of these being from the chronic bursitis patients. Bizarre nuclear phenomena were not as common as in the rheumatoid synovium cultures and RNA and DNA distribution was normal.

Multinucleated cells and nuclear abnormalities are found in established cell lines. However, their presence in early and late cultures of rheumatoid synovium was striking. Stanfield and Stephens (1963) found multinucleated giant cells in 50 per cent. of synovium cultures from 70 rheumatoid arthritics and in 25 per cent. from 102 non-rheumatoid subjects. Some factors that induce the appearance of multinucleated cells in cultures of mammalian cells are bovine serum albumin in the medium, an acid medium (pH 5.6 to 6.0), x-ray treatment, and virus infections (Mitamura, 1959). In this work, rheumatoid and non-rheumatoid synovia were cultured under similar conditions. Using phenol red as an indicator as well as pH meter tests, the pH

of the supernates of the cultures was found to be over 7.0. Foetal calf serum, which was in the medium, has a low albumin content; it contains more than 45 per cent. of a glycoprotein ("Fetuin") which Puck (1960) found to have no influence on the induction of multinucleated cells. It has been reported that increased DNA and peculiar combinations of abnormal intranuclear DNA masses and large nuclei are present in tumours and in cell infections caused by DNA viruses (Leuchtenberger and Leuchtenberger, 1960).

The presence of a latent virus or infectious agent could be inferred if spontaneous, rapid degeneration of the rheumatoid cultures had occurred soon after their proliferation. However, they were generally maintained up to 58 days when non-specific degeneration occurred.

Non-rheumatoid cultures were nearly all maintained for more than 100 days. This could be attributed either to innate differences or to demands of the synovial cells.

Summary

Cultures *in vitro* of rheumatoid synovia proliferated earlier but with few exceptions could not be subcultured as long as non-rheumatoid synovia. Multinucleated giant cells and bizarre nuclear phenomena were also more frequent in early and late rheumatoid synovium cultures. The only exceptions in the non-rheumatoid synovium cultures came from two patients with chronic bursitis in which the growth pattern resembled that of rheumatoid synovia to a limited extent.

Early spontaneous degeneration of the cultures that might imply that they harboured a latent virus did not occur and acridine orange preparations did not show ribonucleic acid or deoxyribonucleic acid inclusion bodies.

Primary cultures of some rheumatoid synovia showed the presence of rheumatoid factor in plasma and round cells, and in one instance only in a fibroblast-like cell.

The variations in growth pattern observed in these studies could be due to specific differences or the requirements of rheumatoid synovial cells in tissue culture.

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La synoviale rhumatoïde arthritique et l'arhumatoïde en culture cellulaire

RÉSUMÉ

Des cultures *in vitro* de la synoviale rhumatoïde proliféraient assez vite mais, avec quelques exceptions, on ne pouvait pas les repiquer pour un temps aussi long que les synoviales arhumatoïdes. Des cellules géantes à noyau multiple et des phénomènes nucléaires bizarres étaient aussi plus fréquents dans les cultures synoviales

rhumatoïdes précoces et tardives. Les seules exceptions parmi les cultures synoviales arhumatoïdes vinrent de deux malades atteints de bursite chronique, où le mode de croissance se paraissait un peu à celui de la synoviale rhumatoïde.

Une dégénérescence spontanée rapide des cultures, qui aurait indiqué qu'elles hébergeaient un virus latent, ne se produisit pas, et des préparations d'orange d'acridine ne révélèrent pas d'inclusions d'ARN ou d'ADN.

Des cultures primaires de certaines synoviales rhumatoïdes accusaient la présence du facteur rhumatoïde dans les histiocytes et dans les cellules rondes et, dans un cas seulement, dans une cellule fusiforme.

Ces variations dans le mode de croissance observé dans ce travail pourraient être dues à des différences spécifiques dans les exigences des cellules synoviales rhumatoïdes en culture.

La sinovia reumatoide artrítica y la non-reumatoide en cultivo celular

SUMARIO

Cultivos *in vitro* de la sinovia reumatoide proliferaron más pronto pero, con algunas excepciones, no se la pudo subcultivar por un tiempo tan largo como la sinovia non-reumatoide. Células gigantes con núcleos múltiples y fenómenos nucleares extraños fueron también más frecuentes en los cultivos sinoviales reumatoides tempranos y tardíos. Las únicas excepciones en los cultivos sinoviales non-reumatoides vinieron de dos enfermos con bursitis crónica, donde el modo de crecimiento se pareció algo al de la sinovia reumatoide.

Una degeneración espontánea temprana de los cultivos, lo que hubiera indicado que albergasen un virus latente, no se produjo, y preparaciones de naranja de acridina no revelaron inclusiones de ácido ribonucleico o de ácido deoxiribonucleico.

Culturas primarias de ciertas sinovias reumatoides acusaron la presencia del factor reumatoide en los histiocitos y en las células redondas y, en un caso sólo, en una célula fusiforme.

Las variaciones en el modo de crecimiento observadas en esta investigación pueden deberse a diferencias específicas en las exigencias de las células sinoviales reumatoides de cultivo.