Search for viruses in rheumatoid macrophage-rich synovial cell populations

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SUMMARY The adherent cells remaining after short-term culture of synovial fluid and synovial membrane cells from rheumatoid and non-rheumatoid patients were examined for the presence of a productive virus infection and for various viral antigens. Labelling was carried out with \(^3\)H-thymidine and \(^3\)H-uridine followed by sucrose density gradient centrifugation of the culture supernatant. Only in 1 case was there incorporation of \(^3\)H-uridine into material of density 1.21 g/cm\(^3\). Viral antigens were tested for by indirect immunofluorescence with antisera to rubella virus, the retroviruses RD-114 and simian sarcoma associated virus, early adenovirus type 2 antigens, late adenovirus type 2 antigens, SV-40 T antigen, and in 1 case measles virus. No cell showed immunofluorescence with any antiserum except the early adenovirus type 2 antiserum, which stained the cytoplasm of about half the synovial cell cultures, some from rheumatoid and some from non-rheumatoid patients.

A possible viral aetiology of rheumatoid arthritis has been suggested (Denman, 1975; Barland, 1973; Hamermer, 1975; Marmion and Mackay, 1977), but it has not yet been supported by clear experimental evidence. Virological investigations until recently have concentrated on the long-term culturing of fibroblastic cells. These are derived from synovial membranes of fluids and are thought to be either type A or B lining cells (Smith, 1971). These studies included attempted detection of cytopathic and non-cytopathic viruses, viral genomes, and neoantigens, and no evidence was found which implicated any virus (Person et al., 1973; Grayzel, 1973; Norval and Marmion, 1976).

An alternative hypothesis (Talal, 1975; Denman et al., 1976; Marmion and Mackay, 1977; Fundenberg and Wells, 1976) suggests that the primary and central change is in the effector cells of the immune system (T or B lymphocytes or monocyte-derived macrophages) and that the rheumatoid process involves a loss of the normal immunoregulatory control rather than a normal immune reaction against an altered target cell such as a virus-infected fibroblast. This loss of immunoregulatory control could be due to a viral infection of the immune effector cells (Messner, 1974).

Recent virological studies on lymphocytes from synovial fluids and membranes have included an examination for retroviruses, but these were not shown to be present by methods such as nucleic acid hybridisation, fusion, and cocultivation with cells permissive for primate retroviruses, immunofluorescence, and radioimmunoassay (Norval et al., 1979; Hart et al., 1979).

The other type of effector cells of the immune system remaining to be subjected to experimental scrutiny is the monocyte derived macrophage. These are the adherent cells present within the first few days of culture of synovial fluids as described by Mackay et al., (1974) which do not seem to multiply and cannot be subcultured. Most often they are overgrown after variable periods of time by the fibroblastic cells. In addition disaggregated synovial membranes yield a heterogeneous mixture of fibroblasts, macrophages, and undifferentiated esterase-positive cells on primary cultures, and again the fibroblasts generally overgrow the other cells. Traynoff et al. (1976) have described methods for identifying the various populations of synovial mononuclear cells on the basis of morphology, sudanophilia, and several cell markers, and they classified such cells into synovial lining cells, monocyte-derived macrophages, monocytes, lymphocytes, and lymphoblasts.

The role of the synovial macrophages in the joint is not clear. It has been suggested that they may be bearing foreign antigens, as antibody dependent lymphocyte cytotoxicity to adherent cells has been
demonstrated in short-term cultures (Neill, personal communication). It is of interest that macrophage-
lymphocyte clusters have been described in auto-
logous cell mixtures obtained from the synovial
fluid of rheumatoid patients (Hepburn et al., 1974).
Webb et al. (1975) showed that such clusters could
be formed with heterologous lymphocytes also.
This suggested that there may be an antigen on the
macrophages towards which the lymphocytes are
sensitive, although there was no stimulation of the
lymphocytes in these clusters. It is possible that such
an antigen may be virally induced or coded.
Thus it was thought worthwhile to examine the
synovial macrophages for the presence of a productive
viral infection by means of short-term cultures and
labelling with $^3$H-uridine and $^3$H-thymidine. In
addition these cells were examined for various
viral antigens by immunofluorescence.

Materials and methods

CELL CULTURE
Synovial fluids from patients with classical or definite
rheumatoid arthritis (RA), other forms of inflam-
atory joint disease, and osteoarthritis (OA) were
treated within 2 hours of extraction from the patient
with 80 U/ml hyaluronidase for 30 min at 37°C.
The cells were deposited by centrifugation at 800 g
for 10 min and washed once in Earles-based Eagle's
complete medium (EE). Short-term cultures were
set up using 1–2 $\times 10^7$ viable cells/ml EE; 10 ml
into 100 ml flasks for labelling with $^3$H-thymidine
and $^3$H-uridine, and 1 ml into each tube containing
coverslips (22 $\times$ 6 mm) for immunofluorescence.
Flying coverslips were used to estimate the number of
adherent cells, and 1 ml cell suspension was suf-
ficient to prepare 8 coverslips. All cultures were
incubated for 3 hours at 37°C in air containing 5%
CO$_2$. The non-adherent cells were removed at this
stage by washing thoroughly twice in EE.
Synovial membranes obtained at synovectomy
were disaggregated within 2 hours of the operation
as described by Abrahamsen et al. (1975), and the
single cell suspensions were then handled as above.

COUNT OF ACID ESTERASE-POSITIVE
ADHERENT CELLS
The flying coverslips were fixed in Baker's formol
calcium and the acid esterase staining procedure was
carried out as described by Mueller et al. (1975).
The adherent count (AC) was determined by counting
the acid esterase-positive cells (AEPC) on 10 mm$^2$
of the coverslip and calculating as follows:

\[ AC/\text{ml cell suspension} = 10 \times 132.8 \times 8 \times \text{AEPC count}. \]

VIRUS DETECTION
After washing the cell sheet to remove the non-
adherent cells, the flasks were labelled by adding
10 ml EE containing 5% fetal calf serum and either
(5-$^3$H)-uridine or (methyl-$^3$H)-thymidine (Amer-
sham), both at a final concentration of 10 mCi/ml.
Incubation was continued for a further 20 hours at
37°C. On 3 occasions attempts were made to induce
a virus by adding cyclohexidime (10 µg/ml) at same
time as the radioactive medium.
Detection of virus in the culture supernatant was
performed as outlined by Norval and Marmion
(1976).

IMMUNOFLUORESCENCE
This was carried out as described previously (Hart
and Marmion, 1977) with coverslips fixed in acetone
after the 3 hour incubation. The specific antisera
used were all prepared in rabbits and have been
described before together with appropriate control
sera. They were anti-rubella virus (Thomas strain)
grown in RK$_{13}$ cells (Hart and Marmion, 1977); anti-RD114 grown in RK$_{13}$, and anti-simian
sarcoma virus grown in KNKR cells (Hart et al.,
1979); anti-adenovirus type 2 grown in RK$_{13}$ cells;
treated with cytosine arabinoside (10 µg/ml) and
used early in infection, and anti-adenovirus type 5
grown in RK$_{13}$ cells used late in infection (Jones
et al., 1979).

In one experiment guinea-pig anti-measles serum
(Flow Laboratories) was used. A weak but definitely
positive reaction was detected specifically on measles
infected Vero cells at 1/20 dilution. This serum was
used at 1/10 dilution for the synovial cells. A few
immunofluorescent tests also included hamster anti-
SV 40 T antigen serum kindly donated by Dr E.
Rogers.
Fluorescein-conjugated anti-rabbit Ig (Wellcome
Laboratories) was used at 1/16 dilution: fluorescein-
conjugated anti-guinea-pig and anti-hamster (Nor-
dic Diagnostic Laboratories) were used at 1/8
dilution.

Results

CULTURE TECHNIQUES
Cells from synovial fluids or membranes of 14
patients with rheumatoid arthritis (RA), 4 with
osteoarthritis (OA), 5 with other non-RA conditions,
and 1 undiagnosed were used and the adherent
cell population separated after culturing for 3 hours
at 37°C. The clinical details are listed in Table 1.
In cases where there were sufficient cells, the cultures
were labelled with $^3$H-thymidine and $^3$H-uridine,
and, after concentration by precipitation with
(NH$_4$)$_2$SO$_4$, the culture supernatants were analysed.
by sucrose density gradient centrifugation. The acid-insoluble counts per minute in each fraction were determined and plotted against the density of the sucrose to find out if there was a peak of incorporation at any particular density throughout the gradient. As may be seen from Table 1, only in one case, 780127, was there a peak of radioactive insoluble material after labelling with $^3$H-uridine, which occurred at density 1.21 g/cm$^3$. This is shown in Fig. 1. Labelling of the same culture with $^3$H-thymidine did not produce such a peak, supporting the idea that there might have been production of an RNA-containing virus by the adherent synovial cells from this patient. The density, 1.21 g/cm$^3$, is similar to that found on $^3$H-uridine labelling of measles virus produced from Vero cells (Norval and Smith, 1979).

With some synovial specimens there were not sufficient cells to enable the labelling experiment to be carried out, and only immunofluorescence was done.

**Table 1**  
Clinical and laboratory details of patients whose synovial specimens were used as a source of adherent cells. Results indicating the count of acid esterase positive adherent cells/ml in the population, and labelling of culture supernatants with $^3$H-thymidine and $^3$H-uridine followed by sucrose density gradient centrifugation are shown. In addition immunofluorescence using a variety of viral antisera was carried out on all the specimens.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Diagnosis</th>
<th>Tests for rheumatoid factor</th>
<th>Specimen</th>
<th>Count of acid esterase positive adherent cells/ml</th>
<th>Labelling of culture supernatants with $^3$H-thymidine $^3$H-uridine</th>
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* 780131 and 780133 were also treated with 10 mg/ml cycloheximide at the same time as adding $^3$H-thymidine or $^3$H-uridine; 780132 was treated with cycloheximide at the same time as adding $^3$H-uridine.

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**IMMUNOFLUORESCENCE**

Coverslips containing adherent cells from all the specimens listed in Table 1 were fixed and stained with specific viral antisera to rubella virus, RD-114 (a feline endogenous retrovirus), SSAR (a simian sarcoma retrovirus), and adenovirus type 2. With the first 3 antisera no specific immunofluorescence was seen, though occasionally there was a non-specific staining, but this could be easily recognised as the control anti-cell sera reacted similarly. In addition results with the antiserum to the late adenovirus type 2 antigens were uniformly negative. However, with the antiserum to the early adenovirus type 2 antigens about half the specimens examined showed weak uniform non-granular cytoplasmic immunofluorescence, which was not present when the control anti-cell serum was used. This immunofluorescence had no particular pattern being present in the adherent cells from some synovial membranes and some fluids, and from approximately equal numbers of RA patients and non-RA.
antigen was of the properties. Very little et (Hepburn et al., 1974) after this work that an antigen was present on the macrophage surface towards which the lymphocytes were sensitised (Hepburn et al., 1974). This result was confirmed by Runge (1976), who measured lymphocyte stimulation after culturing synovial macrophages with autologous peripheral blood lymphocytes from RA patients and controls. A small degree of stimulation was observed in the rheumatoid cultures, but this was not significantly different from that observed in non-RA cultures. It was suggested that there may be non-specific lymphocyte activation by immune complexes associated with the macrophages rather than a specific synovial antigen expressed on the macrophages.

On the other hand the work of Neill (personal communication) does support the idea that some rheumatoid synovial cells at very early passes may express unique antigens. These were detected by an antibody-dependent cytotoxicity technique using synovial cells of mixed types as targets and autologous serum. On subculture, which would eliminate the macrophage population, the cytotoxicity became negative. Other work using similar techniques and also giving negative results has involved target synovial cells maintained in culture through several subcultures, so that no macrophages were likely to be present (Griffiths et al., 1976).

In this present study an attempt was made to look for virus production and some viral antigens on the adherent synovial cells remaining after a short-term culture. It was hoped in this way to examine a macrophage-rich population of synovial cells which are eliminated by the usual methods of in-vitro culture. A productive virus infection was not demonstrated apart from one instance in which there was incorporation of \(^{3}H\)-uridine into material of density 1.21 g/cm\(^3\), the same as measles virus. However, by immunofluorescence using antisera to measles serum no measles antigens were expressed on the adherent cells from this patient. The culture was not analysed further.

Further, when the adherent cells were examined by immunofluorescence for rubella virus antigens, retrovirus antigen, SV-40 T antigen, and adenovirus type 2 late antigens, they were all negative. Thus a unique antigen, induced or coded for by these viruses, was not expressed on the synovial macrophage population. Of course many other virus groups remain, but the ones tested here represent good candidates, being implicated in persistent infections and tumorigenicity.

There was some immunofluorescent staining with antiserum to early adenovirus type 2 antigens in the cytoplasm of the adherent synovial cells from some patients. This did not seem to correlate with the diagnosis of the patient or the type of specimen. It may represent expression of some adenovirus genes in normal human tissue which has been described recently in placentas by Jones et al. (1979), and which is under investigation.

Three cultures, 780131, 780132, and 780133, were also reacted with anti-SV 40 T antigen serum but with negative results.

Finally, measles antiserum was used on adherent cells from patient 780127 to check whether the peak obtained on sucrose density gradients after labelling with \(^{3}H\)-uridine represented productive measles infection. No measles antigens were detected, and no further identification of the material within the radioactive peak was made.

Discussion

Very little has been published thus far on the properties of the synovial monocyte-derived macrophages, though there have been suggestions that they may express unique antigens or phagocytose specific immune complexes and thus may account for the immune processes seen in RA.

Rheumatoid synovial macrophages and lymphocytes were shown to form rosettes or clusters in both autologous and heterologous systems, but, as the lymphocytes were not stimulated by this process, there was no indication from this work that an antigen was present on the macrophage surface towards which the lymphocytes were sensitised (Hepburn et al., 1974). This result was confirmed by Runge (1976), who measured lymphocyte stimulation after culturing synovial macrophages with

Fig. 1 Acid-insoluble counts per minute in sucrose density gradient fractions from 780127 adherent cell culture supernatant after labelling with \(^{3}H\)-thymidine (●●●●●●●●) and \(^{3}H\)-uridine (●●●●●●).
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We thank the surgeons and clinicians at the Princess Margaret Rose Hospital and the Northern General Hospital Hospital and Mrs Leonora Hislop for providing the synovial specimens used in this study.

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References


