Immunopathology of subcutaneous rheumatoid nodules

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Abstract

Nodules obtained from five patients with classical seropositive rheumatoid arthritis were studied by an immunofluorescence technique using polyclonal antibodies to IgG, IgA, IgM, C3c, and fibrin, and monoclonal antibodies to the terminal (C5b-9) complement complex (reaction with a neoantigen in C9 revealed during activation), DR antigens, T cells, macrophages, and interdigiting cells.

In all instances the central necrotic areas stained strongly for fibrin and more weakly for IgG, IgA, IgM, C3, and terminal complement complex. The surrounding palisading cells reacted with antibodies to DR and macrophages. In the peripheral granulomatous tissue most of the lymphocytes reacted with the antibodies to T cells, whereas various amounts of the larger mononuclear cells were stained by antibodies to DR antigens, macrophages, and interdigitating cells. In all instances the walls of some of the smaller vessels in the granulomatous tissue stained for fibrin, C3, and terminal complement complex. Plasma cells were not seen except for scattered IgM cells in one nodule. These results support the view that the palisading cells are derived from macrophages, and indicate that there is vasculitis with activation of C3 and the terminal complement pathway in the granulomatous tissue.

Subcutaneous rheumatoid nodules are found in about 20% of patients with rheumatoid arthritis. These patients almost always have serum samples which are positive for rheumatoid factor, and the nodules are usually found in areas which may be subjected to external pressure. The pathogenetic mechanisms behind the formation of the nodules are largely unknown, but proteolytic enzymes, trauma, immune complexes, and genetic factors have been proposed as pathogenetic factors.

Histologically, rheumatoid nodules consist of three zones, a central necrotic area surrounded by palisading cells, which are again surrounded by granulomatous tissue with numerous vessels and various degrees of infiltration by cells. Several studies have been performed to determine the composition of the necrotic material and the types of cell involved. Conventional staining has shown that the necrotic material contains at least fibrin, the palisading cells are similar to macrophages, and that the granulomatous tissue contains various kinds of mononuclear cells. Recent studies with monoclonal antibodies specific for various cell markers have supported the view that many of the palisading cells are monocytes, as they are usually HLA-DR and CD11b (OKM 1) positive. Many of the cells in the granulomatous area seem to be T cells. However, it is still debatable whether the palisading cells may be fibroblasts, and whether vasculitis occurs in the granulomatous tissue.

This study looked for evidence of complement activation in the granulomatous tissue with a monoclonal antibody to a C9 neopeptope appearing during the activation of the terminal complement pathway. In addition, the palisading cells were examined with a new set of monoclonal antibodies to obtain more precise information about their origin.

Materials and methods

RHEUMATOID NODULES

Subcutaneous nodules obtained from eight patients with classical seropositive rheumatoid arthritis, admitted to the Oslo Sanitetsforenings Rheumatism Hospital, were studied. The nodular tissues were immersed in phosphate buffered saline (PBS) immediately after sampling and stored at 4°C for up to two hours before quick-freezing in Tissue-Tec OCT compound (Lab-Tek Products Division, Miles Laboratories, Naperville, IL, USA), using dry ice, acetone, and isopentane. They were then stored at −70°C until use.

IMMUNOFLUORESCENCE STUDIES

Frozen sections, washed for five minutes in PBS and fixed for ten minutes in acetone at 4°C, were examined with the polyclonal antisera and monoclonal antibodies listed in table 1. When a direct immunofluorescence technique was used, with specific antibodies labelled with fluorescein isothiocyanate (FITC), the sections were incubated with the antibody for 30 minutes, washed twice in PBS, and then mounted in Citifluor mounting medium with glycerol/PBS (Citifluor, London, UK). When an indirect technique was used the sections were incubated overnight at 4°C with the primary, unlabelled monoclonal antibody, washed twice with PBS, incubated with FITC labelled rabbit antimouse immunoglobulin for 30 minutes, washed twice more with PBS, and then mounted as described above.

To obtain a strong signal for the detection of T cells a mixture of antibodies for CD3 and CD5 was used. When the existence of CD5 positive B cells was reported in normal subjects and patients with rheumatoid arthritis, ten
Table 1  Antisera and antibodies used

<table>
<thead>
<tr>
<th>Specificity*</th>
<th>Source</th>
<th>Fluorescein to protein ratio</th>
<th>Mouse Ig class/ subclass</th>
<th>Final IgG concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal rabbit antisera</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Fc IgG-FITC</td>
<td>Behringwerke, Marburg, Germany</td>
<td>3:0</td>
<td>IgG2a</td>
<td>40:0</td>
</tr>
<tr>
<td>Anti-Fc IgA-FITC</td>
<td>Behringwerke, Marburg, Germany</td>
<td>2:0</td>
<td>IgG1</td>
<td>6:0</td>
</tr>
<tr>
<td>Anti-C3c-FITC</td>
<td>Behringwerke, Marburg, Germany</td>
<td>2:6</td>
<td>IgG2a</td>
<td>1:2</td>
</tr>
<tr>
<td>Anti-fibrinogen/fibrin-FITC†</td>
<td>Dakopatts A/S, Glostrup, Denmark</td>
<td>1:5</td>
<td>IgG1</td>
<td>1:10</td>
</tr>
<tr>
<td>Anti-Fc IgM-FITC</td>
<td>Dakopatts A/S, Glostrup, Denmark</td>
<td>2:3</td>
<td>IgM</td>
<td>1:5</td>
</tr>
<tr>
<td>Anti-mouse IgG-FITC</td>
<td>Produced in this laboratory‡</td>
<td>3:0</td>
<td>supernatant</td>
<td></td>
</tr>
<tr>
<td>Monoclonal mouse antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 (Leu 4)</td>
<td>Becton Dickinson, Mountain View, CA, USA</td>
<td></td>
<td>IgG2a</td>
<td>40:0</td>
</tr>
<tr>
<td>Anti-CD5 (Leu 1)</td>
<td>Becton Dickinson, Mountain View, CA, USA</td>
<td></td>
<td>IgG1</td>
<td>6:0</td>
</tr>
<tr>
<td>Anti-CD3 (Leu 1)</td>
<td>Becton Dickinson, Mountain View, CA, USA</td>
<td></td>
<td>IgG2a</td>
<td>1:2</td>
</tr>
<tr>
<td>Anti-HLA-DR-FITC</td>
<td>Becton Dickinson, Mountain View, CA, USA</td>
<td></td>
<td>IgG2a</td>
<td>1:2</td>
</tr>
<tr>
<td>Anti-CD11b (OKM1)</td>
<td>Ortho Diagnostic Systems, Raritan, NJ, USA</td>
<td></td>
<td>IgG1 1:2</td>
<td>dilution of culture</td>
</tr>
<tr>
<td>Anti-macrophage (RFD2)</td>
<td>Kindly provided by Dr T Lea</td>
<td></td>
<td>IgM 1:5</td>
<td>supernatant</td>
</tr>
<tr>
<td>Anti-interdigitating cell (RFD1)</td>
<td>Free Hospital School of Medicine, London, UK</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*FITC=fluorescein isothiocyanate.
†Refined to a antibody to fibrin in the text, as fibrin is more likely to be present in deposits than fibrinogen.
‡As described by Molines et al. 

synovial tissues were tested as control samples, both with the mixture and with each of the two components separately. Although the fluorescence was weaker in the latter, the quantitative evaluations did not differ. The cells detected with the mixture of antibodies are therefore referred to as CD3 and CD5 positive T cells.

The specificity of the antisera and antibodies was examined by testing the performance on selected tissues known to contain the antigens. For the antisera to immunoglobulins and fibrinogen the specificity was further tested by blocking experiments performed with purified antigens. The specificity of the various antibodies to membrane antigens was also tested on mononuclear cells isolated from peripheral blood. Controls with FITC labelled rabbit antimouse immunoglobulin alone were included for each biopsy sample and were consistently negative. Control experiments were also performed with FITC labelled rabbit antimouse immunoglobulin after incubation with normal mouse IgG at concentrations which should contain the immunoglobulin subclasses in amounts corresponding to those in the specific monoclonal antibodies. These control experiments were also always negative.

For each biopsy sample one section was stained conventionally with haematoxylin and eosin. This section was examined first to determine whether the tissue was representative with a clearly identifiable nodular structure. If this was not so new sections were cut until representative areas were obtained. In three of the eight nodules, representative areas were not found and these nodules were not studied further.

The immunofluorescence microscopic examinations were performed with a Leitz Orthoplan incident light microscope. The mean number of cells with positive immunofluorescence counted in five 0.1 mm² areas was recorded according to the following scale: 0 = no positive cells observed; 1 = positive cells observed, but less than one per 0.1 mm²; 2 = average of one to ten positive cells per 0.1 mm²; 3 = average of 11-100 positive cells per 0.1 mm²; and 4 = average of >100 positive cells per 0.1 mm². For quantification of extra-cellular deposits an arbitrary grading from 0 to 4 was used.

Results

CHARACTERISATION OF MONONUCLEAR CELLS

No cells were found which reacted with antibodies to IgG or IgA, whereas antibodies to IgM reacted with cytoplasm in a few cells which

Figure 1  Sections of rheumatoid nodules stained with fluorescein isothiocyanate conjugated antibodies to DR (A) and macrophage (RFD2) (B). The palisading area is shown to the left and the peripheral area to the right/top right of the figure.
were similar to plasma cells in two of the five patients (table 2). Thus plasma cells are rare in these rheumatoid nodules. The necrotic cell area is not included in table 2 as virtually no cells were seen here.

The antibody to DR reacted strongly with a large number of cells in the palisading cell area and the granulomatous area (fig 1A). Similar results are obtained with antibodies specific for macrophages, although the number of positive cells in the peripheral area was lower than with the antibody to DR (fig 1B). With IgM RFD1, no cells were stained in the palisading cell area, whereas in two of the five nodules, scattered positive cells were seen in the granulomatous area. The RFD1 antibody is directed against interdigitating cells, macrophage related cells which may be identical to dendritic cells. Thus the number of cells of this type was considerably lower than that of the macrophages.

With the mixture of the two antibodies to T cells, a large number of T cells were found in the granulomatous area in all patients, especially around small vessels. Only a few positive cells were found in the area of palisading cells.

EXTRACELLULAR DEPOSITS
There was weak and diffuse immunofluorescence in the necrotic area and no fluorescence in the palisading area when antisera to the immunoglobulin classes were used (table 3). The granulomatous area was also negative, except for one nodule in which there was a weak granular

Table 3 Extracellular deposits detected by immunofluorescence staining in sections of rheumatoid nodules

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Central necrotic area*</th>
<th>Palisading area*</th>
<th>Peripheral granulomatous area*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-IgG</td>
<td>1+, diffuse</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-IgA</td>
<td>1+, diffuse</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>1+, diffuse</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-C3c</td>
<td>1+, diffuse</td>
<td>0</td>
<td>2-3+ granular, in walls of scattered small vessels</td>
</tr>
<tr>
<td>Anti-terminal complement complex</td>
<td>1+, diffuse</td>
<td>0</td>
<td>1-2+ granular, in walls of scattered small vessels</td>
</tr>
<tr>
<td>Anti-fibrin</td>
<td>3+, diffuse</td>
<td>0</td>
<td>3+ in vessel walls</td>
</tr>
</tbody>
</table>

*no fluorescence; (1-3+) arbitrarily graded strength of fluorescence.

Figure 2 Sections of rheumatoid nodules stained with fluorescein isothiocyanate conjugated antibodies to C3c (A), terminal complement complex (B), and fibrinogen/fibrin (C). Staining of vessels in the peripheral (granulomatous) area of the nodules is shown.
fluorescence with antibodies to IgM in scattered vessel walls. Deposits of complement were looked for by two antibodies, one directed against C3c, reacting both with native and activated C3. A weak and diffuse fluorescence was observed in the necrotic area and no fluorescence was seen in the palisading area using this antibody. In the peripheral granulomatous area there was granular fluorescence in the walls of small vessels in all nodules (fig 2A). A weaker granular fluorescence was obtained with a monoclonal antibody to the terminal complement complex, C5b-9, reacting with a neoepitope in C9 which is revealed during activation (fig 2B). With antibodies to fibrin there was a strong fluorescence in the central necrotic area and in numerous vessel walls in the granulomatous area (fig 2C).

Discussion

This study confirms that the palisading cells are DR positive and react with a number of antibodies to macrophages, supporting the view that palisading cells are derived from macrophages. In addition, the results with the antibody to interdigitating, or dendritic, cells suggest that few such cells are present in the nodules. In contrast with the other monoclonal antibodies used, this antibody is of the IgM class, and the rabbit antimouse immunoglobulin antiserum which was used as the second antibody in the detection system reacted in a weaker manner with IgM than with IgG (results not shown). However, some clearly positive cells were occasionally seen in this study, suggesting that the results are reliable. This, together with the almost complete lack of plasma cells seen here and elsewhere, suggests that a local humoral response is not taking place in the nodules. Altogether, these data support the view of Duke et al. that the inflammatory process in nodules differs from that in rheumatoid synovitis.

Probably the most important finding in our study is that fibrin and complement are found in the vessel walls in granulomatous tissue. The reaction with antibodies to terminal complement complex indicates that a true activation involving the terminal complement pathway is taking place in situ, not merely a passive influx of serum proteins in an inflamed area. Another argument against the latter possibility is that in a series of 70 biopsy samples from patients with various kinds of rheumatoid synovitis, staining with antibodies to terminal complement complex in vessels was not seen in only eight biopsy samples, and with antibodies to C3c in only two biopsy samples. The same antibodies were used as in this study, supporting the idea that the inflammatory process is different in these two locations.

It may seem surprising that immunoglobulin, complement, and fibrin were observed together in the vessel walls in only one patient. This is, however, a common observation in patients with vasculitis. It may suggest that complement activation is initiated by the deposition of antigen-antibody complexes, such complexes may only be present for a short time, whereas complement and fibrin deposits persist. Another explanation for this observation is that an alternative pathway of complement activation is taking place.

Signs of vasculitis are not uncommon in extra-articular tissues in rheumatoid arthritis. The development of rheumatoid nodules on sites which are often subjected to external pressure may thus be the result of a combination of a general immunological process in the vessel walls and a local mechanical factor from the external pressure. Alternatively, the immunological process may, as recently suggested by Ziff, be local and secondary to damage to small blood vessels.

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