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Studies on fibroectin in inflammatory *vs* non-inflammatory polymorphonuclear leucocytes of patients with rheumatoid arthritis. I. Immunofluorescent and flow cytometric analysis

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SUMMARY

Using indirect immunofluorescence and flow cytometry, we studied the reactivity of an antibody to human fibronectin with human polymorphonuclear leucocytes (PMNL). Our main objective was to compare the intensity of reaction of this antibody with inflammatory *vs* non-inflammatory PMNL. We used peripheral blood PMNL as a source of non-inflammatory cells and PMNL isolated from the synovial fluid of patients with rheumatoid arthritis as a source of inflammatory cells. Our findings revealed considerably brighter staining of the inflammatory PMNL. Using flow cytometry as a method of measurement, a difference in fluorescence intensity of at least 40 channels (log scale) was observed in all 12 patients studied when comparing peripheral blood with synovial fluid PMNL. In inflammatory PMNL, fibronectin was found both at the intracellular and membrane levels of the cell whereas fibronectin could be detected only intracellularly in non-inflammatory PMNL.

Keywords fibronectin polymorphonuclear leucocytes inflammation

INTRODUCTION

Various types of cells synthesize and secrete fibronectin. These include cells which are as diverse as fibroblasts, endothelial cells, chondrocytes, hepatocytes and leucocytes (for review see Hynes & Yamada, 1982). Among leucocytes, macrophages and neutrophils are the cells which have been reported to produce fibronectin (Alitalo, Hovi & Vaheri, 1980; Weissmann *et al.*, 1980; Hoffstein, Weissmann & Pearlstein, 1980). Recently, it has also been shown that both cell types carry receptors for fibronectin on their surface (Bevilacqua *et al.*, 1981; Pommier *et al.*, 1984). However, the function(s) of these receptors as well as the significance of fibronectin synthesis by polymorphonuclear leucocytes (PMNL) and macrophages remain to be clarified at this present state of our knowledge.

Macrophages and PMNL exert key roles in the inflammatory process. Furthermore, it has been suggested that fibronectin may also contribute in some way to inflammation since it can function as an opsonin and a chemotactic agent (Saba & Jaffe, 1980; Czop, Kadish & Austen, 1981, 1982; Pommier *et al.*, 1983; Norris *et al.*, 1982; Postlethwaite *et al.*, 1981). In the light of this, we believe it is important to fully investigate the link which might exist between inflammation and fibronectin production by macrophages and PMNL.

Our objective in this first report was to investigate any difference which might occur in

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fibronectin levels present in inflammatory *vs* non-inflammatory leucocytes, as assessed by an indirect immunofluorescence procedure using an antibody to human fibronectin and flow cytometry. Leucocytes isolated from the synovial fluid of patients with rheumatoid arthritis (RA) were used as a source of inflammatory cells as opposed to peripheral blood leucocytes which were used as a source of non-inflammatory cells. These cells were chosen in view of the fact that fibronectin concentrations in synovial fluids of patients with RA may reach levels as high as 1 mg/ml (Clemmensen & Andersen, 1982). Although it has been suggested that there is an increased local production of fibronectin by synovial cells (Scott *et al.*, 1981), no information exists on its production by synovial fluid leucocytes.

Our findings revealed considerably stronger immunofluorescent staining of the inflammatory *vs* the non-inflammatory PMNL, with relatively little staining of the mononuclear fraction of the total leucocyte population. Fibronectin was detected both inside the cytoplasm and on the surface of the cells. In another study which is presented as a joint report (Ménard *et al.*, 1985), we found that production and stocking of fibronectin is increased in inflammatory when compared to non-inflammatory PMNL.

MATERIALS AND METHODS

Patient population. Patients selected for these studies had either definite or classical RA diagnosed according to American Rheumatism Association criteria (Ropes *et al.*, 1958). All patients were on short acting non-steroidal anti-inflammatory therapy and none were on long acting therapy. Normal controls were recruited among healthy hospital personnel and were sex and aged matched with RA patients.

Preparation of cells. Peripheral blood leucocytes were prepared from 50 ml of venous blood collected in the presence of 10 u/ml of sterile, preservative free sodium heparin. Twenty to 60 ml of

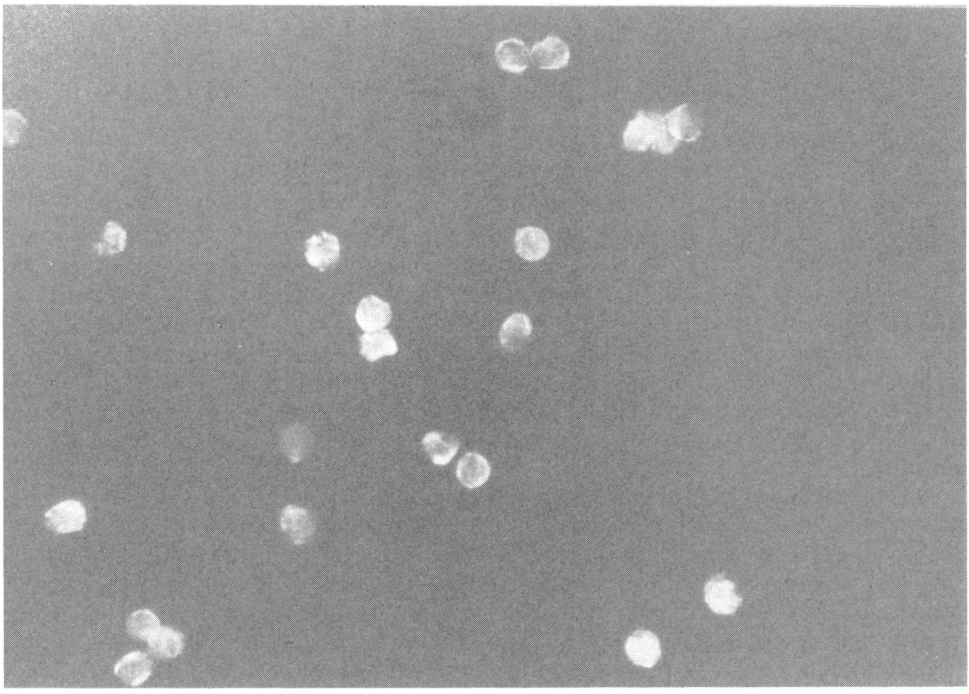

Fig. 1. Immunofluorescent staining of RA synovial fluid PMNL by an indirect immunofluorescence procedure using an antibody to human fibronectin.

synovial fluid were obtained by knee puncture in heparinized syringes from 12 patients with active RA. Synovial fluid and peripheral blood samples were immediately placed on ice and all subsequent purification steps were performed with ice cold buffers. PMNL were prepared by dextran sedimentation to remove the majority of erythrocytes, followed by Ficoll-Hypaque centrifugation. The residual erythrocytes were removed by a short incubation (10 min) wth ammonium chloride. The PMNL were kept in calcium and magnesium free Hanks' balanced salt solution to minimize cell aggregation. Contaminating monocytes were detected by non-specific esterase staining (Burstone, 1965) and were found to be less than 1%.

Immunofluorescence. Cells were fixed with 3·5% paraformaldehyde in phosphate-buffered saline (PBS) pH 7·4 only or with paraformaldehyde followed by permeabilization with acetone for 45 min at −20°C. The cells were then washed in 0·5% bovine serum albumin (BSA) and reacted with a gamma globulin fraction of a monospecific rabbit antiserum to human fibronectin (Cappel, West Chester, Pennsylvania, USA) diluted 1/250 in 0·1% BSA and incubated for 1 h at 4°C. A final incubation was performed with a fluorescein labelled gamma globulin fraction of a goat anti-rabbit gamma globulin antiserum (Cappel) diluted 1/180 in 0·1% BSA. Rabbit gamma globulins were used in the control experiment. Fluorescence intensity was either analyzed by fluorescence microscopy or by a flow cytometer. In this latter instance, an Epics V cell sorter (Coulter Electronics, Inc., Hialeah, Fl) was used and operated at 488 nm with a power output of 300 mW. In each experiment, 10,000 cells were analyzed.

Fibronectin depletion of synovial fluids. Fibronectin was selectively removed from synovial fluid by affinity chromatography on gelatin–Sepharose 4B columns (Pharmacia Fine Chemicals, Dorval, Canada). A 5 ml sample of synovial fluid was applied twice to a 10 ml column. Effective removal of the fibronectin was verified by testing with a rabbit antibody to human fibronectin (Cappel) in the Ouchterlony double diffusion technique.

RESULTS

Fluorescence microscopy

In an indirect immunofluorescence procedure, reactivity of inflammatory and non-inflammatory leucocytes with an antibody to human fibronectin was studied. Leucocytes isolated from the synovial fluid of patients with active RA was used as a source of inflammatory cells whereas

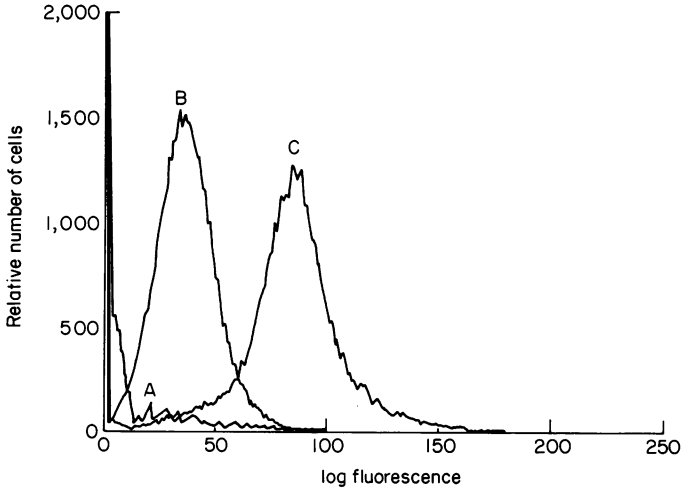

Fig. 2. Histogram illustrating flow cytometric analysis of fluorescence intensity. The negative control in A, peripheral blood PMNL in B and synovial fluid PMNL in C.

leucocytes isolated from peripheral blood of age and sex matched normal individuals or of the patients were used as a source of non-inflammatory cells. A strong reactivity of the antibody was consistently found with inflammatory PMNL as shown in Fig. 1. In some cells, the fluorescence was diffusely distributed whereas in others there existed a denser peripheral distribution. The mononuclear cell population of synovial fluid as well as the non-inflammatory leucocytes of peripheral blood exhibited a fluorescent staining intensity which was too weak to be photographed. No significant difference was observed between patients' and normals' peripheral blood PMNL. Flow cytometric analysis on all cell populations was next undertaken.

Flow cytometric analysis

In Fig. 2 are shown representative results of an experiment comparing paired samples of PMNL isolated from a patient's peripheral blood and synovial fluid, and analysed by flow cytometry. This histogram illustrates the relative fluorescence intensity (log scale) of the negative control in A, of the patient's peripheral blood PMNL in B and of the synovial fluid PMNL in C. Peak C represents the cells which were photographed in Fig. 1. As previously stated, fluorescence intensity of peak A and B was too weak for photography. Peak channel fluorescence intensity was at 37 for the peripheral blood PMNL and at 83 for the synovial fluid PMNL. A difference of at least 40 channels was constantly obtained in all 12 patients studied when using paired samples of patients' peripheral blood and synovial fluid PMNL. No difference was seen when comparing fluorescence intensity of normals' and rheumatoid patients' peripheral blood PMNL. Negative controls were performed using non-immune rabbit gamma globulins in replacement to the antibody to fibronectin.

Fig. 3 illustrates the outcome of an inhibition experiment designed to verify the specificity of the immunofluorescent staining measured by flow cytometry. Synovial fluid PMNL giving a peak channel fluorescence intensity of 60 (peak A) were used for this experiment. The antibody was incubated 1 h at 4°C with 100 μg of fibronectin before reacting with the cells. As can be seen, most of the fluorescence observed with synovial fluid PMNL (peak A) was abolished by prior incubating the antibody with soluble fibronectin (peak B).

We next proceeded to investigate whether reactivity of the antibody to fibronectin was localized intracellularly or on the cell membrane. In order to approach this question we used paraformaldehyde fixation of the cells to investigate for membrane bound fibronectin and paraformaldehyde fixation followed by acetone permeabilization to look for intracellular fibronectin. The findings are presented in Figs 4 & 5. Results obtained with peripheral blood PMNL are shown in Fig. 4 where peak A represents the paraformaldehyde fixed cells only and peak B the permeabilized cells. As can

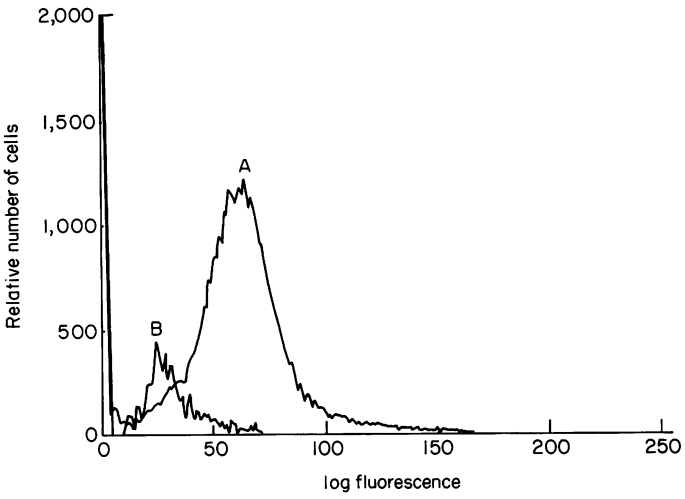

Fig. 3. Histogram illustrating flow cytometric analysis of fluorescence intensity. Synovial fluid PMNL in A and inhibition of fluorescence by pre-incubating the antibody with soluble fibronectin in B.

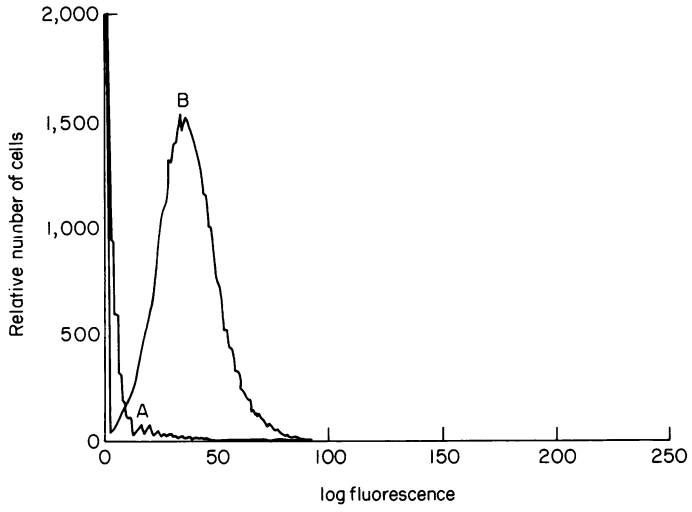

Fig. 4. Histogram illustrating flow cytometric analysis of fluorescence intensity. Paraformaldehyde fixed peripheral blood PMNL in A and paraformaldehyde fixed and acetone permeabilized peripheral blood PMNL in B.

be seen, there is virtually no membrane bound fibronectin on these cells whereas a certain amount can be detected inside the cell. Results differ with synovial fluid PMNL (Fig. 5) where again peak A represents the unpermeabilized cells and peak B the permeabilized cells. Relatively strong fluorescent staining is now obtained with unpermeabilized cells suggesting that in this case, there exists membrane bound fibronectin along with intracellular fibronectin.

The last question which we addressed in this first part of our study was to see if PMNL are able to 'pick up' extracellular synovial fluid fibronectin. The possibility existed that the high concentration of fibronectin present in synovial fluid would lead either to membrane binding and/or cellular internalization of this glycoprotein. Normal peripheral blood PMNL were incubated for 3 h at 37°C in a synovial fluid having a fibronectin concentration of 1·1 mg/ml. After the incubation

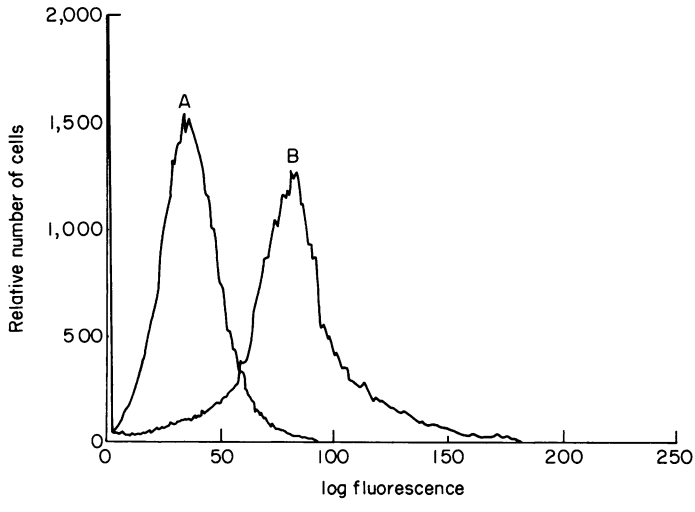

Fig. 5. Histogram illustrating flow cytometric analysis of fluorescence intensity. Paraformaldehyde fixed synovial fluid PMNL in A and paraformaldehyde fixed and acetone permeabilized synovial fluid PMNL in B.

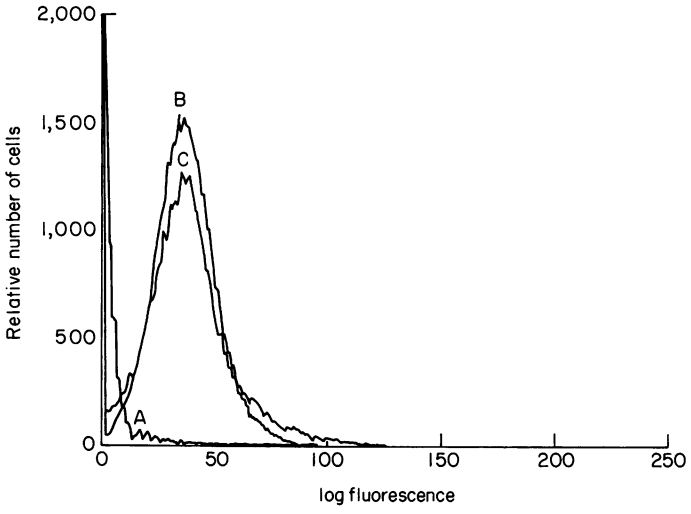

Fig. 6. Histogram illustrating flow cytometric analysis of fluorescence intensity using paraformaldehyde fixed and acetone permeabilized peripheral blood PMNL. The negative control in A, PMNL incubated in the presence of fibronectin depleted synovial fluid in B and PMNL incubated in the presence of fibronectin rich synovial fluid in C.

period, the cells were centrifuged and washed three times with PBS at 4°C. Indirect immunofluorescence was then performed and the results of the flow cytometry measurements are shown in Fig. 6. The negative control is shown in A. Peak B represents the cells incubated with fibronectin depleted synovial fluid. Peak C shows the result obtained with the cells incubated in fibronectin rich synovial fluid. No significant difference in fluorescence intensity was obtained between B and C. The outcome of this experiment suggests that a high concentration of fibronectin in synovial fluid is not likely to affect the intensity of the immunofluorescence we measured in synovial fluid PMNL.

DISCUSSION

The studies which are presented in this report were designed to test the hypothesis that fibronectin production by leucocytes might be increased in an inflammatory environment. Studies based on the assessment of the intensity of immunofluorescent staining using a fluorescein labelled antibody to human fibronectin and flow cytometry, produced evidence which tends to support this possibility. PMNL isolated from inflammatory synovial fluids of patients with RA were strongly reactive with the antibody. In contrast, non-inflammatory peripheral blood PMNL were only weakly reactive. Fibronectin was found to be distributed both inside and on the cell membrane in inflammatory PMNL. This is in contrast to non-inflammatory PMNL where fibronectin was found only inside the cell. No attempt was made in the present study to further characterize the distribution of fibronectin at an ultrastructural level. However, experiments are underway which directly address this question.

The contrasting finding between inflammatory and non-inflammatory PMNL in terms of surface fibronectin is of interest. The fact that membrane bound fibronectin is readily identified on inflammatory PMNL and not on non-inflammatory PMNL is intriguing. This finding could simply be related to the overall higher amount of fibronectin present in inflammatory PMNL. However, the excess of fibronectin present in the synovial fluid could also have lead to the binding of this glycoprotein to the PMNL surface either non specifically or through the fibronectin receptors which are now known to exist on these cells (Bevilacqua *et al.*, 1981; Pommier *et al.*, 1984). The experiments reported in Fig. 6 do not tend to support this possibility since incubating

non-inflammatory PMNL in fibronectin rich synovial fluid did not lead to the appearance of stronger immunofluorescent staining of the cells. It was also important to investigate the possibility that extracellular fibronectin could be internalized by the PMNL of synovial fluid. This could have at least partly explained the results we obtained when comparing inflammatory and non-inflammatory PMNL. Indeed, it is well known that fibronectin may form complexes with collagen and fibrin–fibrinogen products. It has also been shown to be responsible for cryoprecipitate formation in synovial fluids (Beaulieu, Valet & Strevey, 1981) and, as previously stated, it has been shown to exert opsonin activity. The results presented in Fig. 6 directly address this question and the findings do not suggest that synovial fluid fibronectin is 'picked up' by PMNL. Nevertheless, it remains possible that the *in vitro* experimental conditions we used in this study did not adequately reflect the actual *in vivo* situation. Such a possibility cannot be totally excluded. However, in another report which is presented simultaneously with this study (Ménard *et al.*, 1985) we have found evidence that fibronectin synthesis is increased in inflammatory PMNL. It is likely therefore that the higher fibronectin content of inflammatory PMNL is a direct consequence of the increased synthesis by these cells.

In this study, we did not address the question of whether the strong reactivity of PMNL with the antibody to fibronectin is restricted to RA synovial fluid or not. A prospective study is presently underway on this specific point using various sources of inflammatory PMNL. Furthermore, we did not investigate in any depth the monocyte–macrophage population of the synovial fluid. The total mononuclear leucocyte population was only weakly reactive and it is proposed in future studies to isolate the monocyte–macrophage population in order to conduct studies on this particular fraction of the total cell population. Finally, another aspect which will need to be studied is the effect of anti-inflammatory therapy, both short and long acting, on fibronectin production by PMNL.

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