Terminal Complement Proteins C5b-9 Release Basic Fibroblast Growth Factor and Platelet-derived Growth Factor from Endothelial Cells

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Summary

Interactions between endothelium and vascular smooth muscle cells play a major role in the biology of the blood vessel wall. Growth factors released from endothelial cells control in part the normal and pathological proliferation of vascular smooth muscle cells. Endothelial deposits of C5b-9 proteins, the membrane attack complex of complement (MAC), have been found in a variety of pathological tissues in which cell proliferation is an early characteristic abnormality, including atherosclerosis. We have explored a possible bridging role for terminal complement C5b-9 proteins in eliciting focal signals for cell proliferation by releasing growth factors from endothelial cells. We found that both bovine aortic and human umbilical vein cells respond to the MAC by releasing basic fibroblast growth factor and platelet-derived growth factor. These mitogens stimulate DNA synthesis in Swiss 3T3, vascular smooth muscle, and glomerular mesangial cells. Based on these findings, we propose that complement-induced release of mitogens from endothelial cells is a novel pathogenic mechanism for proliferative disorders.

In addition to its cytolcic properties, the membrane attack complex of complement (MAC) is now recognized as a mediator of a range of cellular processes in the absence of cell death (for a review see reference 1). Recent work from our laboratory suggested that focal initiation of cell proliferation may be a new pathobiological function of the complement system (2). The endothelium represents a particularly relevant model to further extend these findings. On the one hand, endothelial cells, located at the interface between blood and tissues, are natural targets of activated complement. On the other hand, endothelial cells synthesize several potent mitogens, such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), which are released in response to stimuli not yet completely understood (for a review see reference 3). Using an in vitro method of generating C5-9 complexes from purified complement components, we were able to study mitogenic activity generated by endothelial cells without the confounding influence of membrane-bound antibody, complement fragments C3a and C5a, and/or exogenously added mitogens. We report here that in two types of endothelial cells, one derived from bovine aortas and the other from human umbilical veins, C5b-9 proteins provide a potent stimulus for the release of two polypeptide mitogens synthesized by these cells, bFGF and PDGF.

Materials and Methods

Cell Culture. Bovine aortic endothelial cells (BAEC-65), human umbilical vein endothelial cells (HUVEC), and rat aortic smooth muscle cells (RASM) were kindly provided by Dr. M. A. Gimbrone, Jr. (Brigham and Women's Hospital). Swiss 3T3 cells were kindly supplied by Dr. H. Green (Harvard Medical School). Rat mesangial cells (RMC) were kindly provided by Dr. J. Bonventre (Massachusetts General Hospital, Boston, MA). BAEC-65 were seeded in 35-mm dishes and grown to confluence in DME supplemented with 10% bovine calf serum (37°C, 95% air/5% CO2). Swiss 3T3 cells were grown in 96-multiwell plastic culture plates and grown to confluence in DME-10% bovine calf serum. 24 h after reaching confluence, the cells were made quiescent by incubation for 24 h in DME-0.5% calf serum. HUVEC were cultured in 199/HBSS containing 20 mM NaCO3, 20 mM Hepes, 2 mM L-glutamine, 20% heat-inactivated FCS, 50 μg/ml endothelial cell growth factor (ECGF) (Biomedical Technologies, Inc., Stoughton, MA), and 100 μg/ml heparin (Sigma Chemical Co., St. Louis, MO).

Abbreviations used in this paper: BAEC, bovine aortic endothelial cell; bFGF, basic fibroblast growth factor; [Ca2+]i, intracellular calcium; HUVEC, human umbilical vein endothelial cell; MAC, membrane attack complex of complement; PDGF, platelet-derived growth factor; RASM, rat aortic smooth muscle cell.
RASM were grown in DME supplemented with 10% bovine calf serum, and RMC in RPMI supplemented with 15% FCS (37°C, 95% air/5% CO2). For mitogenic assays, the cells were plated in 96-multiwell plates, made quiescent, and assayed exactly as described above for 3T3 cells.

MAC Formation on Endothelial Cells and Assay for Mitogenic Activity in the Conditioned Medium. Complement protein C5b6 was made from purified C5 and C6, using factor B, bovine venom factor (CVF) (all purchased from Quidel, San Diego, CA), and recombiant factor D (gift of T. White, Metabolic Biosystems, Mountain View, CA) (4). Ni2+ (1.0 mM) was used to stabilize the CVFBB convertase (5). The C5b6 was fractionated by HPLC on a DEAE column (AP-1 Protein Pak 8HR; Waters, Milford, MA), as described (2). One unit of C5b6 was defined as the amount of C5b6 required to produce 50% lysis of 5 × 107 human RBC when incubated in a total volume of 300 μl with C7 (0.1 μg), C8 (0.5 μg), and C9 (0.5 μg) (2).

To form the MAC on endothelial cells, purified human terminal complement components diluted in DME were added sequentially as follows: 400 μl of C5b6 containing n number of hemolytic units, 400 μl of C7 (30 μg/ml), and 3 min later, 400 μl of C8 plus C9 (30 μg/ml each) (C7, C8, and C9 were from Quidel). After 45 min, the culture medium was removed, spun down, and the supernatant added to indicator cells to test for mitogenic activity.

Mitogenic Assays. Mitogenic activity was detected by adding dilutions of the conditioned media to indicator cells, and measuring [3H]thymidine incorporation into DNA, as described (2).

Neutralization of Mitogenic Activity with Specific Antibodies. Conditioned medium from MAC-treated BAEC and HUVEC were collected after 45 min and incubated with neutralizing antibodies: 100 μg/ml goat anti-human PDGF (Upstate Biotechnology Inc., Lake Placid, NY), and 50 μg/ml rabbit anti-recombinant human bFGF (kindly provided by Dr. P. D’Amore, Children’s Hospital, Boston, MA). The antigen antibody complexes were immunoprecipitated by incubation with protein G-Sepharose (60 μg/ml, Sigma Chemical Co.), followed by centrifugation. To control for nonspecific effects of the antibodies, an irrelevant goat and rabbit antibody was used. Cross-reactivity between anti-PDGF and anti-bFGF was ruled out by analyzing the effect of anti-bFGF on PDGF and of anti-PDGF on bFGF-stimulated 3T3 cells.

bFGF ELISA. A commercially available solid phase ELISA specific for human bFGF and that cross-reacts 100% with bovine bFGF was performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The ELISA uses a mouse anti-human bFGF monoclonal antibody, and a rabbit polyclonal anti-bFGF antibody conjugated to horseradish peroxidase. Dilutions of recombinant human bFGF were used for the standard calibration curve.

Measurement of Intracellular Ca2+ Activity ([Ca2+]i). Endothelial cells were plated at a density of 6 × 103 cells/cm2 on 12-mm glass coverslips that had been placed in the wells of 24-well sterile culture plates. The cells were grown to confluence, and on the day of the experiment, loaded for 30 min at 37°C with 5 μM fura-2-AM dissolved in DMSO-20% pluronic acid, and washed and placed in serum-free DME medium. The coverslips were placed in a water-jacketed chamber on a light-shielded, heated stage of an inverted fluorescence microscope (37°C ± 0.5°C). Fluorescence measurements were performed in a dual excitation spectrofluorimeter (model CM2; SPEX Industries, Edison, NJ) (6). The excitation wavelengths were 340 and 380 ± 1.8 nm. A dichroic mirror deflected the excited light (400 nm cut-off) to the perfusion chamber on the stage of an inverted epifluorescence microscope (Nikon, Nikon, Japan). The fura-2 emission signals were collected for each wavelength at 500 ± 5 nm. [Ca2+] was determined by the equation: [Ca2+] = Kd × (R - Rmin)/(Rmax - R) (7).

Results
Detection of Mitogenic Activity in Conditioned Medium from BAEC and HUVEC. To investigate whether the MAC would induce the release of mitogens from endothelial cells, BAEC were sequentially exposed to human purified complement components to form the MAC. First, the cells were incubated in serum-free medium containing purified C5b6 protein and C7 for 3 min. C8 and C9 were then added to form the MAC. After 45 min, the culture medium was removed, spun down, and the supernatant added to indicator quiescent Swiss 3T3 cells to measure DNA synthesis by [3H]thymidine incorporation. As shown in Fig. 1 a, medium conditioned by MAC-treated BAEC stimulated DNA synthesis severalfold, indicating that a potent mitogenic activity was released from the endothelial cells in response to the MAC.

Release of mitogenic activity by MAC-treated endothelial cells was rapid, achieving maximal levels within 10 min after exposure to the MAC. Furthermore, replacement of the conditioned medium every 10 min for 2 h after formation of the MAC, failed to detect any sustained release of mitogens after the initial 10 min. During the first 2 h after release, the activity was not degraded by endothelial cells, since no difference was detected in the mitogenic activity harvested from cultures 10, 20, and 60 min, and 2 h after exposure to the MAC.

Activation of complement is highly regulated. Endothelial cells, like most mammalian cells, have two well-characterized membrane-bound proteins, decay accelerating factor (DAF) and CD59, that restrict complement activation at the plasma membrane (8). Regulation by DAF and CD59 at the C3 and C8/C9 steps, respectively, is "species restricted," meaning that DAF and CD59 are most effective at inhibiting homologous complement and less effective in heterologous systems like the bovine cells/human complement used in the experiments described thus far. To further assess the potential relevance in human diseases of the phenomenon of MAC-induced release of mitogens from endothelial cells, experiments were repeated using a homologous system composed of HUVEC and human complement. The results show that a potent mitogenic activity is also released by the MAC from HUVEC (Fig. 1 b).

The ability of the MAC to release mitogenic activity from endothelial cells was dose dependent: increasing the concentration of C5b6, which is limiting in MAC formation, increased the mitogenic activity of the cell supernatant (Fig. 2 a). In BAEC, four functional units of C5b6 released mitogenic activity equivalent to ≈80% of the activity obtained with 10% calf serum, used as positive control. The mitogenic response of the indicator 3T3 cells to the conditioned medium was also dose dependent, varying with the concentration of cell supernatant used (Fig. 2 b).
Both bFGF and PDGF Contribute to the Mitogenic Activity Released by the MAC from BAEC and HUVEC. Both bFGF and PDGF are known potent mitogens for the 3T3 cells used in our mitogenic assays. It is known that bFGF, but not PDGF, is heat sensitive and has a high affinity for heparin, thus adsorbing tightly to heparin-Sepharose resins (9). Heat (56°C for 10 min), or exposure to heparin-Sepharose, reduced the mitogenic activity released by the MAC from BAEC by 30 to 40% (data not shown), indicating the possible contribution of bFGF. This was confirmed by direct quantification of bFGF using an ELISA specific for human bFGF. This assay detected an increased concentration of bFGF in the conditioned media of MAC-treated HUVEC as compared with control cells. The amount of bFGF in the conditioned media was proportional to the mitogenic activity of the media and dependent on the concentration of C5b6 used to form the MAC (Table 1).

To further elucidate the nature and relative contribution of the mitogens released from endothelial cells in response to the MAC, we used specific neutralizing anti-bFGF and anti-PDGF antibodies, insolubilized with protein G coupled to Sepharose beads. The mitogenic activity released by the MAC from both BAEC and HUVEC was blocked ≈60% by anti-PDGF and ≈30–40% by anti-bFGF. Moreover, the mitogenic activity was completely blocked when the conditioned media were sequentially exposed to both neutralizing antibodies (Fig. 3, a and b, respectively). The blocking effect was not due to nonspecific effects of the antibodies, or the protein G-Sepharose immune-immobilization procedure because no neutralizing effect was detected with irrelevant species, class- and type-matched antibodies (Fig. 3).

Mechanism of MAC-induced Release of Mitogens from Endothelial Cells. The conditioned media from BAEC and HUVEC expressed high mitogenic activity only when the cells were exposed to all terminal components sequentially in a manner that leads to formation of the MAC (Fig. 1, inset). Increased mitogenic activity was not observed when cells were incubated with (a) each terminal complement component alone; (b) C5b6 + C7; (c) C5b6 + C7 + C8; (d) any other combination of the complement components that does not result in formation of the MAC; or (e) a mixture derived from the sequential addition of C5b6 + C7 + C8 + C9 incubated together for 5 min before contact with the endothelial cells (C5b678-9 Prior, Fig. 1 inset). Under the latter conditions, the short half-life of the C5b6-7 complex in the fluid phase precludes effective MAC insertion into the plasma.
membrane. Thus, the release of mitogens from endothelial cells seems to require assembly of the MAC in the cell membrane.

Insertion of the MAC into the plasma cell membrane increases the leak of $\text{Ca}^{2+}$ from the extracellular medium and transiently increases $[\text{Ca}^{2+}]_{i}$ (10–12). To further investigate whether insertion of the MAC into the plasma membrane of the endothelial cell is a necessary condition for growth factor release, we determined in parallel the effect of different concentrations of the MAC on the cytosolic-free $\text{Ca}^{2+}$ $[\text{Ca}^{2+}]_{i}$ of fura-2–loaded endothelial cells and on the release of mitogens from those cells. The results of these experiments showed that mitogens were released into the media only at MAC concentrations that induced effective MAC insertion into the membrane as revealed by a transiently increased cytosolic $[\text{Ca}^{2+}]_{i}$, a marker of MAC-pore formation in the plasma membrane (Fig. 4). Furthermore, the mitogenic activity released by the MAC was proportional to the increased $[\text{Ca}^{2+}]_{i}$, as shown in Fig. 4.

Since insertion of MAC pores into the plasma membrane can lead to colloid-osmotic cell lysis of the target cells, we investigated the relationship between MAC-induced cytolysis and the release of mitogenic activity by MAC-treated endothelial cells. Cell viability and lysis were assessed by the release of $\text{^{51}Cr}$, and the uptake of trypan blue into the cells. Exposure of the cells to the MAC at concentrations resulting in significant release of mitogenic activity did not affect either the release of $\text{^{51}Cr}$ or the uptake of trypan blue. Furthermore, follow-up of the endothelial cells for up to 48 h after treatment with the MAC failed to detect any significant morphologic differences when compared with control cells. The results of these experiments indicate that release of mitogenic activity from endothelial cells is a sublytic effect of the MAC.

In response to the MAC, nucleated cells shed membrane vesicles that are enriched in MAC proteins, a mechanism that contributes to limit the life span of the MAC pore and to restrict colloidosmotic lysis (1, 13). To investigate whether mitogens could be released from the MAC entrapped in membrane vesicles shed in response to the MAC, conditioned media from MAC-treated endothelial cells were assayed for mitogenic activity before and after ultracentrifugation at 25,000 $g$. Others have shown that these conditions are sufficient to bring down the membrane vesicles (13). No difference in the mitogenic activity was detected when the conditioned media were assayed before and after ultracentrifugation, indicating that the growth factors released apparently were neither entrapped inside membrane vesicles nor attached to their membranes.

bFGF binds with low affinity to heparan sulfate proteoglycans on the cell surface and extracellular matrix (14). Proteoglycan binding appears to protect bFGF from degradation and provides a reservoir of active bFGF. Release of bFGF from cell surface and extracellular matrix occurs by (a) proteolysis of the proteoglycan core protein; (b) partial degradation of the heparan sulfate component with heparinase; and (c) treatment with heparin, which binds bFGF with high affinity, and competes strongly with the low affinity extracellular binding site for bFGF. Incubation of cultured endothelial cells with heparin removes active bFGF from the cell-associated

### Table 1. Presence of bFGF in Conditioned Media of MAC-treated HUVEC

<table>
<thead>
<tr>
<th>Source of conditioned media</th>
<th>C5b6 (U)</th>
<th>bFGF (pg/ml)</th>
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<tbody>
<tr>
<td>Untreated cells</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>C5b67-treated cells</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>C5b6789-treated cells (MAC)</td>
<td>2</td>
<td>27</td>
</tr>
</tbody>
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A quantitative ELISA specific for human bFGF was used to measure the concentration of bFGF in the conditioned media of control and MAC-treated cells.
Figure 3. bFGF and PDGF are released by the MAC from both BAEC and HUVEC. Endothelial cells were exposed to the MAC as in Fig. 1. Conditioned medium from MAC-treated BAEC (a) and HUVEC (b) were collected after 45 min, incubated with neutralizing antibodies, and the antigen antibody complexes were immunoprecipitated, as described in Materials and Methods. Exposure of a conditioned medium to both antibodies was done sequentially, i.e., the medium was first incubated with anti-bFGF and immunoprecipitated, and then the procedure was repeated with anti-PDGF. Controls with irrelevant class- and type-matched irrelevant antibodies were performed exactly as with specific neutralizing antibodies. (*) The bar represents the results obtained testing either rabbit or goat irrelevant antibodies.

Figure 4. Effect of increasing concentrations of the MAC on cytosolic free-calcium concentration and release of mitogens from HUVEC. (Top) BAEC cultured in gelatin-coated glass coverslips were loaded with fura extracellular sites partially depleting the cell surface and extracellular matrix reservoirs (15). To differentiate whether the MAC releases mitogens from intracellular pools or from the extracellular binding sites, BAEC were incubated for 5 min at 37°C, and washed three times with DME medium containing 100 μg/ml heparin, before exposure to the MAC. No difference in mitogenic activity was detected in media conditioned by BAEC pretreated and untreated with heparin. These results indicate that under our experimental conditions, the MAC is releasing bFGF predominantly from an intracellular source.

Mitogens Released by the MAC from Endothelial Cells Stimulate Proliferation of Vascular Smooth Muscle Cells. Growth factors released from endothelial cells play a major role in the biology of the vascular wall by controlling the normal and pathological proliferation of vascular smooth muscle cells. To de-
Table 2. Conditioned Medium from MAC-treated BAEC Stimulation DNA Synthesis in RASM and Rat Glomerular Mesangial Cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Control</th>
<th>MAC (4 U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]Thymidine uptake</td>
<td></td>
</tr>
<tr>
<td>Aortic smooth muscle</td>
<td>820 ± 50</td>
<td>1,800 ± 79</td>
</tr>
<tr>
<td>Glomerular mesangial</td>
<td>780 ± 48</td>
<td>2,800 ± 86</td>
</tr>
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</table>

Cells were grown to confluence, made quiescent, and exposed to conditioned media from BAEC cells that were and were not treated with the MAC. [3H]Thymidine uptake was assessed 36 h later as shown in Fig. 1.

Discussion

We have documented that interaction of the MAC with endothelial cell membranes results in the release of both bFGF and PDGF, two well-characterized growth factors synthesized by these cells. Conclusive evidence for the major contribution of these two growth factors to the mitogenic activity present in media conditioned by MAC-treated endothelial cells was provided by complete blockade of the mitogenic activity by the sequential addition of neutralizing anti-bFGF and anti-PDGF antibodies (Fig. 3). The presence of bFGF was further confirmed by the direct ELISA measurement of bFGF (Table 1). Which isoforms of PDGF are released in response to the MAC, and whether other growth regulatory molecules synthesized by endothelial cells are also released in small amounts together with PDGF and bFGF, is not known at present and is under investigation.

Formation of the MAC with the same concentrations of all terminal complement components, released a more potent mitogenic activity from BAEC than from HUVEC (Fig. 1). Since the number of cells in a confluent monolayer of BAEC and HUVEC (6.5 × 10⁵ ± 10⁴ and 10⁶ ± 1.8 10⁴, respectively) does not account for this difference, the observation may represent a lower sensitivity to the MAC in the human cells due to the more efficient action of CD59, a complement regulatory protein that limits formation of the MAC in a species-specific manner; e.g., C5b-9 is more efficiently limited by human than by heterologous CD59. It could also reflect different amounts and/or specific activities of the mitogens released from either cell type. We have previously shown that the MAC can deliver mitogenic signals directly to 3T3 cells (2). However, the mitogenic activity present in the supernatants of MAC-treated endothelial cells is not due to MAC complexes carried over from the endothelial cells media onto the 3T3 cells because (a) the mitogenic activity can be completely blocked with anti-bFGF and anti-PDGF antibodies (Fig. 3); and (b) the mitogenic activity present in the supernatants of MAC-treated endothelial cells could be preserved for several days at 4°C, and for at least 2 wk by freezing the samples; given the very short half-life of the C5b6-7 complex in fluid phase (seconds), it is extremely unlikely that any MAC insertion could occur after those time intervals. In conclusion, a direct mitogenic effect of the MAC is not responsible for the mitogenic activity present in conditioned media of MAC-treated endothelial cells.

Both PDGF and bFGF synthesized by endothelial cells are potent mitogens for mesenchymal cells. PDGF is secreted in response to thrombin, cell injury, and other stimuli (19). bFGF, in contrast, is a cell-associated mitogen that lacks a signal peptide characteristic of proteins secreted via the exocytic pathway (20, 21). This nonglycosylated growth factor is stored in a soluble active form in the cell cytoplasm and also is attached to the cell surface via glycosyl-phosphatidylinositol (GPI) residues (22). No stimulus for bFGF release has been characterized, and it is believed that it is liberated through breaks in the plasma membrane when cells are mechanically damaged (23). The results of the experiments presented here indicate that insertion of the MAC into the plasma membrane and a MAC-induced membrane lesion are a necessary condition for the release of growth factors (Fig. 4). This lesion, however, must be transient and very short-lived since the release of growth factors occurs in the absence of any detectable cell lysis or compromise of the cell viability for at least 48 h. The nonlethal transient changes in membrane permeability produced by the MAC (24, 25) that we and others have previously documented, may support different, not necessarily exclusive, mechanisms for release of PDGF and bFGF: (a) the cells may undergo a very rapid and transient swelling that may open permeability pathways for the release of soluble cytoplasmic compounds; (b) the MAC-induced ionic changes, including the transient increase in intracellular Ca²⁺, may activate the exocytic pathways; and (c) mitogens may leave the cell through the MAC pore itself. In addition, signal transduction through CD59 could also contribute to the release of growth factors from endothelial cells. CD59, a GPI-anchored complement regulatory protein complexed to protein tyrosine kinases, has been shown to activate metabolic pathways in other cell types (26). The possibility that the MAC-induced release of mitogens observed in our experiments could require increased expression of mRNA and/or protein synthesis is unlikely because maximum release oc-
curred as early as 10 min after exposure of the cells to the MAC. In the longer term, the MAC could trigger expression of mRNA and synthesis of growth regulatory molecules. This may occur either by a direct effect of the MAC or by an autocrine and/or paracrine action of molecules released during its initial interaction with the target cell membrane. The results of the work presented in this paper indicate that MAC-mediated release of growth factors from endothelium may be a pathogenic mechanism in proliferative disorders in which complement activation and deposition of the MAC have been clearly established. These include atherosclerosis, in both its classical and accelerated forms, mesangial cell proliferative glomerulonephritis, and rheumatoid arthritis.

Immunohistochemical methods have consistently demonstrated MAC complexes in atherosclerotic lesions (27), and the severity of cholesterol-induced atheroma is markedly reduced in rabbits deficient in C6 (28). Plasma and platelet growth factors do not fully account for the abnormal proliferation of smooth muscle cells that characterizes the atheromatous lesions, implying that other factors are involved. Our finding that conditioned media from MAC-treated endothelial cells strongly stimulates DNA synthesis in rat vascular smooth muscle cells (Table 2) indicates that MAC-induced release of bFGF and PDGF from endothelium may contribute significantly to the proliferative response of smooth muscle cells in atheromas. Synergism between bFGF and PDGF would further potentiate their mitogenic effect.

Experimental and clinical evidence indicate that complement and growth factors are involved in the onset of mesangial cell proliferation in proliferative glomerulonephritis (29). In the work reported here, we have shown that mitogens released by the MAC from endothelial cells stimulate the proliferation of mesangial cells (Table 2). Floege et al. (30) have recently found that antibody-sensitized mesangial cells lysed by complement also release active bFGF into the culture medium. Thus, release of growth factors from endothelial and from mesangial cells may act synergistically to induce mesangial cell proliferation, expansion of the mesangial matrix and, ultimately, glomerulosclerosis. It has been recently reported that bFGF released by mechanical injury of cultured endothelial cells stimulate synovial cell proliferation (31). It is conceivable that in rheumatoid arthritis, where complement activation in the synovial fluid and deposition of the MAC in the synovial surface have been well documented (20), MAC-induced release of mitogens may play a major role in the abnormal proliferation of synovial cells that characterizes this condition.

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