Role of Fcγ receptors in the activation of neutrophils by soluble and insoluble immunoglobulin aggregates isolated from the synovial fluid of patients with rheumatoid arthritis

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Abstract

***Objectives*—Synovial fluid from patients with rheumatoid arthritis contains both soluble and insoluble immunoglobulin aggregates which activate reactive oxidant production in human neutrophils. The objectives were to determine the roles played by Fcγ receptors in activation of neutrophils by these complexes.**

***Methods*—Pronase treatment was used to remove FcγRIII from the neutrophil surface and blocking monoclonal antibodies were used to prevent the binding of complexes to FcγRII and FcγRIII.**

***Results*—When FcγRIII was removed from the cell surface by pronase treatment, activation by the soluble aggregates did not occur [mean (SD) inhibition 89 (16)%, n = 6] whereas activation via the insoluble aggregates was less affected [34 (16)%, n = 6]. Blocking the binding to FcγRIII with antibodies decreased activation in response to the soluble aggregates [mean (SD) inhibition 71 (22)%, n = 8] but again had a lower effect on activation by the insoluble aggregates [40 (17)%, n = 9]. When binding to FcγRII was blocked, activation via the soluble aggregates was substantially inhibited [mean (SD) 93 (13)%, n = 8] whereas that via the insoluble aggregates was inhibited to a much lesser extent [28 (38)%, n = 9]. When FcγRII and III were simultaneously blocked, activation by the insoluble aggregates was only inhibited by 45% [(19), n = 5].**

***Conclusion*—These data thus indicate that activation of human neutrophils by soluble immunoglobulin aggregates from rheumatoid synovial fluid occurs via cooperative occupancy of both FcγRII and III: perturbation of binding to either of these receptor classes will abrogate activation.**

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In addition to their crucial role in host defence, it is appreciated that inappropriate infiltration and activation of neutrophils into tissues can result in tissue damage in inflammatory conditions such as rheumatoid arthritis. Much evidence now exists in previous reports to suggest that neutrophil activation has occurred within synovial joints[1-7] and hence it is of potential pharmacological interest to understand the molecular processes which activate and regulate neutrophil function within such diseased joints. The major neutrophil-activating factors within synovial fluid appear to be immune complexes/immunoglobulin aggregates[8-12] which are capable of activating neutrophils via interactions with their plasma membrane Fc receptors.

Neutrophils possess receptors recognising the Fc portions of IgG and IgA[13,14] and of these the Fcγ receptors are the most clearly defined. Three types of FcγR can be present.[15] FcγRI (CD64) is not present on blood neutrophils but its expression is up-regulated upon exposure to cytokines such as γ-interferon[16]; this receptor is also detected at low levels on neutrophils isolated from the synovial fluid of some patients with rheumatoid arthritis.[5] FcγRII (CD32) and FcγRIII (CD16) are both present on the surface of blood neutrophils at levels of about 7–15 000 and 100–200 000 per cell, respectively.[17] There is much debate as to the role of FcγRII and FcγRIII in neutrophil function. Neither of these bind monomeric IgG, but they bind dimers, trimers, immune complexes and opsonised particles. It is currently believed that FcγRIII binds complexes, but this binding does not activate phagocytosis, degranulation or the respiratory burst: FcγRII occupancy, however, is believed to result in neutrophil activation.[18,19] Unlike FcγRII, FcγRIII is held on the neutrophil plasma membrane via a glycerophosphoinositol anchor which is cleaved upon activation,[20] and may also be released experimentally via treatment of neutrophils with pronase, elastase and phospholipase C.

We have recently shown that synovial fluid from patients with rheumatoid arthritis contains both soluble and insoluble immunoglobulin aggregates which are capable of activating reactive oxidant production by neutrophils.[9] However, several lines of evidence indicate that these aggregates activate neutrophils via distinct mechanisms. Firstly, the soluble aggregates only activate neutrophils that have been primed in vivo or in vitro by GM-CSF or γ-interferon. Secondly, the soluble aggregates activate a transient (2–4 minutes) burst of oxidase function in primed cells whereas the insoluble aggregates stimulate a slower (15–20 minutes) activation in primed

or unprimed cells. Thirdly, activation via the soluble aggregates is staurosporine-insensitive (and hence probably protein kinase C-insensitive), whereas that activated by the insoluble aggregates is staurosporine-sensitive.[21] Thus these observations indicate that the soluble and insoluble aggregates activate neutrophils via processes which differ in their receptor/signal transduction pathways. The aim of this work therefore was to determine the roles of FcγRII and FcγRIII in neutrophil activation via these immunoglobulin aggregates isolated from the synovial fluid of patients with rheumatoid arthritis.

Materials and methods

PREPARATION OF NEUTROPHILS

Neutrophils were isolated from heparinised venous blood of healthy volunteers using Mono Poly-resolving Medium exactly as described in the manufacturers instructions.[22] The process involved the separation of neutrophils via density gradient centrifugation at 600 g for 25 minutes. The neutrophil band was removed and subjected to hypotonic lysis to eliminate contaminating erythrocytes. After purification, neutrophils were washed and resuspended in RPMI 1640 medium (containing 20 mM HEPES) and their purity (>97%) and viability (>95%) assessed by May-Grünwald/Giemsa staining and trypan blue exclusion, respectively. Cells were counted using a Fuchs-Rosenthal haemocytometer slide after a 100 fold dilution, and used within 5 hours of preparation.

PREPARATION OF SYNOVIAL FLUID

Synovial fluid was collected by aspiration of joints from rheumatoid arthritis patients with knee effusions and collected into heparinised tubes. The fluid was then centrifuged at 600 g for 10 minutes to remove the infiltrated cells. The cell free fluid was then either used immediately or else stored in aliquots at −20°C, before fractionation by centrifugation at 11 600 g for 2 minutes.[9] The resultant supernatant was retained whilst the pellet was resuspended to an equivalent volume in RPMI 1640 medium. In all, synovial fluid samples from 9 different patients with seropositive rheumatoid arthritis were examined.

NEUTROPHIL PRIMING

Suspensions of neutrophils were incubated for 1 hour at 37°C in RPMI 1640 medium at 10^7 cells/ml. Control suspensions contained no further additions whilst primed neutrophils were supplemented with 50 U/ml recombinant (r) GM-CSF.[23] After incubation under these conditions the ability of the cells to generate reactive oxidants was assessed.

MEASUREMENT OF REACTIVE OXIDANT GENERATION

Chemiluminescence was performed on suspensions of neutrophils (5×10^5/ml) suspended in RPMI 1640 medium containing 10 μM luminol in a total volume of 1 ml, at 37°C.[24] After the addition of stimuli, photon emission was measured at 37°C using either a 25 channel LKB Wallac 1251 luminometer or else a single channel LKB 1250 luminometer.

ANALYSIS OF NEUTROPHIL MEMBRANE RECEPTOR EXPRESSION

The monoclonal antibodies used were IV3 (anti-CD32, from Mederex) and Leu 11b (anti-CD16, from Becton Dickinson) which recognise the intermediate and low affinity receptors respectively, of the Fc portion of IgG (that is, FcγRII and FcγRIII). 31D8 was a gift from Dr Krause, Hartford Hospital, Connecticut, USA: 31D8 positive cells avidly bind formylpeptides, whereas 31D8 negative cells do not respond functionally to fMet-Leu-Phe.[25,26] For immunostaining of isolated neutrophils,[27] cells were suspended in PBS/1% BSA (globulin-free)/0·1% sodium azide, pH 7·2, and receptor expression measured using a standard indirect immunofluorescence technique using FITC-labelled goat-(anti-mouse) immunoglobulin as a second layer. Both first and second layer antibodies were added at saturating concentrations, and in all experiments non-immune mouse IgG of the appropriate isotype was included as a class specific first layer control. Stained cells were fixed in 1% paraformaldehyde in PBS and analysed using a Becton Dickinson FACS Analyser 1 and a Consort 30 computer and software. Fluorescence distributions represent a total of 5000 gated events (cells), with the mean fluorescence proportional to the number of specific antigenic sites per cell.

PRONASE TREATMENT

Suspensions of primed neutrophils (1×10^7) were incubated in the presence of 50 μg/ml pronase for 30 minutes at 37°C, to cleave GPI-linked membrane receptors. Cells were primed in order to mobilise sub-cellular pools of receptors on to the plasma membrane before addition of pronase. Membrane receptor expression was subsequently analysed by immunostaining and chemiluminescence determined as described above.

MONOCLONAL ANTIBODY BLOCKING STUDIES

Monoclonal antibodies 197 (anti-FcγRI/CD64, Mederex), IV3 (anti-FcγRII/CD32, Mederex) and Leu 11b (anti-FcγRIII/CD16, Becton Dickinson) were used at saturating concentrations (2·5 μg/ml), after the preservative azide was removed by washing with PBS through Millipore 30 kDa filters (Ultrafree-MC filter unit, low-binding PLTK membrane). Antibodies were added to neutrophil suspensions (2×10^6/ml) and incubated for 10 minutes at 37°C prior to addition of rheumatoid immune complexes and analysis of chemiluminescence.

Results

THE EFFECT OF PRONASE TREATMENT ON NEUTROPHIL MEMBRANE EXPRESSION AND FUNCTION

When primed neutrophils were treated with pronase (50 μg/ml for 30 minutes) their membrane expression of 31D8 and FcγRIII was considerably decreased (fig 1) compared with cells incubated in the absence of the enzyme [mean (SD) decrease in expression = 89% (2), n = 3, and 76% (3), n = 3, for 31D8 and FcγRIII respectively). However, pronase treatment did not significantly affect FcγRII or CD11b receptor expression (mean (SD) decrease in expression = 0·7% (0·6), n = 3, and 9% (8), n = 3, for FcγRII and CD11b respectively). Thus, pronase treatment cleaved FcγRIII and the receptor recognised by monoclonal antibody 31D8 (which are GPI-linked), without markedly affecting FcγRII or CD11b (which are attached to the plasma membrane via a hydrophobic transmembrane anchor).

When primed neutrophils were incubated with pronase and then stimulated with fMet-Leu-Phe (fig 2A), the chemiluminescence response was inhibited by 60% (mean (SD) inhibition = 62% (13), n = 6) compared with responses of cells incubated in the absence of pronase. PMA stimulation of oxidant production was not markedly affected by pronase treatment (mean (SD) inhibition = 16% (7), n = 5, fig 2B) as was expected because PMA activates protein kinase C and does not utilise a plasma membrane receptor. The precise receptor recognised by monoclonal antibody 31D8 is not certain but previous work has clearly shown that 31D8-positive cells are fMet-Leu-Phe responsive, whereas 31D8-negative cells are not.[25 26]

We have previously shown that rheumatoid synovial fluid contains soluble and insoluble IgG-containing immune complexes.[9] Protein A affinity chromatography removes both types of complex and the flow through from this column does not activate the respiratory burst of neutrophils. This treatment thus removes all respiratory burst activating factors from synovial fluid and hence these two types of complex are the only factors within synovial fluid that can activate the respiratory burst in our experiments. Furthermore, because they bind protein A, they are likely to interact with neutrophils via Fcγ receptors.

The soluble and insoluble immunoglobulin aggregates from synovial fluid of patients with rheumatoid arthritis were then separated by centrifugation.[9] Activation of primed neutrophils with the soluble aggregates resulted in a chemiluminescence response which reached a maximal value by 3–4 minutes and then declined (fig 3A). However, this oxidase activity was largely absent in neutrophils which had been incubated with pronase (mean (SD) inhibition = 89% (16), n = 6). Activation of primed cells with the insoluble immunoglobulin aggregates from synovial fluid resulted in a slower activation of oxidase activity which reached a maximal value by 15–20 minutes after addition (fig 3B). However, cell suspensions treated with pronase still generated substantial levels of reactive oxidants in response to these insoluble complexes (mean (SD) inhibition = 34% (16), n = 6): similar values for oxidant production in pronase treated cells were obtained using these complexes at 50% (v/v). Because FcγRII expression is not affected by pronase treatment the inhibition of oxidant production via the soluble immunoglobulin aggregates is likely to be due to the cleavage of FcγRIII. Thus FcγRIII is required for mediating oxidant production in response to the soluble immunoglobulin aggregates, but this receptor plays a less important role in oxidant production stimulated by the insoluble immunoglobulin aggregates.

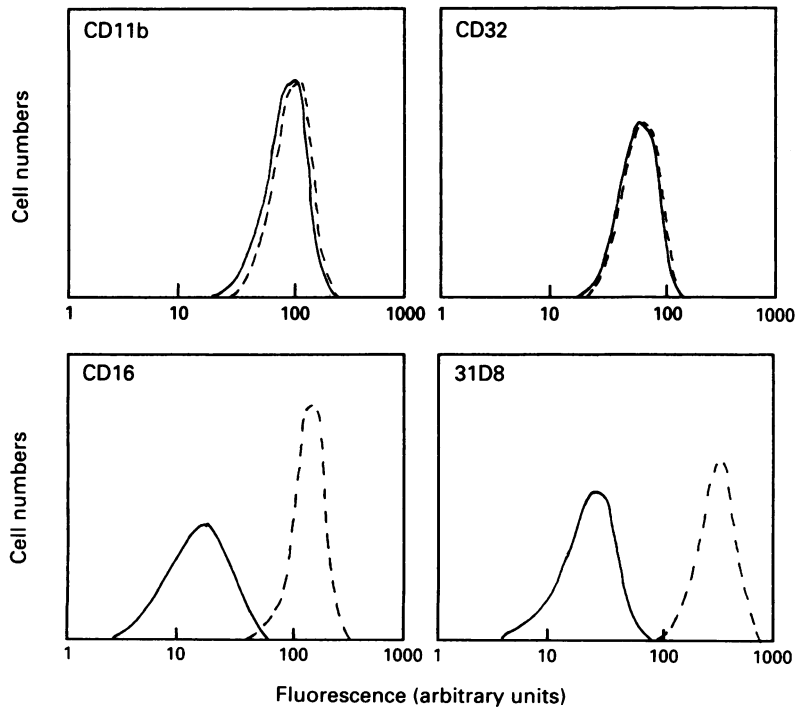

Figure 1 Plasma membrane receptor expression after pronase treatment. Control bloodstream neutrophils in RPMI (10^7 cells/ml) were incubated for 1 hour at 37°C in the presence of GM-CSF (50 U/ml) Cells were then further incubated in the presence or absence of pronase (50 μg/ml) for 30 minutes at 37°C. Receptor expression (CD32, CD16, 31 D8 and CD11b) was quantified by immunostaining (– – – Primed, —— Pronase treatment). Similar results were found in at least five further experiments.

THE EFFECT OF BLOCKING MONOCLONAL ANTIBODIES ON NEUTROPHIL OXIDANT PRODUCTION

A more specific method for assessing the role of FcγR receptors in neutrophil function utilised blocking monoclonal antibodies against a particular FcγR receptor. When the soluble immune complexes were isolated from cell free synovial fluid and used to stimulate primed neutrophils, the presence of IV3 (FcγRII) and Leu 11b (FcγRIII) inhibited the mean (SD) chemiluminescence responses by 93% [(13), n = 8] and 71% [(22), n = 8] respectively (fig 4A). Thus blocking the binding of these soluble aggregates to FcγRII completed abrogated oxidase activation. When the isolated insoluble immunoglobulin aggregates were used to stimulate primed cells, blocking with IV3 and Leu 11b again resulted

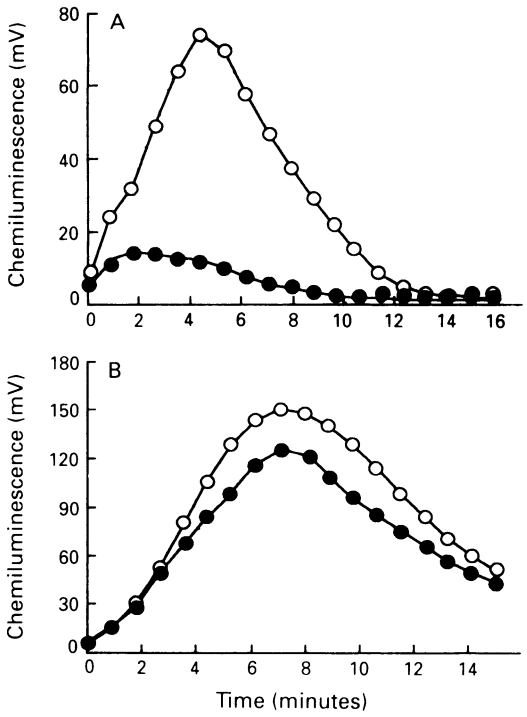

Figure 2 The effect of pronase treatment on fMet-Leu-Phe and PMA induced neutrophil chemiluminescence. Control bloodstream neutrophils in RPMI (10^7 cells/ml) were incubated in the presence of GM-CSF (50 U/ml) for 60 minutes at 37°C. The primed cells were incubated for a further 30 minutes at 37°C in the presence (●) or absence (○) of pronase (50 μg/ml). At time zero, 5×10^5 cells were stimulated with (A) μM FMLP or (B) 0·1 μg/ml PMA (final concentrations), and the chemiluminescence measured using an LKB 1251 luminometer. Both assays were performed at 37°C in the presence of 10 μM luminol in RPMI to a final volume of 1 ml. Similar results were found in at least four further experiments.

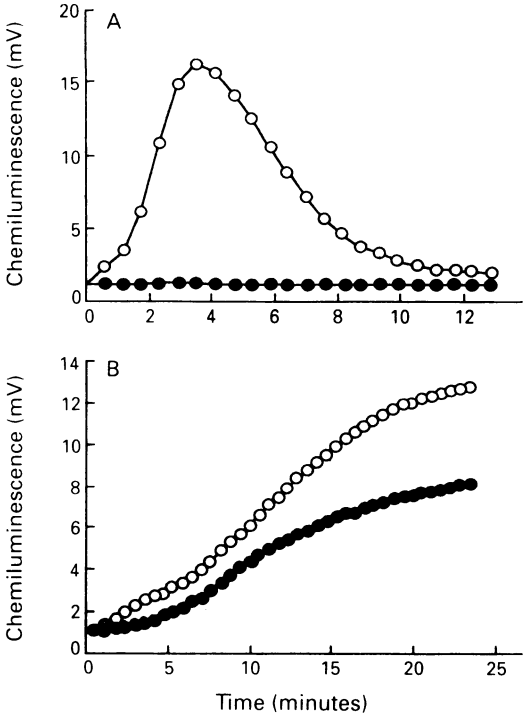

Figure 3 The effect of pronase treatment on neutrophil chemiluminescence activated by soluble and insoluble immunoglobulin aggregates from rheumatoid synovial fluid. Control bloodstream neutrophils in RPMI (10^7 cells/ml) were incubated in the presence of GM-CSF (50 U/ml) for 60 minutes at 37°C. The primed cells were incubated for a further 30 minutes at 37°C in the presence (●) or absence (○) of pronase (50 μg/ml). At time zero, 5×10^5 cells were stimulated with (A) 10% (v/v) final concentration of the supernatant of cell free synovial fluid, or (B) 10% (v/v) final concentration of the pellet of cell free synovial fluid, and the chemiluminescence measured using an LKB 1251 luminometer. The pellet and supernatant were derived by centrifugation of the cell free synovial fluid for 2 minutes at 11 600 g. Assays were performed at 37°C in the presence of 10 μM luminol in RPMI to a final volume of 1 ml. Similar results were found in at least five further experiments.

in a much lower inhibition (fig 4B). For example, when binding to FcγRII was blocked, mean (SD) inhibition was only 28% [(38), n = 9] whilst blocking the binding to FcγRIII resulted in 40% [(17), n = 9]. Similar results were obtained using the insoluble immune complexes at 10 or 50% (v/v) final concentration. Blocking both FcγRII and III simultaneously only inhibited activation by the insoluble aggregates by 45% [(19), n = 5]. Doubling the concentrations of antibodies did not significantly increase the level of inhibition.

These experiments indicate that both FcγRII and FcγRIII are important in the activation of neutrophil oxidant production by soluble immunoglobulin aggregates. The role of these receptors in neutrophil activation by insoluble aggregates is less defined. The monoclonal antibodies used had no inhibitory effect on fMet-Leu-Phe or PMA stimulated neutrophil activation, and 197 (anti-FcγRI) failed to inhibit the chemiluminescence response to fMet-Leu-Phe, PMA or soluble or insoluble immunoglobulin aggregates (data not shown).

Discussion

The combined use of depleting the neutrophils of FcγRIII by pronase treatment and the use of blocking monoclonal antibodies has shown that FcγRII and FcγRIII are both required to varying extents for the activation of primed neutrophils by the soluble and insoluble immunoglobulin aggregates. Treatment of primed neutrophils with pronase resulted in about 80% depletion of FcγRIII from the cell surface, but did not affect FcγRII expression. Stimulation of cells with fMet-Leu-Phe after pronase treatment was considerably decreased (as would be predicted because 31D8 expression is closely linked to fMet-Leu-Phe responsiveness[25 26]) whereas activation by PMA was largely unaffected. This indicates that pronase treatment did not non-specifically affect neutrophil responsiveness. It is thus of great interest that pronase treatment largely abolished the ability of neutrophils to generate reactive oxidants in response to the soluble immunoglobulin aggregates isolated from the synovial fluid of patients with rheumatoid arthritis. Conversely, activation via the insoluble aggregates was inhibited to a much lower extent by pronase treatment. The use of blocking monoclonal antibodies largely confirmed the results of pronase treatment in that activation via the soluble aggregates largely requires FcγRIII and FcγRII. However, the use of both monoclonal antibodies together only inhibited activation by the insoluble complexes by about 50%.

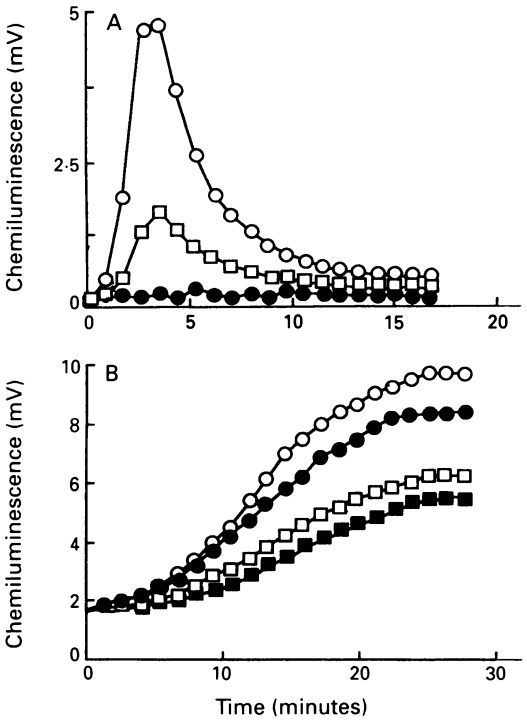

Figure 4 The effect of blocking monoclonal antibodies on neutrophil chemiluminescence activated by soluble and insoluble immunoglobulin aggregates from rheumatoid synovial fluid. Control bloodstream neutrophils in RPMI (2×10^6 cells/ml) were incubated in the presence of GM-CSF (50 U/ml) for 60 minutes at 37°C. The primed cells were incubated for a further 10 minutes at 37°C in the absence (○) or presence of Leu 11b (anti-FcγRIII, □) or IV3 (anti-FcγRII, ●), or both antibodies together (■). At time zero, 2×10^5 cells were stimulated with (A) 10% (v/v) final concentration of the supernatant of cell free synovial fluid, or (B) 10% (v/v) final concentration of the pellet of cell free synovial fluid, and the chemiluminescence measured using an LKB 1251 luminometer. The pellet and supernatant were derived by centrifugation of the cell free synovial fluid for 2 minutes at 11 600 g. Assays were performed at 37°C in the presence of 10 μM luminol in RPMI to a final volume of 1 ml. Similar results were found in at least seven further experiments.

There is much debate in previous reports regarding the role of FcγRII and FcγRIII in neutrophil function. On the one hand, it has been proposed that occupancy of FcγRII is required for activation of reactive oxidant production, degranulation and phagocytosis, whereas FcγRIII is only required for the binding of immune complexes without subsequent activation.[18 19 28] On the other hand, some recent evidence has suggested that both FcγRII and FcγRIII are involved in activation of the respiratory burst by immune complexes.[29 30] However, if FcγRIII is required for binding, then blocking occupancy of this receptor may indirectly affect the efficacy by which FcγRII can generate intracellular signals in response to immune complexes. Hence, FcγRII and FcγRIII probably function cooperatively to bind immune complexes and generate intracellular signals which lead to cell activation. Such cooperation between these two receptors is also suggested because FcγRIII occupancy can enhance subsequent FcγRII-dependent phagocytosis.[31]

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