Terminal Complement Complex C5b-9 Stimulates Mitogenesis in 3T3 Cells

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Abstract

The membrane attack complex of complement (MAC) can induce reversible changes in cell membrane permeability resulting in significant but transient intracellular ion changes in the absence of cell lysis. Because ion fluxes and cytosolic ion changes are integral steps in the signaling cascade initiated when growth factors bind to their receptors, we hypothesized that the MAC-induced reversible changes in membrane permeability could stimulate cell proliferation. Using purified terminal complement components we have documented a mitogenic effect of the MAC for quiescent murine 3T3 cells. The MAC enhances the mitogenic effects of serum and PDGF, and also stimulates cell proliferation in the absence of other exogenous growth factors. MAC-induced mitogenesis represents a novel effect of the terminal complement complex that could contribute to focal tissue repair or pathological cell proliferation locally at sites of complement activation. (J. Clin. Invest. 1993. 91:1974–1978.) Key words: complement • C5b-9 • mitogenesis • cell proliferation • growth factors

Introduction

The complement system is composed of 12 activation proteins and at least 13 regulatory proteins that function as either fluid phase or membrane-attached inhibitors. There are two major pathways of complement activation, classical and alternative, which converge at the level of the C3 step. Thereafter both pathways share a common sequence through the late components C5, C6, C7, C8, and C9, leading to the generation of the membrane attack complex of complement (MAC).1 Assembly of the MAC is initiated upon cleavage of C5 and generation of C5b. C5b reacts with C6 and C7 forming C5b-7 which binds to the cell membranes; then, binding of C8 occurs and the resulting C5-8 complex incorporates several molecules of C9 to form C5b-9, the MAC. The number of C9 molecules per complex (n) is believed to be one to two in homologous cell/serum systems and three to four in heterologous systems (1). The MAC is a transmembrane pore which is functionally expressed as an increased membrane permeability (2, 3). The cation leak through the MAC can lead to colloidosmotic swelling and lysis of the target cell or, at sublytic concentrations, to large but reversible changes in the internal composition of the cell (4). Ca2+ ions that leak through the MAC transiently increase the internal Ca2+ concentration with different functional consequences to the target cell (5–8). In erythrocytes, recruitment of a Ca2+-activated K+ channel balances the internal cation content preventing cell swelling (9), and Ca2+-dependent vesiculation contributes to terminate the complement lesion (10).

It is well established that the response of growth-arrested cells to a variety of growth factors and pharmacological mitogens involves a series of ionic changes such as a rapid increase in cytosolic free Ca2+ ([Ca2+]i), activation of Ca2+-sensitive K+ channels with membrane hyperpolarization, and stimulation of Na+/H+ exchange which results in alkalization of the cytosol (11–13). These changes that occur within minutes after mitogenic stimulation are believed to play a critical role in the initiation of DNA synthesis that begins many hours later. We investigated whether the transient changes in membrane permeability induced by the MAC could also induce cell proliferation. We report here that in Swiss 3T3 cells, the MAC enhances the mitogenic effect of serum and PDGF, and also stimulates cell proliferation in the absence of exogenous growth factors. The phenomenon of MAC-induced mitogenesis may represent a novel role for the complement system: directed regulation of cell proliferation.

Methods

Cell culture. Mouse Swiss 3T3 cells (kindly provided by Dr. H. Green, Department of Physiology, Harvard Medical School), were used between passages 17 and 32. The cells were grown in DME supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, at 37°C in 95% air/5% CO2. For mitogenic assays, the cells were seeded in 48-well plates at 104 cells per well. When they reached confluence, usually between 3 and 4 d, the medium was replaced with DME 0.5% serum to make them quiescent, and mitogenesis assays were performed 24 ± 2 h later.

Measurement of intracellular Ca2+ activity. Swiss 3T3 cells were plated at a density of 6 × 104 cells/cm² on 12-mm glass coverslips, which had been placed in the wells of 24-well sterile culture plates. The cells were grown to confluence, as described above. The day of the experiment, the cells were loaded with 5 µM fura-2 for 30 min at 37°C, washed, and placed in serum-free DME medium containing 0.1% gelatin. The coverslips were placed in a water-jacketed chamber on a light shielded, heated stage of an inverted fluorescence microscope (37±0.5°C). Fluorescence measurements were performed in a CM2 dual excitation spectrophuorimeter (SPEX Industries Inc., Edison, NJ) (14). The excitation wavelengths were 340 and 380±1.8 nm. A dichroic mirror deflected the excited light (400 nm cut-off) to the perfusion channel of the stage of an inverted epifluorescence microscope (Nikon). The Fura-2 emission signals were collected for each wavelength at 500±5 nm. Cells were individually calibrated in situ by exposure to Ca2+-free buffers containing LaCl3. Intracellular free calcium ([Ca2+]i) was determined by the equation: [Ca2+]i = Kd × Q/R

1. Abbreviations used in this paper: [Ca2+]i, intracellular free calcium; MAC, membrane attack complex of complement.

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Complement components/assays. C5b6 was made from purified C5 and C6, using factor B, cobra venom factor (all purchased from Quidel, San Diego, CA), and recombinant factor D (gift of T. White, scios, Mountain View, CA) (16). Ni²⁺ (1.0 mM) was used to stabilize the cobra venom factor Bb convertase (17). The C5b6 was fractionated by HPLC on a DEAE column (AP-1 Protein Pak 8HR; Waters Associates, Milford, MA). The reaction mixture was applied in buffer A (60 mM NaCl, 10 mM sodium phosphate < pH 7.6) and eluted with a linear 60-min gradient of buffer A and buffer B (500 mM NaCl, 10 mM sodium phosphate, pH 7.6). The C5b6 eluted as a distinct peak with a characteristic absorption of spectra at 220, 250, and 280 nm (Diode Array Spectrophotomer 1040A; Hewlett-Packard Co., Avondale, PA) and a reproducible retention time of 39–40 min.

The C5b6 pool was titrated the same day of the experiment using human red blood cells. 1 U of C5b6 was defined as the amount of C5b6 required to produce 50% lysis of 5 × 10⁷ human red blood cells when incubated in a total volume of 300 μl with C7 (0.1 μg), C8 (0.5 μg), and C9 (0.5 μg) (Quidel).

Uptake of [³H]thymidine. 3 h after exposure of quiescent Swiss 3T3 cells to a mitogenic stimulus, 1 μCi/ml of [³H]thymidine was added to the wells, and the cells maintained at 37°C/5% CO₂ for an additional 21 h. Then the cells were washed three times with DME medium and the acid-precipitable radioactivity was extracted with cold 10% TCA. After neutralization with 0.3 N NaOH, aliquots were counted in a Tri-Carb Scintillation counter (Packard Instrument Co., Downers Grove, IL). For negative controls, the cells were kept in 0.5% serum, and for positive controls they were stimulated with 10% serum. In all experiments, each control and experimental point was performed in quadruplicate (four wells). The protein content of each well, measured by the Comassie-Plus Protein Assay Reagent as per manufacturer’s instructions (Pierce Chemical Co., Rockford, IL), was used to assess the number of cells/well. When the dispersion of the protein readings is <5%, incorporation data are expressed as counts per minute per well.

Results

The MAC transiently increases intracellular Ca²⁺. Cultured Swiss 3T3 cells can be made to enter reversibly the nonproliferative G₀ phase of the cell cycle by the withdrawal of serum. When supplied with the appropriate growth factors, the cells reenter G₁ and progress into the cell cycle. Because the early ionogenic changes that occur in response to growth factors are very similar to those induced by the MAC, we hypothesized that the MAC could provide a stimulus for cell proliferation. To test this hypothesis, confluent serum-deprived quiescent Swiss 3T3 cells were exposed to the MAC assembled from purified human C5b6 plus C7 (3 min at 37°C), and subsequent addition of C8 and C9. To correlate the hemolytic titer of the MAC with reversible nonlethal ionogenic changes in the 3T3 cells, we measured the effect of the MAC on the intracellular Ca²⁺ concentration of these cells. When Fura-2–loaded 3T3 cells were exposed to 1 U of C5b6 and 9 μg of C7 (3 min at 37°C), addition C8 and C9 (9 μg each) resulted in a transient increase in intracellular Ca²⁺ (Fig. 1). Similar to the effect of growth factors such as PDGF and EGF (12), the MAC at the concentrations used promoted a rapid rise in [Ca²⁺], that reached a peak before declining to a new level, slightly higher than the unstimulated value. The effect of the MAC on [Ca²⁺], shown in Fig. 1 was expected from the work of others (18); however, this is the first demonstration of a transient rise in [Ca²⁺], induced by the MAC assembled from purified components and at the single cell level. The advantage of using purified components is that it avoids the early, MAC-independent, oscillations on [Ca²⁺], observed when the complex was activated from C8-deficient sera (19). The concentrations of C5b6 used for subsequent experiments was correlated with its hemolytic activity in our standardized assay.

The MAC enhances the mitogenic effect of serum. Synthesis of DNA by quiescent fibroblasts was stimulated by serum in a dose-dependent manner (Fig. 2, lower curve). When terminal complement components were present in the medium to form the MAC simultaneously with the addition of serum, the mitogenic effect was significantly enhanced, as detected by the increased incorporation of [³H]thymidine (Fig. 2, upper curve). The stimulatory effect was not produced by C5b6 alone, nor by

**Figure 1.** Effect of the MAC on the free intracellular calcium of Swiss 3T3 cells. Fura-2–loaded 3T3 cells were preexposed to 1 U of C5b6 and 9 μg of C7 in a volume of 400 μl for 3 min. The arrow indicates the time when C8/C9 (9 μg each, in a volume of 200 μl) were added.

**Figure 2.** Effect of the MAC on the mitogenic effect of serum. Cells (4 × 10⁴) were plated in 48 multiwell plastic culture plates. 24 h after reaching confluency, the cells were made quiescent by incubation for 12–18 h in DME-0.5% calf serum. Then the cells were stimulated with different concentrations of serum in the presence and absence of the MAC. Complement components and different concentrations of serum diluted in DME were added sequentially as follows: 200 μl of C5b6 (1 U), 200 μl of C7 (9 μg), and 3 min later 200 μl of the appropriate serum dilution to obtain final concentrations of 0, 0.1, 0.25, 0.5, 1.2, and 5% with and without C8 + C9 (9 μg each). DNA synthesis was determined by measuring the uptake of [³H]thymidine. Each point represents the mean±SEM of quadruplicate values obtained in one representative experiment which was performed four times with comparable results. (Inset) Lack of effect of single or combined complement components in mixtures that do not form the MAC. The serum concentration was 2.5%, and maximum [³H]thymidine uptake was 4,200 cpm/well.
the individual complement components alone or by any combination that did not include the serial addition of C5b6, C7, and C8 + C9 (inset, Fig. 2). Thus, the stimulatory effect of the terminal complement components for the mitogenic effect of serum required the formation of the MAC.

The MAC enhances the mitogenic effects of PDGF. Because the principal mitogen present in whole blood serum is PDGF, we determined the effect of the MAC on the mitogenic effect of purified PDGF. These experiments were conducted in serum-free medium containing 0.1% gelatin, 30 ng/ml IGF, and 10 μg/ml transferrin, the empirically determined minimum requirements for PDGF-stimulated cells to enter into the growth cycle in the absence of serum. The MAC significantly enhanced the mitogenic effect of PDGF (Fig. 3). Similar to the effect on serum-stimulated mitogenesis, the effect of PDGF was not potentiated by either individual complement components or any combination of these components that does not form the MAC. These results indicate that cells targeted by the MAC are sensitized to the mitogenic effect of PDGF.

The MAC is mitogenic in the absence of exogenous growth factors. In some experiments the MAC stimulated the uptake of [3H]thymidine in the absence of any serum or PDGF, suggesting a possible mitogenic effect of the MAC by itself. Indeed, exposure of quiescent fibroblasts to the MAC in serum and PDGF-free media, stimulated DNA synthesis in a dose-dependent manner (Fig. 4).

Growth-arrested cells reinitiate DNA synthesis ~12 h after mitogenic stimulation. Thus, kinetic differences in the progression of the cells through the cell cycle could explain the increased uptake of [3H]thymidine observed in the presence of the MAC. This possibility was explored by following the time course of thymidine uptake in the presence of either PDGF or the MAC. The results shown in Fig. 5 indicate that increased DNA synthesis and not different kinetics of [3H]thymidine incorporation explain the effect of the MAC observed in our experiments.

To assess whether the transient increase in intracellular Ca2+ induced by the MAC would suffice to initiate the mitogenic response, quiescent cells were exposed to a concentration of the Ca2+ ionophore A23178 that promotes an increased in-

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**Figure 3.** Effect of the MAC on the mitogenic effect of PDGF. Uptake of [3H]thymidine by quiescent 3T3 cells exposed to the same concentrations of the complement components as in Fig. 2, but to PDGF instead of serum (experimental procedures as described for Fig. 2). DME used for this experiment was serum free and contained 0.1% gelatin, 30 ng/ml IG, and 10 μg/ml transferrin (DMS**+**). Final concentrations of PDGF were 0, 0.1, 0.5, 1, 2.5, and 5 ng/ml. Each point represents the mean±SEM of quadruplicate values obtained from one representative experiment of four experiments performed.

**Figure 4.** Mitogenic effect of the MAC in the absence of other exogenous mitogens. Quiescent Swiss 3T3 cells placed in DME**++** were exposed sequentially to complement components to form the MAC. The concentrations of C5b6 were 1 and 3 U, and those of C7, C8, and C9 were as described for Fig. 2. Results are expressed as relative values of the [3H]thymidine uptake into cells stimulated with 10% calf serum. Each bar depicts the mean±SEM of the relative thymidine incorporation calculated from two sets of quadruplicate values obtained in two experiments.

**Figure 5.** Comparison of the kinetics of [3H]thymidine uptake induced by the MAC and PDGF. Uptake of [3H]thymidine by Swiss 3T3 cells was measured at different time intervals after mitogenic stimulation of quiescent 3T3 in serum-free medium. (○) DME**++**; (△) C5b6 (1 U); (●) PDGF (2.5 ng/ml); (■) C5b-9 (1 U C5b6 + C7 + C8 + C9, 15 μg/ml each).
ternal Ca\(^{2+}\) comparable to that in Fig. 1. To mimic the transient increase observed with the MAC the ionophore was left in the medium for a time interval of 3 min and then aspirated and replaced by exactly the same medium without A12378. The transient Ca\(^{2+}\) influx produced with the ionophore did not increase DNA synthesis or enhance the mitogenic response to PDGF (2.5 ng/ml).

**Discussion**

The results presented in this paper are consistent with a mitogenic effect of the MAC: It sensitized 3T3 cells to the mitogenic stimulus of serum and PDGF, and also stimulated cell proliferation when assembled in the cell membrane in the absence of any exogenously added growth factors. These experiments suggest that control of cell proliferation may be a previously unrecognized function of the complement system.

Two major types of growth factors receptors have now been characterized: receptor tyrosine kinases (PDGF, EGF, and FGF) (20), and G protein coupled receptors (\(\alpha\)-thymosin, S-HT) (21). Activation of growth factor receptors' transmembrane signaling molecules stimulates PI3, phospholipase C, which in turn activates the phosphatidylinositol cascade of second messengers generating IP3 and diacylglycerol. Diacylglycerol is the physiological activator of protein kinase C, which accounts for some of the early mitogenic events, including activation of Na\(^+\)/H\(^+\) exchanger, and phosphorylation of the ribosomal S6 protein. Associated with these biochemical phenomena, mitogens also induce a rapid increase in [Ca\(^{2+}\)], which may come from intracellular stores, and/or from influx of extracellular Ca\(^{2+}\) through channels in the plasma membrane (12).

Fragments of complement factors B and C3 can influence B lymphocyte proliferation (22, 23) through specific membrane receptors. A novel aspect of MAC-stimulated mitogenesis is that it would not be restricted to a specific cell type because the MAC inserts into the lipid bilayer of the cell membrane and does not require specific membrane receptors. Although the transduction of intracellular signals by the MAC is not completely understood, it has been established that several pathways of intracellular signaling are activated by the MAC, including protein kinase C (24, 25), the adenlylcyclase system which raises cyclic AMP (19), and the Ca\(^{2+}\)-activated K\(^+\) efflux (9), which is stimulated by the MAC-induced increase in [Ca\(^{2+}\)]. Thus, there are several points of convergence where the MAC could interact synergistically with the mitogenic pathways to promote cell proliferation. One common cellular effect that could mediate the MAC-induced mitogenesis is the increased [Ca\(^{2+}\)], particularly since increased [Ca\(^{2+}\)] seems to be a necessary condition for the MAC to induce cell proliferation (Fig. 1). However, MAC-induced mitogenesis is not only related to the changes in [Ca\(^{2+}\)] since an ionophore-induced influx of Ca\(^{2+}\) comparable in magnitude and duration to the one induced by the MAC failed to stimulate DNA synthesis. This result implies that additional effects of the MAC contribute to its mitogenic effect. Rozenburg shored that mellitin, an amphipathic pore-forming polypeptide that increases Na influx also stimulates DNA synthesis in quiescent Swiss 3T3 cells (26). Whether the mitogenic effect of the MAC is solely the consequence of Ca\(^{2+}\) and other MAC-induced ionic changes, or whether it also directly stimulates chemical signaling pathways in a manner that short-circuits the receptor-initiated reactions, is not known at present.

We propose that activation of complement and formation of the MAC may transmit local mitogenic signals. The significance of the MAC regulating cell proliferation lies in the fact that complement is uniquely adapted to deliver a focal mitogenic stimulus: generation of the MAC is tightly controlled by the specificity and restriction of complement activation. MAC stimulated mitogenesis could normally contribute to focal tissue repair. Under circumstances of abnormal complement activation, the MAC may provide a direct stimulus (Fig. 4) and/or synergistic action with other growth factors (Figs. 2 and 3) for the pathological proliferative response that characterizes many diseases in which deposition of the MAC has been immunohistochemically documented. These include: the mesangial cells in proliferative glomerulonephritis (27), the synovial cells in rheumatoid arthritis (28), and also the smooth muscle cells of the atheromatous plaque (29). It is also intriguing to speculate that the deregulated hematopoiesis characteristic of the disease paroxysmal nocturnal hemoglobinuria may result from mitogenic stimulation by the MAC. In paroxysmal nocturnal hemoglobinuria, the deficiency of complement regulatory proteins normally present on the plasma membrane leads to unrestricted complement activation at the surface of the PNH cells.

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**References**


