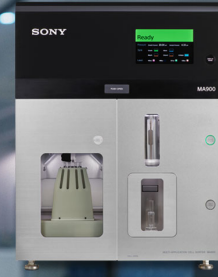


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C5a and C5a^{desArg} Enhance the Susceptibility of Monocyte-Derived Macrophages to HIV Infection¹

Laco Kacani,^{2*} Zoltán Bánki,^{*} Jörg Zwirner,[†] Harald Schennach,[‡] Zsuzsa Bajtay,[§] Anna Erdei,[§] Heribert Stoiber,^{*} and Manfred P. Dierich^{*}

Mononuclear phagocytes, which include circulating blood monocytes and differentiated tissue macrophages, are believed to play a central role in the sexual transmission of HIV infection. The ability of HIV to productively infect these cells may be influenced by action of exogenous or host-derived substances at the site of viral entry. Given the potent capacities of inflammatory mediators to stimulate anaphylatoxic and immunomodulatory functions in mucosa, the effects of complement-derived anaphylatoxins on the susceptibility of monocytes and monocyte-derived macrophages (MDM) to HIV-1 infection were examined. In our *in vitro* system, the susceptibility to infection was up to 40 times increased in MDM that had been exposed to C5a or C5a^{desArg}, but not to C3a or C3a^{desArg}, for 2 days before adding of virus. By contrast, the treatment with complement anaphylatoxins did not affect HIV replication in fresh monocytes. Stimulatory effect of C5a and its desArg derivative on HIV infection correlated with the increase of TNF- α and IL-6 secretion from MDM. All these functional effects of C5a and C5a^{desArg} were reversible by treatment of cells with the mAb that functionally blocks C5aR. Taken together, these results indicate that C5a and C5a^{desArg} may increase the susceptibility of MDM to HIV infection through stimulation of TNF- α and IL-6 secretion from these cells. *The Journal of Immunology*, 2001, 166: 3410–3415.

The complement system has been shown to be involved in several clinical situations, such as sepsis, burn injury, adult respiratory distress syndrome, autoimmune diseases, and infection with HIV. Contact of this pathogen with human serum results in activation of the classical pathway of the complement cascade and deposition of C3 fragments on the surface of virus, even in the absence of HIV-specific Abs (1–3). In addition, activation of complement via either the classical or alternative pathways leads to the N-terminal cleavage of components C3, C4, and C5, resulting in the generation of anaphylatoxins C3a, C4a, and C5a. These molecules are powerful mediators of inflammation and possess immunomodulatory activities. C5a, the best characterized of the anaphylatoxins, is a potent proinflammatory mediator that induces chemotactic migration, enhances cellular adhesion, stimulates oxidative metabolism, and liberates lysosomal enzymes and numerous inflammatory mediators, including histamine and cytokines (4). C3a is spasmogenic, stimulates release of PGE₂ from macrophages, induces degranulation and chemotaxis of eosinophils, attracts mast cells, and possesses proinflammatory properties, which partially overlap with C5a (5). Because of the potential damage to the host that would result from excessive inflammation, the biological activities of C3a and C5a are tightly

controlled. A plasma enzyme carboxypeptidase N rapidly removes C-terminal arginyl residues from C3a and C5a and thereby converts anaphylatoxins into their desArg forms. Whereas C5a^{desArg} still exerts significant proinflammatory effects, C3a^{desArg} is generally regarded to be devoid of any biological activity (6, 7).

Various biological effects of C3a and C5a are initiated by high-affinity binding of these anaphylatoxins to cell surface receptors. Both C3aR and C5aR (CD88) were recently cloned and belong to the rhodopsin subfamily of G protein-coupled receptors with seven-transmembrane segments (8, 9). Expression of C5aR and C3aR was thought to be restricted to cells of myeloid origin, until recent studies have demonstrated the constitutive expression of C5aR in epithelial, endothelial, and parenchymal cells of many organs (10–12). Most recently, evidence for the expression of C5aR in human B and T lymphocytes has been presented (13, 14).

HIV replicates more efficiently in activated cells, and viral levels consistently increase when the immune system of HIV-infected individuals is activated by exogenous stimuli such as opportunistic pathogens. This increase in the rate of viral replication is associated with cellular activation and expression of HIV-inducing cytokines, and leads to acceleration in the course of HIV-induced disease (15). Proinflammatory cytokines were shown to stimulate HIV replication, and the blockade of these factors by receptor antagonists, or by Abs against their receptors, consistently and occasionally completely suppresses viral replication (16). On the other side, recent studies demonstrated inhibition of HIV replication in blood monocytes and alveolar macrophages preincubated in the presence of proinflammatory cytokine TNF- α (17). In this study, we investigated the influence of complement-derived anaphylatoxins on HIV replication in monocytes and monocyte-derived macrophages (MDM).³ Furthermore, we examined the generation of proinflammatory cytokines in monocytic cells during cultivation in the presence of anaphylatoxins. Our data indicate

*Institut für Hygiene und Sozialmedizin, Leopold-Franzens-Universität, Ludwig-Boltzmann-Institut für AIDS-Forschung, Innsbruck, Austria; [†]Department of Immunology, University of Göttingen, Göttingen, Germany; [‡]Central Institute for Blood Transfusion, General Hospital, Innsbruck, Austria; and [§]Department of Immunology, Eötvös Loránd University, Budapest, Hungary

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² Address correspondence and reprint requests to Dr. Laco Kacani, Institut für Hygiene und Sozialmedizin, Fritz Pregl-Strasse 3, Leopold-Franzens-Universität, A-6020 Innsbruck, Austria. E-mail address: Laco.Kacani@uibk.ac.at

³ Abbreviations used in this paper: MDM, monocyte-derived macrophage; TCID₅₀, 50% tissue culture infectious dose.

that C5a and C5a^{desArg} induce TNF- α and IL-6 secretion from MDM, thereby increasing the susceptibility of these cells to HIV infection.

Materials and Methods

Reagents

All chemicals were obtained from Sigma (St. Louis, MO), unless indicated otherwise. Ficoll was purchased from Pharmacia (Piscataway, NJ). Human C3a, C5a, and their desArg derivatives were from Calbiochem (Darmstadt, Germany). TNF- α and IL-6 ELISA quantikine kits were obtained from Endogen (Woburn, MA). Mouse mAb hC3aRZ1 and hC3aRZ2 directed against human C3aR and blocking anti-C5aR mAb S5/1 were kindly provided by Dr. O. Götze (Göttingen, Germany).

Preparation of PBMC, monocytes, and MDM

PBMC were separated from peripheral blood of healthy human donors, as described previously (18). Monocytes were isolated by adherence of PBMC on gelatin-coated petri dishes (18). MDM were monocytes cultivated for 5 days in the presence of RPMI 1640/5% heat-inactivated normal human AB serum (complete RPMI).

Infection assay

Macrophage-tropic strain Ba-L was obtained from MRC (Hertfordshire, U.K.). Virus stocks were prepared by expansion of inoculum in MDM from healthy HIV-negative donors. Supernatants were clarified, sterile filtered, and ultracentrifuged (20,000 rpm, 1 h, 4°C), and virus was quantified by capture ELISA for HIV-1 p24 Ag (p24). The 50% tissue culture infectious dose (TCID₅₀) was determined in cultures of monocytes and MDM from at least three donors, and one TCID₅₀ corresponded to 56–121 pg of p24 per ml.

Infection assay was performed in 96-well plates, as described previously, with minor modifications (19). Briefly, monocytes or MDM (10⁵ cells/100 μ l/well) were preincubated in the presence or absence of different concentrations of anaphylatoxins for 2 days. Thereafter, cells were washed with complete RPMI, and 4-fold dilution series (three replicates per dilution) of HIV_{Ba-L} were added to each well. After 4 h at 37°C, the medium was aspirated, cells were washed three times, and fresh complete RPMI was added. In some experiments, MDM were exposed to anaphylatoxins after viral pulse. Cells were cultivated up to 14 days and infection was screened at different time points by p24 capture ELISA in culture supernatants.

To determine the kinetics of anaphylatoxin-mediated effects, MDM were incubated for 30 min, 2 h, 8 h, 16 h, 24 h, and 48 h in the presence of C5a or C5a^{desArg} (1 μ g/ml). Thereafter, cells were washed and cultivated in complete medium without anaphylatoxins up to 48 h. Subsequently, MDM were exposed to HIV_{Ba-L} and infection assay was performed as described above.

The TCID₅₀ (reciprocal of the virus dilution that causes 50% of wells to be infected) was calculated by using the ID-50 software freely available from the National Center for Biotechnology Information (National Library of Medicine, National Institutes of Health, Bethesda, MD).

FACS analysis

Indirect immunocytochemical staining and cytofluorometric analysis of monocytes and MDM were performed as described previously (18). Briefly, fresh monocytes and monocytes cultivated in complete RPMI for 4 days were detached from six-well culture plates using 5 mM EDTA, gently scraped, and collected by centrifugation. Following mAb were used: anti-C3aR mAb hC3aRZ1 and hC3aRZ2 directed against human C3aR; anti-C5aR mAb S5/1 and isotype-specific controls S-S.1 (IgG2a) and 1B7.11 (IgG1; both obtained from American Type Culture Collection, Manassas, VA). Cells were washed twice in PBS and resuspended in 50 μ l of diluted mAb (2 μ g/ml) in PBS/1% BSA. Thereafter, cells were incubated for 30 min at 4°C, washed with PBS, and resuspended in FITC goat anti-mouse Ig polyclonal Ab (Dako, Glostrup, DK). After incubation for 30 min on ice, cells were fixed in ice-cold PBS containing 2% paraformaldehyde. Finally, samples were analyzed on FACSscan flow cytometer (Becton Dickinson, Mountain View, CA), using forward and side scatter characteristics to identify monocytes and MDM among contaminating lymphocytes and dead cells.

p24 capture ELISA

Amount of HIV in culture supernatants was determined on the basis of p24 level, which was measured by capture ELISA, as described elsewhere (18).

Measurement of cytokine production

TNF- α and IL-6 were determined in supernatants of cell cultures at 2, 8, 24, and 48 h after beginning of incubation. Commercial ELISA kits specific for TNF- α and IL-6 (Endogen) were used. In our hands, the detection limits of these ELISA were 15,625 pg/ml for both TNF- α and IL-6, respectively. All measurements were performed according to the manufacturer's instructions, in duplicates for each sample.

Results

In our initial experiments, we examined the effects of C3a and C5a treatment on HIV infection of PBMC and monocytes. Preincubation for up to 2 days or coinubation of PBMC with anaphylatoxins did not influence the infectivity or kinetics of HIV replication (not shown). Similarly, preincubation of monocytes in the presence of C3a^{desArg} or C5a^{desArg} did not affect HIV infection in these cells. Moderate, but statistically nonsignificant, increase of viral infectivity was detected with monocytes incubated in the presence of >10 ng/ml C5a or C3a (Fig. 1).

In contrast, treatment of MDM with C5a significantly enhanced HIV infection. The exposure of MDM to C5a at concentrations \geq 62.5 ng/ml decreased the amount of virus necessary for productive infection >40 times. Similar enhancement was observed with MDM prestimulated with C5a^{desArg}, although >10-fold higher amounts of this anaphylatoxin were required to reach the same

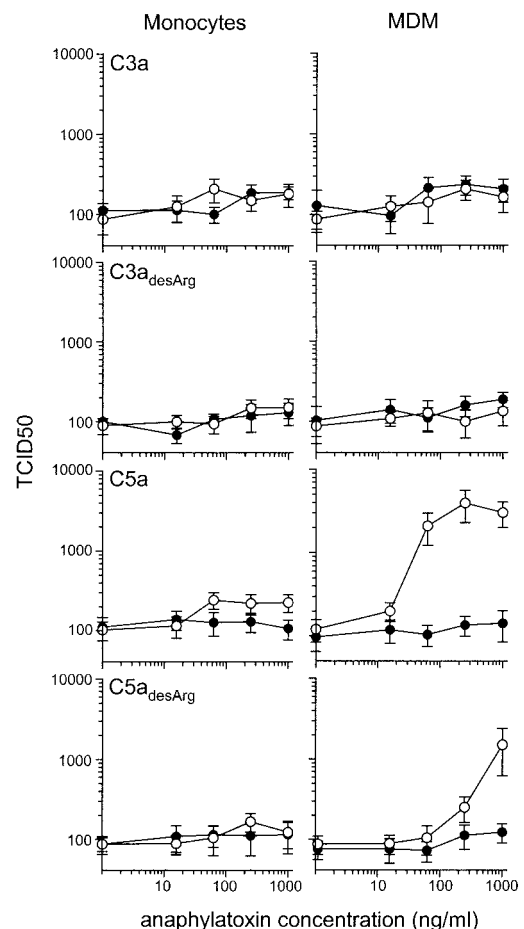


FIGURE 1. Modulation of HIV infection by anaphylatoxin treatment of monocytes (*left*) and MDM (*right*). Cells were either treated with anaphylatoxins at different concentrations for 48 h before HIV infection (○), or infected with HIV and then cultivated in the presence of anaphylatoxins (●). HIV infection was determined on day 7, as described in *Materials and Methods*. Calculated TCID₅₀ \pm SEM from at least four independent experiments are given.

effect when compared with C5a treatment. Conversely, the coin-cubation with C5a or C5a^{desArg} after viral pulse did not exert any effect on HIV replication in the whole concentration range tested (Fig. 1).

Because an increase of susceptibility usually results in faster infection, we next studied the HIV replication kinetics in MDM treated with different anaphylatoxins. In the presence of optimal dose of virus (100 TCID₅₀), the infection was established 2 days earlier in cultures treated with C5a than in control wells (Fig. 2). Treatment of MDM either with C5a^{desArg} (Fig. 2), or C3a and C3a^{desArg} (not shown) did not affect the kinetics of HIV replication in these cells.

Anaphylatoxin-mediated activation of monocytic cells usually occurs very fast. Therefore, transient effects of C5a and C5a^{desArg} on HIV infection in MDM were examined. Significantly higher susceptibility of MDM to HIV infection was detected following ≥ 8 h of cultivation in the presence of C5a or after ≥ 16 h exposure to C5a^{desArg} (Fig. 3).

To determine whether an increase of susceptibility to HIV was due to the quantity of available anaphylatoxin receptors, the surface expression of C3aR and C5aR during differentiation of monocytes was measured by flow cytometry. Upon cultivation in the presence of medium supplemented with 5% human AB serum, MDM expressed significantly higher amounts of both C3aR and C5aR than fresh monocytes. Expression of both anaphylatoxin receptors reached peak levels on day 4 (Fig. 4). Thus, MDM display more C3aR and C5aR on their surface than fresh monocytes, indicating that unresponsiveness of monocytes to C3a and C5a treatment may be associated with low density of anaphylatoxin receptors on their surface.

To further analyze promoting effect of C5a and C5a^{desArg} on HIV infection, the secretion of cytokines by monocytes and MDM upon cultivation in the presence of anaphylatoxins was investigated. Because proinflammatory cytokines were referred to enhance HIV replication in monocytes/macrophages (15, 20), we measured the secretion of TNF- α and IL-6 by these cells during 48-h cultivation period in the presence of C5a and C5a^{desArg} derivative. Monocytes released substantial levels of TNF- α and IL-6 already 2 h after start of incubation. Secretion of TNF- α decreased continuously with time, whereas IL-6 production remained stable during whole incubation period. However, the TNF- α and IL-6 secretion from monocytes was not modulated by C5a and C5a^{desArg}, when compared with nonstimulated cells (Fig. 5).

In contrast to monocytes, MDM spontaneously secreted only marginal levels of TNF- α and IL-6 during whole incubation pe-

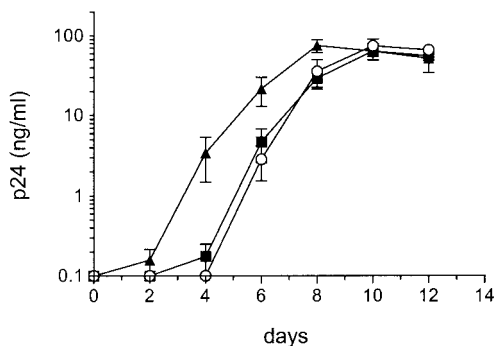


FIGURE 2. Kinetics of viral infection in cultures of MDM that were incubated for 2 days in the absence (○) or in the presence of 1 μ g/ml C5a (▲) or 1 μ g/ml C5a^{desArg} (■). Subsequently, cultures were infected with 10 ng p24/ml of HIV-1_{Ba-L} strain, and p24 amounts were determined in supernatants harvested on days 2, 4, 6, 8, 10, and 12. A typical result obtained with one of four independent experiments (donors) is presented.

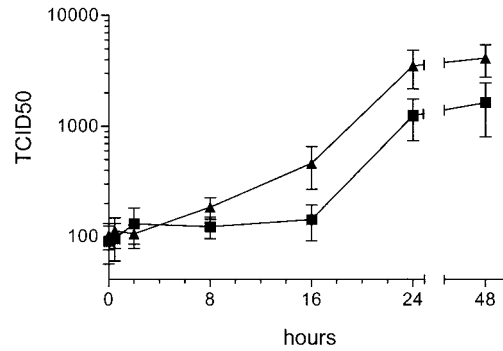


FIGURE 3. Kinetics of anaphylatoxin-mediated effects on susceptibility of MDM to HIV infection. MDM were incubated in the presence of 1 μ g/ml C5a (▲) or 1 μ g/ml C5a^{desArg} (■). At indicated time points, cell cultures were washed and incubated in the fresh medium up to 48 h before viral pulse. Finally, cultures were infected with 10 ng p24/ml of HIV-1_{Ba-L}, and p24 amounts were determined on day 7. A representative experiment of four is shown.

riod. The treatment of MDM with C5a caused significant and dose-dependent increase in secretion of both TNF- α and IL-6, with the peak at 8 h for TNF- α and at 24 h for IL-6, respectively. In addition, C5a^{desArg} exerted basically a similar influence on TNF- α generation, although 10 times higher concentrations were necessary to reach the same effect than in case of C5a (Fig. 6). By contrast, C5a^{desArg} had no effect on IL-6 secretion from MDM.

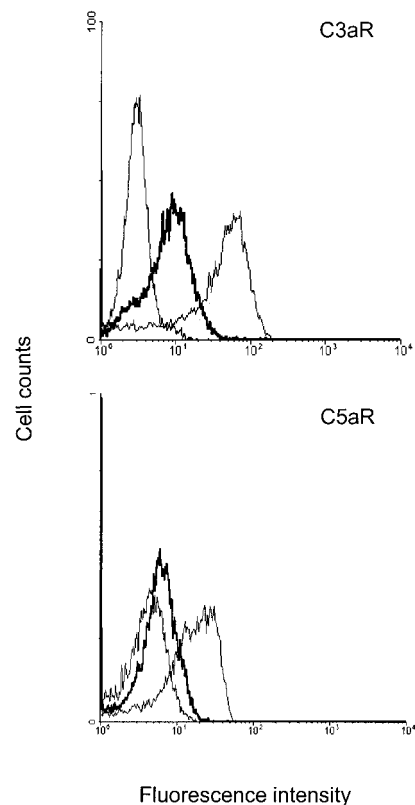


FIGURE 4. Comparative immunofluorescence analysis of C3aR and C5aR expression by fresh monocytes (thick-lined histogram) and by MDM incubated for 4 days in the presence of RPMI 1640/5% normal human AB serum (shaded histogram). Anti-C3aR mAb hC3aRZ1 and anti-C5aR mAb S5/1, as well as isotype IgG1 mAb 1B7.11 and IgG2a mAb S-S.1 (thin-lined histogram), respectively, were used.

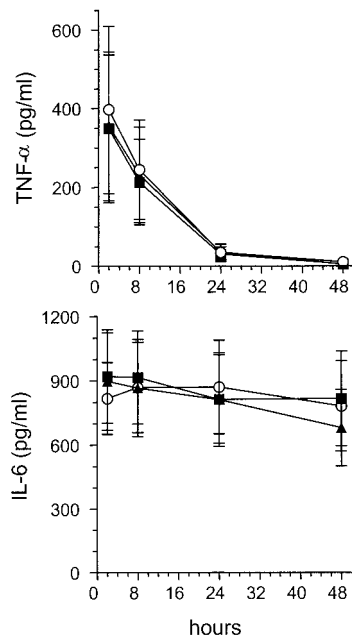


FIGURE 5. Kinetics of TNF- α (upper) and IL-6 (lower) secretion from monocytes incubated in the absence (\circ) or in the presence of 100 ng/ml C5a (\blacktriangle) or 1 μ g/ml C5a (\blacksquare). Data represent means \pm SEM from four independent experiments (donors), each performed in duplicate.

Finally, we investigated effects of the C5aR blockade on viral replication, as well as on secretion of cytokines in MDM treated with C5a or C5a^{desArg}. C5aR-blocking mAb S5/1 (5 μ g/ml) reversed the stimulatory effect of C5a or C5a^{desArg} on HIV infection (Fig. 7). Furthermore, this mAb exerted similar inhibitory effects on secretion of TNF- α and IL-6 by MDM stimulated with C5a or C5a^{desArg}. Whereas TNF- α production by MDM was significantly blocked in the whole concentration range (\leq 1 μ g/ml C5a), inhibitory effect on IL-6 secretion was observed up to 250 ng/ml of C5a (Fig. 7). At the highest concentration tested (1 μ g/ml C5a), only partial inhibition of anaphylatoxin-mediated effects was observed using 5 μ g/ml of mAb. Similarly, blocking anti-C5aR mAb re-

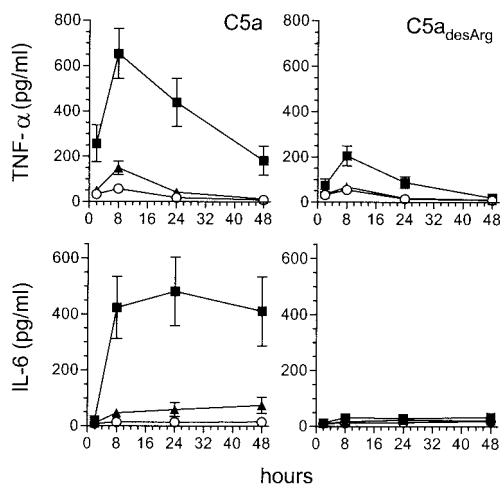


FIGURE 6. Kinetics of TNF- α (upper) and IL-6 (lower) production by MDM stimulated with C5a (left) or C5a^{desArg} (right). MDM were incubated in the absence (\circ) or in the presence of 100 ng/ml (\blacktriangle) or 1 μ g/ml (\blacksquare) of particular anaphylatoxin. Data represent means \pm SEM from six independent experiments (donors) performed in duplicate.

versed stimulatory effects of C5a^{desArg} on TNF- α production from macrophages. Thus, these data indicate the reversibility of C5a-mediated activation of MDM through blockade of C5aR with mAb.

Discussion

The present study demonstrates that human C5a, and to a lesser degree also C5a^{desArg} increase the susceptibility of MDM to HIV infection in vitro. This stimulatory effect of C5a and its C5a^{desArg} derivative correlated with the stimulation of secretion of endogenous TNF- α and IL-6, the cytokines that are known to up-regulate HIV replication in an autocrine/paracrine manner (21–23).

Higher likelihood of HIV transmission and acquisition is associated with the occurrence of sexually transmissible diseases, supposing that acute and chronic inflammation plays an important role in the establishment of HIV infection. Therefore, the identification of host-derived substances that influence the ability of HIV to productively infect target cells is critical to the development of effective therapeutic and vaccination strategies. We identified inflammatory anaphylatoxins C5a and C5a^{desArg} as potent stimulatory factors that are able to prime monocyte-derived macrophages for HIV infection in vitro. The treatment of MDM for 2 days with C5a before viral pulse enhanced HIV infectivity up to 40 times and, although to a lesser extent, a similar effect was observed with C5a^{desArg}. Kinetic analysis of HIV replication in this system has shown that exposure to C5a led to the acceleration of infection in MDM. Moreover, the treatment with blocking anti-C5aR mAb reversed the susceptibility of MDM to HIV infection. Of note, we did not detect any modulatory effect on HIV replication when these cells were incubated in the presence of C5a and C5a^{desArg} after HIV pulse. Although the exact mechanism has to be established, it is likely that exposure of MDM to C5a and C5a^{desArg} before adding of HIV facilitates either viral entry or early steps of viral replication.

By contrast, described priming effects of C5a and C5a^{desArg} on MDM were not observed in cultures of PBMC and monocytes. To find the relevant mechanism for this dichotomy, comparative cytofluorometric analysis revealed that MDM express remarkably higher amounts of both anaphylatoxin receptors C3aR and C5aR on their surface than monocytes. These data suggest that the quantity of available anaphylatoxin receptors might be one of the explanations for anaphylatoxin-mediated priming of MDM, but not fresh monocytes, to HIV infection.

C3a and C3a^{desArg} exert only marginal effects on monocytes and MDM. This is consistent with previous reports, demonstrating that C5a (and probably also C5a^{desArg}) is the most important among anaphylatoxins for activation of monocytes/macrophages during inflammatory responses (4). Therefore, we tested various inflammatory mediators (e.g., IL-8, prostaglandins, leukotrienes, histamine, and serotonin) in our system. Surprisingly, we did not succeed to identify any modulatory effect of these substances on viral infection, except of already published resistance of PGE₂-treated MDM to HIV infection (24). Thus, from all inflammatory factors tested, complement-derived anaphylatoxins C5a and C5a^{desArg} seem to be critical inducers of the macrophage activation, thereby mediating higher susceptibility of MDM to HIV infection.

Because exposure of C5aR-expressing cells to anaphylatoxins usually results in the rapid calcium efflux and signal transduction, we tested transient effects of C5a and C5a^{desArg} on priming of MDM to HIV infection. Short-time exposure (up to 2 h) did not affect the susceptibility of MDM, while significant increase of HIV infection was observed after 8 and 16 h of incubation in the presence of C5a and C5a^{desArg}, respectively. From these findings, we

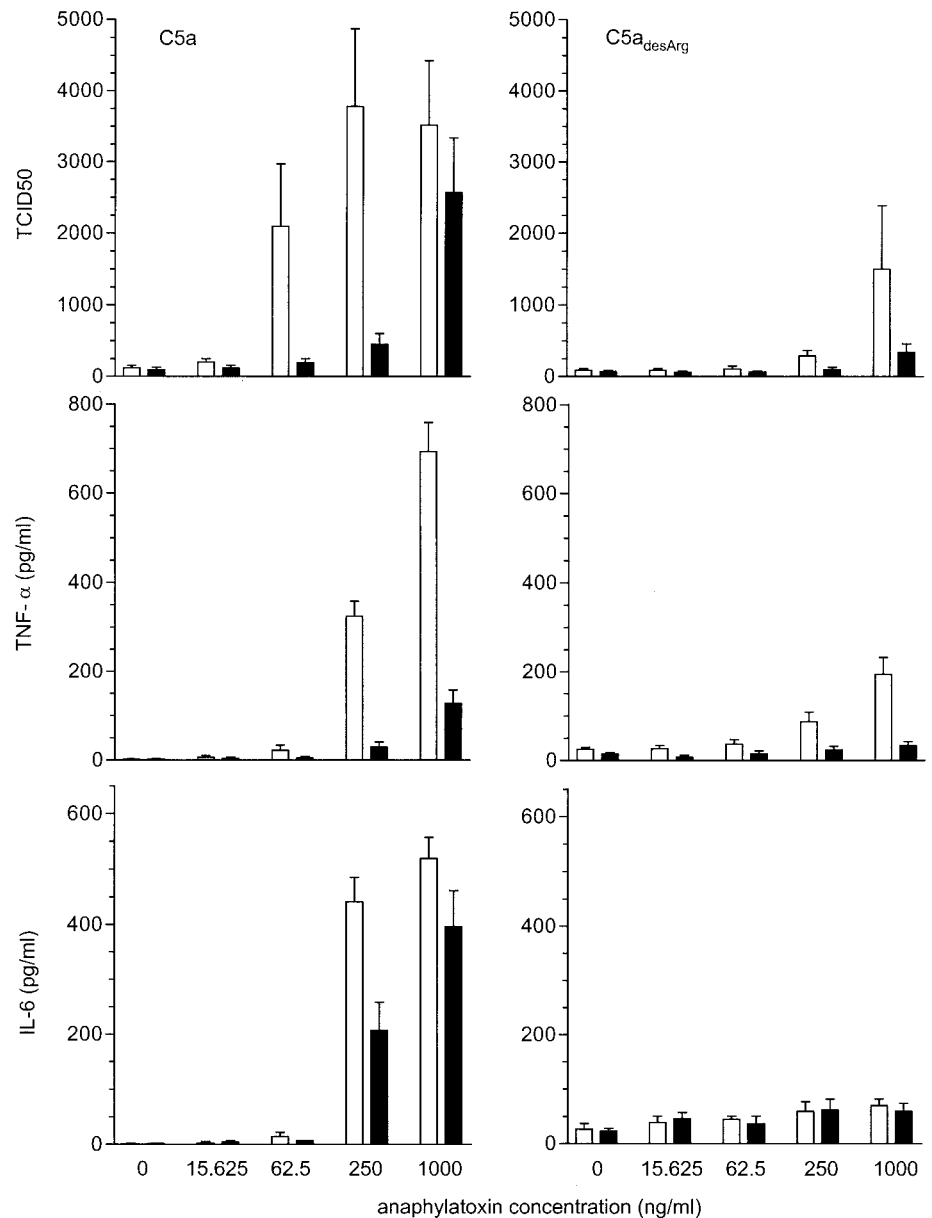


FIGURE 7. Inhibition of C5a and C5a^{desArg} effects on MDM via C5aR blockade. MDM were incubated for 30 min in the absence (□) or in the presence (■) of 5 μg/ml of blocking anti-C5aR mAb S5/1 before treatment with C5a (left) or C5a^{desArg} (right). HIV infection (top) was determined on day 7, and TNF-α (middle) and IL-6 (bottom) secretion were estimated after 8 and 24 h of incubation, respectively. Results represent means ± SEM of four experiments performed in duplicate.

assume that C5a and its C5a^{desArg} derivative did not affect the HIV replication in MDM directly. Therefore, we examined the secretion of proinflammatory cytokines that were reported to increase HIV replication (21–23).

TNF-α and IL-6 have been shown to act in a positive feedback loop on HIV replication, e.g., TNF-α and IL-6 increase HIV replication, and HIV infection of monocytic cells in turn further increases secretion of these cytokines (25, 26). We observed significant and dose-dependent increase of TNF-α and IL-6 secretion by MDM cultivated in the presence of C5a, whereas treatment with C5a^{desArg} effectively enhanced only TNF-α generation in these cells. These results provide evidence that effects mediated by C5a^{desArg} did not originate from the C5a contamination of the C5a^{desArg} preparation. Furthermore, observed differences could reflect diverse efficacy of C5a and C5a^{desArg} to stimulate distinct signaling pathways.

The treatment of MDM with C5aR-blocking mAb before adding C5a or C5a^{desArg} reversed enhancing effect on secretion of TNF-α. These data indicate that modulation of cytokine production was related to higher susceptibility of MDM to HIV infection after

treatment with C5a and C5a^{desArg}. On the other side, C5aR blockade only partially inhibited HIV infection and IL-6 production in MDM treated with the highest concentrations of C5a and C5a^{desArg}. These data imply that anaphylatoxin-mediated enhancement of HIV infectivity may involve other factors, which could substitute for TNF-α (27, 28).

Furthermore, anaphylatoxins did not exert any modulatory effect on secretion of proinflammatory cytokines from monocytes. This is consistent with previous observations that C5a by itself did not induce significant TNF-α and IL-6 production, but considerably increased the LPS-induced synthesis of both cytokines in these cells (27, 29, 30). By contrast, HIV alone or in combination with anaphylatoxins did not provide such a stimulatory effect on synthesis of both TNF-α and IL-6 by monocytes.

Of note, recent publications revealed that TNF-α inhibits HIV infection in blood monocytes and primary macrophages by decreasing the CCR5 expression (17, 31). Our data have shown that peak of TNF-α secretion from MDM was reached after 8 h and decreased continuously to the base level after 48 h, when cultures were infected with the virus. In addition, the amount of secreted

TNF- α reached only one-tenth of concentrations necessary to down-regulate CCR5, as demonstrated by cytofluorometry.

The ability of C5a and C5a^{desArg} to prime macrophages for HIV infection is a new finding. This may be of particular importance, because C5a has been shown to mediate inflammatory responses at sites of HIV entry (4). C5a is generated during acute and chronic infection with bacteria or intracellular pathogens, as well as in autoimmune disorders (5). Bacterial LPS and C5a have been shown to act synergistically in the induction of proinflammatory cytokines TNF- α , IL-1, and IL-6 by monocytes and macrophages (27, 28). Therefore, the generation of complement-derived anaphylatoxins during local inflammation at mucosal surfaces of genital tract may enhance the efficiency of sexual transmission during earliest phases of HIV infection (32).

It was estimated that >90% of cases of HIV infection in developing countries have occurred during sexual activity (33). In experimental model of HIV infection, macrophages together with dendritic cells have been shown to be among the first SIV-infected cells upon vaginal inoculation (34, 35). Macrophages are localized at peripheral tissues, among them in mucosa of rectum, uterus, and cervix, and constitute a first-line defense against viruses and other pathogens. Sexually transmitted diseases, such as syphilis and gonorrhea, may cause ulcerative or inflammatory lesions of penile urethra and vaginal mucosa, thus creating an inflammatory environment at these sites of HIV entry. In accordance with these findings, individuals with sexually transmitted infection are 2 to 5 times more likely to become infected with HIV (36, 37). In our study, we have shown that C5a and its metabolite C5a^{desArg} are potent promoters of HIV infection in MDM in vitro. Because mucosal inflammation increases the risk of HIV transmission (32) and is frequently associated with generation of complement anaphylatoxins, our results provide further remarkable insight into the earliest phase of HIV pathogenesis.

Acknowledgments

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