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C5a and C5a<sub>desArg</sub> Enhance the Susceptibility of Monocyte-Derived Macrophages to HIV Infection<sup>1</sup>

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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<sub>desArg</sub>, but not to C3a or C3a<sub>desArg</sub>, 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<sub>desArg</sub> were reversible by treatment of cells with the mAb that functionally blocks C5aR. Taken together, these results indicate that C5a and C5a<sub>desArg</sub> 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.
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 des-Arg 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 (10^6 cells/100 μl/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, 8, 9 h, 16 h, 24 h, and 48 h in the presence of C5a or C5a desArg (1 μg/ml). Thereafter, cells were washed and cultured 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 immunochemical 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-5/1 (IgG2a) and 1B7.11 (IgG1); both obtained from American Type Culture Collection, Manassas, VA). Cells were washed twice in PBS and resuspended in 50 μl of diluted mAb (2 μg/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 glycoconal 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 coincubation 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 ≥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 level.

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 = SEM from at least four independent experiments are given.
effect when compared with C5a treatment. Conversely, the coinubcation 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 period. 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.
Furthermore, this mAb exerted similar inhibitory effects on IL-6 secretion as well as on secretion of cytokines in MDM treated with C5a or C5adesArg. 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 C5adesArg after HIV pulse. Although the exact mechanism has to be established, it is likely that exposure of MDM to C5a and C5adesArg before addition of HIV facilitates either viral entry or early steps of viral replication.

Finally, we investigated effects of the C5aR blockade on viral replication, as well as on secretion of cytokines in MDM treated with C5a or C5adesArg. C5aR-blocking mAb S5/1 (5 μg/ml) reversed the stimulatory effect of C5a or C5adesArg 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 C5adesArg. Whereas TNF-α production by MDM was significantly blocked in the whole concentration range (≥1 μg/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 μg/ml C5a), only partial inhibition of anaphylatoxin-mediated effects was observed using 5 μg/ml of mAb. Similarly, blocking anti-C5aR mAb reversed stimulatory effects of C5adesArg 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 C5adesArg increase the susceptibility of MDM to HIV infection in vitro. This stimulatory effect of C5a and its C5adesArg 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 C5adesArg 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 C5adesArg. 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 C5adesArg after HIV pulse. Although the exact mechanism has to be established, it is likely that exposure of MDM to C5a and C5adesArg before addition of HIV facilitates either viral entry or early steps of viral replication.

By contrast, described priming effects of C5a and C5adesArg 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 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 C5adesArg 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 C5adesArg. 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 C5adesArg after HIV pulse. Although the exact mechanism has to be established, it is likely that exposure of MDM to C5a and C5adesArg before addition of HIV facilitates either viral entry or early steps of viral replication.

C3a and C3adesArg exert only marginal effects on monocytes and MDM. This is consistent with previous reports, demonstrating that C5a (and probably also C5adesArg) 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 C5adesArg 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 C5adesArg 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 C5adesArg, respectively. From these findings, we
assume that C5a and its C5a\textsubscript{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-\textalpha{} and IL-6 have been shown to act in a positive feedback loop on HIV replication, e.g., TNF-\textalpha{} 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-\textalpha{} and IL-6 secretion by MDM cultivated in the presence of C5a, whereas treatment with C5a\textsubscript{desArg} effectively enhanced only TNF-\textalpha{} generation in these cells. These results provide evidence that effects mediated by C5a\textsubscript{desArg} did not originate from the C5a contamination of the C5a\textsubscript{desArg} preparation. Furthermore, observed differences could reflect diverse efficacy of C5a and C5a\textsubscript{desArg} to stimulate distinct signaling pathways.

The treatment of MDM with C5aR-blocking mAb before adding C5a or C5a\textsubscript{desArg} reversed enhancing effect on secretion of TNF-\textalpha{}. These data indicate that modulation of cytokine production was related to higher susceptibility of MDM to HIV infection after treatment with C5a and C5a\textsubscript{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\textsubscript{desArg}. These data imply that anaphylatoxin-mediated enhancement of HIV infectivity may involve other factors, which could substitute for TNF-\textalpha{} (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-\textalpha{} 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-\textalpha{} and IL-6 by monocytes.

Of note, recent publications revealed that TNF-\textalpha{} inhibits HIV infection in blood monocytes and primary macrophages by decreasing the CCR5 expression (17, 31). Our data have shown that peak of TNF-\textalpha{} 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 cytometry.

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 autoimmune disorders (5). Bacterial LPS and C5a have been shown to mediate inflammatory responses at infection is a new finding. This may be of particular importance, because C5a has been shown to mediate inflammatory responses 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.

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