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In Vitro Analysis of Complement-Dependent HIV-1 Cell Infection Using a Model System

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Previous studies based on the use of human serum as a source of C have provided evidence for the C-dependent enhancement of cell infection by HIV-1. The present study was undertaken to distinguish C from other serum factors and to identify the proteins and the mechanisms involved in C-dependent cell infection by HIV-1. The classical C activation pathway was reconstituted from the proteins C1q, C1r, C1s, C4, C2, C3, factor H, and factor I; each were purified to homogeneity. A mixture of these proteins at physiological concentrations was shown to reproduce the ability of normal human serum to enhance the infection of MT2 cells by HIV-1 at low doses of virus. This enhancing effect was abolished when heat-inactivated serum and C2- or C3-depleted serum were used, and was restored upon addition of the corresponding purified proteins. A mixture of two synthetic peptides corresponding to positions 10–15 and 90–97 of human C receptor type 2 (CD21) as well as soluble CD4 both inhibited the C-dependent infection process. These data provide unambiguous evidence that HIV-1 triggers a direct activation of the classical C pathway in vitro and thereby facilitates the infection of MT2 cells at low doses of virus. These findings are consistent with a mechanism involving increased interaction between the virus opsonized by C3b-derived fragment(s) and the CD21 cell receptors and subsequent virus entry through CD4 receptors. The Journal of Immunology, 1999, 162: 4088–4093.

Complement is a major system of innate immunity that is designed to provide the human body with an initial line of defense against pathogenic microorganisms. However, several pathogens, including herpes simplex virus (1), EBV (2), and vaccinia virus (3) have developed mechanisms to evade the destructive actions of C. Moreover, certain viruses, such as flaviviruses and EBV, have been shown to make use of the C system to initiate infection (reviewed in Ref. 4).

A number of studies performed in the last decade have provided evidence for both Ab-dependent and Ab-independent activation of the classical C pathway by HIV-1, as well as for activation of the alternative pathway (5–11).

Several reports show that HIV infection is correlated in vivo with high levels of C activation, as shown by both a decrease of C components and an increase of activation fragments in the blood (12, 13). However, the overall role of C activation in HIV infection is not fully understood. Although there is evidence that C activation by HIV-1 in the presence of Abs may lead to viral lysis (14), other studies have failed to show C-dependent viral elimination (15). Indeed, various studies support the hypothesis that the efficient destruction of HIV is inhibited in vivo by the C regulatory proteins normally used by host cells to protect themselves against C (16–19).

Moreover, a number of studies performed with various cell lines in vitro indicate that C significantly enhances the infection of C receptor-bearing cells by HIV-1 (5–7, 20, 21), suggesting that C may be involved in the infectious process in vivo. All of these studies have made use of whole human serum as a source of C, which raises the question of the possible implication of non-C serum factors in the observed serum-mediated effect and renders difficult a clear-cut discrimination between the classical and alternative pathways of C. The primary objective of this work was consequently to reconstitute the initial (activation) part of the classical pathway from its purified constituent proteins with the aim of testing the ability of this part of the pathway to mimic the effect of whole serum.

Materials and Methods

Sera and reagents

The normal human serum (NHS)1 used as a C source was a pool obtained from three healthy individuals that were seronegative for HIV-1 and HIV-2. Inactivation of C activity was obtained by heat treatment of NHS for 30 min at 56°C. C3- and C2-depleted human sera were purchased from Calbiochem (Meudon, France). Recombinant soluble CD4 (sCD4) expressed in Chinese hamster ovary cells (22) was kindly provided by D. Klatzmann (Hôpital de la Pitié, Paris, France). Veronal-buffered saline (VBS) contained 5 mM sodium barbital, 1.5 mM CaCl2, 0.6 mM MgCl2, and 150 mM NaCl (pH 7.4).

C proteins

The C1q subunit of C1 was purified from human plasma as described previously (23). Isolation of the proenzyme form of the C1s-C1r-C1r-C1s subunit was performed as described previously (24), except that the C1r plus C1s mixture released by EDTA was dialyzed against 50 mM triethanolamine hydrochloride, 145 mM NaCl, and 2.5 mM CaCl2 (pH 7.4); final purification of the Ca2+ -dependent tetramer was achieved by high-pressure

1 Abbreviations used in this paper: NHS, normal human serum; CR2, C receptor type 2; RT, reverse transcriptase; sCD4, soluble CD4; TCID50, 50% tissue culture infectious dose; VBS, veronal-buffered saline.
gel permeation chromatography on a TSK-G3000 SW column (Pharmacia LKB Biotechnology, Uppsala, Sweden) in the same buffer. Components C4, C2, and C3 were isolated from human citrated plasma according to published procedures (25–27). The human C factors H and I were purified as described by Sim et al. (28). The concentrations of purified Clq, Cls-Clr, C1r, C1s, C1, C2, C3, H, and I were determined spectrophotometrically using values of A (1%, 1 cm) at 280 nm of 6.8, 13.5, 8.3, 10.0, 10.0, 14.2, and 10.0; M_r values were 459,300, 330,000, 205,000, 102,000, 185,000, 150,000 and 75,000, respectively (23, 25, 26, 29–31). The homogeneity and activation state of the purified proteins was assessed by SDS-PAGE using 10% acrylamide gels under reducing and nonreducing conditions. Each of the proteins used in this study was ≥95% pure. All proteins were stored on ice, and the functional activity of components C4, C2, and C3 was checked periodically using appropriate hemolytic assays (32, 33). In the case of C4 and C2, the susceptibility to Cls cleavage was used as a further test of biological activity. To reconstitute the initial part of the classical C pathway, the purified proteins were dialyzed individually against VBS and then mixed at their respective physiological concentrations in serum, namely 200 g/ml (factor I) (11, 30, 34, 35). The protein mixture was incubated for 15 min at 0°C prior to being used for opsonization of the virus.

**Synthetic peptides**

The peptides Leu-Asn-Gly-Arg-Ile-Ser and His-Gly-Asp-Ser-Val-Thr-Phe-Ala corresponding to positions 10–15 and 90–97 of human C receptor type 2 (CR2) (CD21) (36) as well as peptide Ala-Ser-Arg-Pro-Glu-Gly-Tyr from the *Androctonus australis* hemocyanin were synthesized chemically using a stepwise solid-phase method (37) on an Applied Biosystems 430A automated peptide synthesizer (Applied Biosystems, Foster City, CA). Synthesis was conducted on a phenylacetamidomethyl resin with tert-butylloxycarbonyl-protected amino acids (Neosystems, Strasbourg, France). All couplings were performed by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole method, using N-methylpyrrolidone and DMSO as coupling solvents, as recommended by the manufacturer. Deprotection and cleavage of the peptides from the resin was performed using hydrogen fluoride in the presence of 10% anisole as a scavenger. Peptides were purified by semipreparative reverse-phase HPLC on a 30-nm Vyde C18 column (Vydac, Hesperia, CA) (2.2 cm × 25 cm, 10 μm) using a 30-min linear gradient of acetonitrile (5–60%) in 0.1% trifluoroacetic acid. The purity and identity of the peptides were assessed by analytical reverse-phase HPLC and electrospray or by fast atom bombardment mass spectrometry analyses. The experimental mass values of the peptides were 658.39 ± 0.01 Da (Leu-Asn-Gly-Arg-Ile-Ser), 832.22 ± 0.08 Da (His-Gly-Asp-Ser-Val-Thr-Phe-Ala), and 778.5 ± 0.1 Da (Ala-Ser-Arg-Pro-Glu-Gly-Tyr). The calculated average mass values were 658.38, 832.37, and 778.40 Da, respectively. Before use, peptides were dissolved in VBS at concentrations of 10 mg/ml (Leu-Asn-Gly-Arg-Ile-Ser) and 8 mg/ml (His-Gly-Asp-Ser-Val-Thr-Phe-Ala and Ala-Ser-Arg-Pro-Glu-Gly-Tyr).

**Source of virus**

The HIV-RF isolate of HIV-1 was cultivated in H9 cells. Virus was obtained from supernatants of de novo-infected cells, clarified from cells by low-speed centrifugation at 400 × g for 10 min, passed through a 0.45-μm filter, and titrated at 50°C until use. The infective titer of the virus suspensions was determined using MT2 cells as described previously (8). The titer of the HIV-RF stock suspension was 2 × 10^6 of the 50% tissue culture infectious dose (TCID_{50})/ml.

**Target cells**

Cells of the lymphocytic line MT2 (38) expressing both CD4 and CR2 (CD21) (39) and known to be highly sensitive to the cytopathic effect of HIV-1 were used as targets. The cell line was maintained in RPMI 1640 medium (Life Technologies, Cergy, France) supplemented with 10% heat-inactivated FCS (Biosys, Compiègne, France) and antibiotics.

**Infection of MT2 cells with HIV-1**

Infection experiments were realized in triplicate in 24-well plates. Virus opsonization was performed by incubating appropriate dilutions of HIV-1 in culture medium (final volume = 500 μl) for 60 min at 37°C with 50 μl of either NHS or a mixture of the purified C proteins Clq, C1s-Clr, C1r, C1s, C1, C2, C3, factor H, and factor I, as indicated. Control virus samples were incubated with heat-inactivated NHS or with VBS under the same conditions. MT2 cells (3 × 10^5 cells in 1.5 ml of culture medium) were then added to the virus samples and cultured under 5% CO_2 at 37°C for different periods of ≤15 days. Cultures were examined under the microscope for the cytopathic effect of HIV. At day 8, 10, 12, or 15 of culture, the supernatants were collected and tested for reverse transcriptase (RT) activity as described previously (40).

**Results**

*NHS promotes infection of MT2 cells by low doses of virus*

The effect of NHS on the infection of MT2 cells with the human T-lymphotropic virus-RF strain of HIV-1 was initially investigated. Various dilutions of virus with values of TCID_{50} ranging from 0.2 to 100 were preincubated with either NHS or heat-inactivated NHS, then each virus sample was used to infect MT2 cells, and infection was assessed by measuring the RT activity of the culture supernatants at day 10 of culture. As illustrated by the representative experiment shown in Fig. 1, preincubating the virus with NHS promoted significant infection by very low doses of virus, whereas heat-treatment of the serum under conditions known to suppress C activity abolished this enhancing effect. Compared with heat-inactivated serum, the observed enhancing effect of NHS was generally maximal at a TCID_{50} value of 0.2, with an enhancement factor ranging from 23 (as shown in Fig. 1) to ~50. At high doses of virus (TCID_{50} = 100 or higher), significant infection was observed with the untreated virus; preincubation of the virus with NHS had no enhancing effect on infection. On the contrary, as shown in Fig. 1, a slight inhibitory effect was consistently observed under these conditions. All additional experiments described in this study were conducted at a TCID_{50} of 0.2, where virtually no infection was observed upon preincubation with heat-treated serum, whereas significant infection was induced by NHS.

*C3 or C2 depletion abolishes the enhancing effect of human serum*

In an attempt to confirm that the observed enhancing effect of NHS was due to C activation, human sera depleted of the C proteins C2 (a component of the classical pathway) or C3 (common to both the classical and alternative pathways) were tested. As shown in Fig. 2, no significant infection was observed after 12 days of culture.
when the virus was preincubated with C2-depleted serum; C3-depleted serum only yielded a slight infection (20%) compared with NHS. In contrast, the addition of physiological concentrations of purified C2 and C3, respectively, to the C2- and C3-depleted sera, fully restored their ability to promote viral infection and even yielded increased enhancing effects compared with NHS. These data provide further support to the hypothesis that the observed enhancing effect of NHS requires the integrity of the classical C activation pathway, including component C3. To test this hypothesis, additional experiments were conducted using purified proteins from the classical pathway as a source of C.

A mixture of the purified proteins (C1, C4, C2, C3, H, and I) mimics the enhancing effect of NHS

The classical activation pathway of C was reconstituted from its constituent components (C1, C4, C2, and C3) plus the regulatory factors H and I, which are required for further proteolytic cleavage of C3b into fragments iC3b and C3dg. All of these proteins were isolated from human serum and purified to homogeneity. Fig. 3 shows a representative analysis by SDS-PAGE of the purified complement proteins used in this study. The concentration of each protein in the mixture was adjusted to its known physiological level in NHS. MT2 cells were cultured in the presence of a fixed amount of virus (TCID50 = 0.2) initially preincubated with either NHS or the purified protein mixture. As measured from the RT activity of culture supernatants at days 8 (M) and 12 (f) of culture, the whole protein mixture exhibited an ability to promote infection that was comparable with that of NHS (Fig. 4). A protein mixture containing “aged” (hemolytically inactive) C2 did not yield infection (data not shown). Omitting factors H and I in the mixture significantly decreased, but did not abolish, the enhancing effect. These data clearly demonstrated the ability of the reconstituted classical activation pathway of C to mimic the effect exerted by whole serum. The fact that a full enhancement of infection required the presence of factors H and I strongly suggested that the infection of MT2 cells involved CR2 (CD21), as this receptor is known to bind preferentially to the C3b-derived fragments C3dg and, to a lesser extent, iC3b (41). The observation that significant infection occurred when the virus was preincubated in the presence of the components C1, C4, C2, and C3 may be explained by the fact that this mixture is expected to yield fragment C3b, which may itself exhibit low affinity for CR2 (42). However, the significance of the latter effect should be judged in relation to the infection level yielded in the same experiment by the virus preincubated with buffer alone (Fig. 4). In this respect, it should be stressed that viral
C-dependent infection of MT2 cells by HIV-1 requires both the CR2 and CD4 receptors (CD4R)

To demonstrate the involvement of CR2 in the observed C-dependent infection of MT2 cells, two peptides corresponding to residues 10–15 and 90–97 of human CR2, previously shown to block iC3b binding to CR2 (36), were synthesized and tested for their ability to inhibit C-dependent infection. Both peptides were used at a concentration of 300 μM, which was determined previously to yield a complete inhibition of ligand binding to CR2 (36). The virus was first preincubated with NHS, further incubated for 15 min at room temperature in the presence of both peptides, and subsequently used for infection of MT2 cells. As illustrated in Fig. 5, incubation of the virus with the peptides resulted in a background RT activity similar to that observed for cells infected with control virus samples preincubated with buffer or heat-inactivated serum. In contrast, incubation of the virus with a control peptide (Ala-Ser-Arg-Pro-Glu-Gly-Tyr) unrelated to CR2 at a concentration of 600 μM did not alter the C-dependent infection of MT2 cells (Fig. 5). Examination of the cell cultures under the microscope revealed no detectable cytopathic effect of the CR2-derived peptides. These results clearly demonstrate that the observed C-dependent infection is CR2-dependent and consequently involved interaction of the C-opsinized virus with MT2 cells through CR2.

Further studies based on the use of recombinant sCD4 were performed to establish the role of CD4 in the C-dependent infection process. After preincubation with NHS, the virus was incubated with sCD4 (2 μg/ml) for an additional 30 min at 37°C and then used to infect MT2 cells. As shown in Fig. 6, treatment with sCD4 virtually abolished the ability of the C-opsinized virus to infect MT2 cells at low doses, indicating that the observed C-dependent infection process required both CR2 and CD4.

Discussion

The experiments described in the initial part of this work provide further evidence that HIV-seronegative NHS promotes in vitro infection of cells bearing C receptors by low doses of HIV-1; this observation is in full agreement with data obtained previously in various laboratories (5, 7, 8, 20, 43). Although it is generally accepted that the observed enhancing effect is initiated by direct C activation by HIV-1, the fact that all studies performed thus far have been based on the use of whole serum has raised questions about the possible involvement of other non-C factors, such as naturally occurring IgM Abs exhibiting cross-reactivity to HIV (44). In the same way, the use of whole serum did not facilitate discrimination between the different pathways of C. Indeed, C activation by HIV has been reported to involve both the classical and the alternative pathway (7, 8, 10, 45).

The present study provides several lines of evidence indicating that the direct activation of C through the classical pathway is very likely a major factor of the enhancing effect observed in the presence of NHS: 1) no significant effect was observed when C2-depleted serum was used, whereas the addition of purified C2 fully restored the ability of serum to promote virus infection; and 2) most importantly, the effect of whole NHS could be quantitatively reproduced by a mixture of the proteins C1, C4, C2, C3, factor H, and factor I. The fact that each of these proteins was purified to homogeneity removes doubts about the involvement of non-C factors and shows unambiguously that the classical activation pathway per se is able to recognize HIV-1 and consequently trigger a...
series of reactions that promote viral infection. This does not necessarily imply that the enhancing effect measured in the presence of whole serum cannot be due, at least in part, to C activation by other pathways. Indeed, activation of the classical pathway will generate the C3b fragment, which itself is expected to trigger activation of the alternative pathway. Also, the observation that mannan-binding lectin recognizes recombinant glycoprotein 120 and triggers C activation (46) suggests that the recently recognized “lectin pathway” of C activation (47) may take part in the effect exerted by whole serum. However, our data suggest that this effect mainly occurs through activation of the classical pathway.

With respect to the mechanisms involved in cell infection, our data are consistent with a requirement of CR2 on MT2 cells: 1) the presence of factors H and I, which are necessary to generate the iC3b fragment recognized by CR2, is needed to reproduce a full enhancement of infection; and 2) synthetic peptides considered to represent major ligand-binding sites of human CR2 (36) abolish the ability of NHS to stimulate infection at concentrations previously found to prevent iC3b binding to CR2. The use of these specific synthetic peptides provides an efficient means of blocking virus infection and lends further support to previous results obtained with CR2”-cells using mAbs directed to CR2 (8, 44, 48).

Alternatively, our finding that sCD4 blocks C-dependent virus infection is consistent with an infection mechanism involving both CR2 and CD4. Other studies provide evidence for a requirement of both C receptor type 3 and CD4 for cell infection (5, 20). A requirement for CD4 in the C-dependent enhancement of infection was observed for HIV-1-Bru but not for HIV-1-RF (48). In the case of follicular dendritic cells, which express C receptors but lack CD4, C was found to mediate cell binding but not cell entry of HIV-1 (43). Undoubtedly, further investigations using different cell types and virus isolates as well as different approaches are needed to address the question of whether C-dependent HIV infection requires CD4 or may occur through C receptors alone. Our own data appear consistent with a mechanism involving: 1) opsonization of HIV-1 by C3b-derived fragment(s) and subsequent binding of the opsonized virus to CR2 cell receptors; and 2) CD4-mediated virus entry.

In this scheme, the fact that C facilitates cell infection at low doses of HIV-1 would be a consequence of an increased binding of the virus to the target cell, due to the higher efficiency of the opsonization process in terms of virus-cell encounter. This hypothesis is fully consistent with the known ability of microorganisms coated with C3-derived fragments to acquire high binding specificity and avidity for C receptors (4), as well as with modeling studies of the C-dependent infection enhancement process (50). The increased number of virus particles bound to CR2 would in turn allow increased cell infection through a “classical” mechanism involving the neighboring CD4.

A number of non-human retroviruses of avian, rodent, and feline origin have been shown to bind C1 and thereby activate the classical C pathway (51). The present study provides further and unambiguous evidence that the C1 complex of C also has the ability to recognize HIV-1 directly and triggers reactions that facilitate virus infection of C receptor-bearing cells in vitro. Although these conclusions should be extrapolated with care due to the limitations of the particular model system used, they provide further support to the hypothesis that C may play a role in viral infection and/or propagation, especially under situations as those seen in the early stages of infection, where virus concentration is low.

References


