Detection of antibody-dependent complement mediated inactivation of both autologous and heterologous virus in primary HIV-1 infection

Running title: C'MI in primary HIV-1 infection

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Abstract: 148 words

Text: 4,015 words

ABSTRACT

Specific CD8 T-cell responses to human immunodeficiency virus type 1 (HIV-1) are induced in primary infection and make an important contribution to the control of early viral replication. The importance of neutralizing antibodies in containing primary viremia is questioned because they usually arise much later. Nevertheless anti-envelope antibodies develop simultaneously with, or even before, peak viremia. We determined if such antibodies might control viremia by complement-mediated inactivation (C'MI). In each of seven patients studied, antibodies capable of C'MI appeared at or shortly after the peak in viremia, concomitantly with detection of virus-specific T-cell responses. The C'MI was effective on both autologous and heterologous HIV-1 isolates. Activation of the classical pathway and direct viral lysis were at least partly responsible. Since IgG-antibodies triggered the C'MI, specific memory B-cells could also be induced by vaccination. Thus consideration should be given to vaccination strategies that induce IgG antibodies capable of C'MI.

INTRODUCTION

To design a successful vaccine against HIV it is important to determine which arm of the immune response is capable of recognizing and destroying invading virus before infection becomes systemic. Studies of early infection can give clues as to the relevant immune response in controlling viral replication. Primary HIV-1 infection (PHI) is characterized by an uncontrolled viremia, which subsequently settles on a lower steady level. This viral load 'set-point' is a prognostic indicator for the subsequent rate of disease progression (12, 17, 22). It has been long established that cytotoxic T lymphocyte (CTL) activity can be detected concurrent with the initial decrease in plasma viral RNA levels, suggesting that the control of plasma viremia is at least partly due to cell mediated immune responses (5, 13, 24). In contrast, the role of humoral immunity and neutralizing antibodies (Nabs) has remained elusive (21). Although Nabs can be detected as early as 4 weeks after onset of symptoms in some patients (2, 28), they are generally absent or weak until several months after infection (1, 13, 20, 25, 26, 36). Anti-envelope antibodies, however, are generally present from the time-point of initial containment of viremia (1, 15, 16). In addition to direct interference with viral entry, antibodies *in vivo* mediate opsonization, antibody dependent cellular cytotoxicity (ADCC) and complement activation (6, 7). This led us to question whether early anti-envelope antibodies have a role in viral inactivation, not detectable in traditional neutralization assays, by triggering complement activation.

In this study of seven patients who initially presented with symptomatic primary infection, we show that immunoglobulin (Ig) G antibodies to the HIV-1 envelope present

at, or close to, peak viral load, can inactivate virus by direct viral lysis through activation of the classical complement pathway. In all patients the development of such antibodies mirrored the early detection of HIV-1 specific T-cell activity. These complement-activating antibodies can inactivate both autologous and heterologous virus in the majority of the patients. This suggests that it would be wise to incorporate envelope antigens capable of inducing such effector antibodies in potential vaccine candidates.

MATERIALS AND METHODS

Cells and viruses. Human glioma cells NP2, stably transfected with CD4 and CCR5, and 293T cells were grown in Dubelcco's Modified Eagle Medium (DMEM; Invitrogen, Paisley, UK) with 5% Foetal Calf Serum (FCS; Invitrogen, Paisley, UK). Chimeric viruses, and the molecular clone HIV-1_{YU2}, were produced by transfecting 293T cells. HIV-1_{YU2} was also grown in phytohaemagglutinin (PHA) stimulated PBMC, obtained from blood donors. The PBMC were cultured in RPMI-1640 (Invitrogen, Paisley, UK) supplemented with 10% FCS and 20 U/ml interleukin-2 (Roche, Lewes, UK).

Patients. Seven men who have sex with men (27-41 years old) presenting with PHI (5-26 days following onset of symptoms, DFOSx), following sexual exposure, were enrolled. Recent HIV-1 infection was diagnosed by the detection of HIV-1 genomes (PBMC proviral DNA or plasma RNA) in the presence or absence of an evolving HIV-1 antibody profile, which subsequently became fully positive, or by a fully positive HIV-1 antibody test within three months of a negative HIV-1 antibody test (MM4). Blood

samples were obtained weekly for the first month, monthly for three months and then at 3-monthly intervals. At each visit the patients' HIV-1 viral load (Chiron 3.0; Chiron, Emeryville, California, USA) was determined. MM22 commenced antiretroviral therapy at day 26 post-symptoms; only serum samples collected prior to this date were analyzed. The study protocol was approved by The Camden and Islington Community Services Local Research Ethics Committee and written informed consent obtained from all subjects.

Amplification of gp120 and generation of chimeric molecular clones. Viral envelopes were amplified from proviral DNA from patient PBMC as described previously (1). Briefly, the gp120 was amplified using the primers 988L+ 5'-GTAGCATTAGCGGCCGCAATAATAATAGCAATAG-3', 943S+ 5'-CAA TAG(CT)AGCATTAGTAGTAG-3', 609RE- 5'-CCCATAGTGCTTCCGGCCGCTCCCAAG-3' and 628L- 5'-TCATCTAGAGATTTATTACTCC-3' for the first round. For the nested polymerase chain reaction (PCR), primers 626L+ 5'-GTG**GGTCACC**GTCTATTATGGG-3' and 125Y- 5'-CACC**ACGCGT**CTCTTTGCCTTGGTGGG-3' were used, which contain *BstE*II and *Mlu*I sites (bold). The PCR conditions were: 30 cycles of 92 ºC for 45 seconds (s), 45 ºC for 45 s and 68 ºC for 210 s. The amplified DNA-fragment was cloned into pGEM-T Easy (Promega, Southampton, UK) and transferred into pHxB2-MCS-Δ-*env*, by digestion with *BstE*II and *Mlu*I. Plasmid pHxB2-MCS-Δ-*env* allows incorporation of heterologous gp120 sequences from amino acid 38 (seven amino acids after the signal peptide) to six amino acids prior to the gp120/gp41 junction (19). The

resulting molecular clones encode replication competent viruses with gp41 derived from HIV-1_{HxB2}.

Virus titration. Ten-fold serial dilutions of viral stocks were incubated on semi-confluent NP2/CD4/CCR5 cells, seeded in 48-well plates, for 2 hours at 37 ºC. The cells were then washed once and cultured for 72 hours. Infection was detected by p24-immunostaining, as detailed elsewhere (1). Briefly, fixed cells were incubated with mouse anti-HIV-1 p24 monoclonal antibodies (ADP 365 and 266, NIBSC, Potters Bar, UK; 1:40 dilution for 1 hour) followed by a goat anti-mouse Ig antibody conjugated to β-galactosidase (Southern Biotechnology Associates, Birmingham, Alabama, USA; at 2.5 µg/ml for 1 hour). After incubation with X-Gal substrate at 37 ºC, infected cells appear blue and focus-forming units (FFU) are counted using light microscope.

T-cell assays. HIV-specific T-cell responses were assessed using recombinant vaccinia virus-based IFN-γ ELISPOT assays (14). Responses were measured to a control recombinant vaccinia virus (vSC8) and vaccinia recombinants expressing HIV-1 gp160 (vPE16), Gag (vAbT-141.5.1), Pol (vCF21), Nef (vTFnef2) or Tat (vTat) (all derived from clade B viruses). Results are expressed as the mean (of duplicate or triplicate wells) number of protein-specific spot forming cells (SFCs) per 10^6 PBMCs, i.e. the mean number of cells producing IFN-γ in response to stimulation with the recombinant vaccinia virus expressing a given HIV protein minus the mean response observed to the control recombinant vaccinia virus.

Detection of anti-gp120 antibodies by enzyme-linked immunosorbent assay (ELISA). For detection of anti-gp120 Abs in sera, HIV-1_{IIIB} gp120 (NIBSC product No. EVA657, Potters Bar, UK) were bound to 96-well Maxisorb plates (Nalgene, NUNC International) which had been coated with antibody D7324 (10 μg/mL, over night) and pre-blocked with 1% milk powder (Marvel, 1 hour). D7324 (Aalto Bio Reagents, Dublin, Ireland) is a polyclonal antibody that recognizes a conserved epitope in the C-terminus of HIV-1. Serial diluted patient sera (from 1:100) in 100 μL TMT/GS (4% Marvel, 10% goat serum and 0.05% Tween 20 in TBS) was added to duplicate wells with captured envelope and to duplicate wells containing no gp120 (blanks), for background subtraction. Bound antibodies were subsequently detected with an alkaline phosphatase (AP) conjugated goat-anti-human Ig antibody (Harlan SeraLab, UK; diluted 1:2000 in TMT/GS) followed by a substrate solution (Lumi-Phos Plus, Aureon BioSystems GmBH, Austria). Relative light units were measured at 405 nm. Cut off for a positive reaction was set to 100 light units above background. At this cut of value none of more than ten HIV-seronegative control sera tested scored positive.

Complement. Complement was prepared from clotted blood from HIV-seronegative individuals and stored in aliquots at –80 ºC. Complement was inactivated by incubating the serum at 56 ºC for 2 hours. Guinea pig complement was reconstituted in PBS (Sigma, Poole, UK). C3 deficient serum was reconstituted with recombinant human C3 (Sigma, Poole, UK) to physiological concentration (1.2 mg/ml).

Neutralization and C'MI assays. Serial diluted heat-inactivated patient sera were incubated with 100 FFU of HIV-1 in a volume of 100 µL, for 1 hour at 37 ºC. To detect C'MI, a source of complement (HIV-seronegative human serum) was also included and added at a final concentration of 10% (v/v). As a negative control, parallel assays were run with heat-inactivated complement. Following the incubation the serum-antibody-virus cocktail was added to NP2/CD4/CCR5 cells, seeded in 48-well plates. After 2 hours incubation at 37 ºC, the cells were washed once and then cultured for 72 hours. Infection was measured by p24-immunostaining, as described above (see virus titration). The percentage reduction of infection in the presence of antibody (i.e. patient serum) and complement was calculated using the following formula: 100 x [1-(average FFU in the presence of patient serum and complement / average FFU in the presence of complement only)]. The percentage neutralization was calculated using the same formula, but with the virus assayed in the presence of heat-inactivated complement. Each patient-serum was assayed in triplicate, at least twice. Unless otherwise stated, presented data refers to patient-serum activity at a 1:10 dilution, with error bars showing the standard deviation between repeated independent experiments. For determination of serum neutralization end-point titres, FFU obtained in the presence of serial dilutions of patient serum was compared with FFU obtained in the presence of HIV-1 seronegative serum at a 1:10 dilution (both serum sources heat-inactivated). The threshold for a positive neutralization reaction was set to 90%, as reductions below this level are intrinsically variable between experiments (1).

Affinity purification of IgG. IgG was isolated from plasma using the MAbTrap kit (Amersham Biosciences, Little Chalfont, UK).

Detection of viral lysis. Viral lysis by complement and antibody was assessed by quantification of reverse transcriptase (RT) activity in virus suspensions following incubation at 37 °C for 1 hour. HIV-1_{YU2} (~10,000 FFU derived from 293T cells or 1,000 FFU from PBMC) was incubated with heat-inactivated patient sera (or seronegative sera) in the presence of 10% (v/v) complement in a 100 µl volume. RT activity was measured using the Lenti-RT Activity Assay (Cavidi Tech AB, Uppsala, Sweden). The amount of free RT in the samples is measured in a two-step procedure. Immobilized polyA template (in a microtiter plate) is reverse transcribed by free RT in the samples using bromo-deoxyuridine triphospate (BrdUTP) as nucleotide source. Incorporated BrdUMP is quantified in a colorimetric assay using an anti-BrdU antibody conjugated to alkaline phosphatase. Specifically, 50 µl of virus sample diluted 1:5 in the detergent free sample-dilution buffer was applied to duplicate wells for 3 hours. The plates was then washed once with detergent-free wash-buffer and subsequently processed following the supplied protocol for RT quantification. RT release is expressed as percentage increase in RT activity in virus suspensions incubated with complement in the presence of patient sera.

RESULTS

Late development of neutralizing antibodies. For this study we enrolled seven men between 27-41 years of age who initially presented with symptoms characteristic of PHI. To study the development of antibody-mediated neutralization, autologous envelopes (SU, gp120) were cloned into a HIV-1_{HXB2} based vector from patient PBMC at the earliest time-point available (between day 6 and 28 post onset of symptoms of PHI). We have previously shown that viral envelopes directly cloned in this way are representative of a homogeneous population of replicating viruses *in vivo* (1). The time to development of autologous Nabs ranged from 81 to 466 days post onset of symptomatic PHI (Table 1). Two patients, MM19 and MM28, developed intermediate activity (75-85%) by day 287 and day 405, respectively, but did not attain full neutralizing activity (≥90% reduction in virus infection) throughout the study (last sampled at day 701 and 503, respectively). This range of times to development of autologous virus neutralization is similar to that reported previously (1, 28, 36). All patients initiated a decline in viremia within the first month of symptomatic PHI, clearly preceding their development of Nabs (supplemental online material).

HIV-specific T-cell activity is detected from the earliest time-point. Unlike Nab, HIV-specific CTL responses can be detected prior to the peak in acute-phase viral replication, with strong virus-specific CD8 T-cell responses being temporally associated with the decline in primary viremia (5, 13, 24, 37). We confirmed that HIV-specific T-cell responses could be detected in this patient cohort during the acute phase of infection by using recombinant vaccinia virus-based IFN-γ ELISPOT assays to assess T-cell

responses to five HIV proteins: gp160, Gag, Pol, Nef and Tat at the earliest time-points for which PBMC samples were available. In five of the seven patients (MM8, MM19, MM22, MM23 and MM28), T-cell responses were assessed within two weeks of onset of symptoms of PHI, at time-points when the plasma viral load was in excess of 3,000,000 RNA copies/ml – i.e. at or close to the peak in acute viral replication; and in a sixth patient (MM27) the response was tested at a slightly later time-point, when the viral load was in decline. There were no appropriate PBMC samples available for testing from the seventh patient (MM4). In all six patients tested, T-cell responses were detected to at least two of the HIV proteins used at these acute (or very early) time-points (Table 2). Limited sample availability precluded confirmation of the phenotype of the responding T cells, but both we and others (12) have previously shown that the HIV-specific responses detected in these assays are predominantly mediated by $CD8^+$ T cells.

Antibody dependent complement mediated inactivation of virus is also detectable at acute infection time-points. In contrast to the delayed development of Nabs, anti-envelope antibodies were detectable in the first serum samples taken 6-28 days after onset of symptoms, as expected from our previous study (1). We tested whether the presence of complement could augment antiviral activity of early sera and thus if the early anti-envelope antibodies might contribute to the decline in viremia during primary infection. HIV-seronegative human serum was used as a source of complement and heat-inactivated aliquots served as negative controls. Inclusion of complement had a marked effect on the potency and time to development of serum mediated anti-viral activity (Fig.

1a). All activity was ablated if the source of complement was heat-inactivated, with neutralization titers remaining the same as with patient serum alone.

The potency of the C'MI response varied from patient to patient and the profiles fell into three distinct patterns (Fig. 1a). Acute sera from MM4, MM8 and MM19 had weak and varied neutralizing activity in the absence of complement but demonstrated potent viral inactivation (90-100%) in its presence, even at the first sampling time-points. Thus MM4, MM8 and MM19 achieved significant complement dependent 'neutralization' of viral infection by days 17, 12 and 13 respectively. MM23 and MM28 exhibited a different profile; their early sera lacked any detectable neutralizing activity (until day 204 and >503, respectively) but again substantial C'MI activity was evident from the earliest sample at day 9 and 6, respectively. The third pattern was observed for MM27 where weak neutralizing activity was steadily augmented over time by complement, from the first sample at day 28. No C'MI activity was observed with sera from MM22, but we could only analyze sera between days 14-25 since this patient commenced antiretroviral therapy at day 26.

Typical titration curves of C'MI activity at early and chronic infection time-points is shown in Fig. 1b, illustrating that the C'MI activity is detectable at higher serum dilutions (>1:10) than that used in Fig. 1a. At later time-points when 'conventional' neutralizing activity developed the C'MI effect augmented the neutralizing activity. IC_{90} end-point titers were generally enhanced two- to four-fold, irrespective of the C'MI potency at early time-points (Fig. 1b).

Table 3 summarizes the first time-point at which anti-envelope Abs, Nab, C'MI and T-cell activity was detected for each patient. The status of the patients' virus loads at these time-points is also indicated and shows that the majority had detectable C'MI in the acute viremic phase, characterized by high viral loads (>3,000,000 RNA copies/ml). Autologous C'MI and T-cell activity developed at least 2 months earlier than Nab activity, and typically more than 6 months earlier (range 69– >503 days). Apart from MM22, all patients developed antibodies capable of C'MI of autologous virus by the first sampling point. The most striking difference was seen in patient MM28 whose date for first detectable anti-HIV antibody activity was shifted from more than 503 days to day 6. Thus we have shown that antibody dependent C'MI of HIV-1, in contrast to Nabs, arises during acute infection when containment of viral replication is initiated. These results implicate a role for humoral immunity in control of viremia in primary HIV-1 infection.

The C'MI by patient sera is broad and inactivates heterologous HIV-1 isolates. The viruses tested so far were derived from molecular clones encoding autologous, patient-specific envelope. Apart from two patients (MM4 and MM8), who showed weak heterologous neutralizing activity (50%) from 200 days post onset of symptomatic PHI, no significant (>90%) heterologous neutralization was detected in any patient within the first year (Fig. 2a). We tested how broad the C'MI activity is using envelopes from an unrelated seroconverter (by testing responses to MM4's virus, HIV-$1_{4.1.33}$, in the other seroconverters) and the molecular clone HIV-1_{YU2}. In striking contrast to heterologous neutralization, apart from patient MM27, all patients developed heterologous C'MI activity in the earliest sample tested between 9 and 28 days post onset of symptomatic

PHI (Fig. 2a). In all cases the potency of C'MI reached levels similar to that of autologous virus inactivation. A typical profile can be seen for patient MM19, with at least 90% inactivation of HIV-1_{YU2} concomitant with autologous virus inactivation and maintained antiviral activity throughout the study period (Fig. 1a and 2a). The HIV-1_{YU2} virus was often more sensitive to C'MI than HIV-$1_{4.1.33}$ and in some cases inactivation of HIV-1_{YU2} was stronger than that of the autologous virus. For example, MM22 scored less than 40% inactivation of his own virus yet reached almost 100% inactivation of HIV-1_{YU2}. The lack of C'MI of heterologous viruses by serum from MM27 maybe linked to the weak autologous C'MI activity in this patient (Fig. 1a).

Previous reports have suggested that PBMC cultured HIV-1 is relatively resistant to C'MI (30, 32). In agreement with these studies we observed that PBMC-derived HIV-1_{YU2} virus is less susceptible to C'MI compared to 293T-derived virus (Fig. 2b).

Characterization of the antiviral activity. As shown above the antiviral effect is most likely to be mediated by complement because it is lost after heat-inactivation. We sought to determine that this antiviral response is indeed mediated by complement, rather than some other unknown heat-labile component, using purified guinea pig complement. No neutralization of autologous virus with serum from MM8 day 49 was observed in the absence of complement (Fig. 3a). Substitution of human serum as a source of complement with purified guinea pig complement resulted in inactivation profiles similar to C'MI with human serum as source (Fig. 3a). Thus, a purified source of complement

proteins can substitute for human serum in virus inactivation by antibodies early in infection.

The complement cascade *in vivo* can be activated by an antibody dependent (classical), an antibody independent (alternative) or a lectin pathway (4). In order to determine the pathway involved in the observed antiviral effect, we substituted the complement source with sera obtained from C1q and C3 deficient individuals. C1q deficiency results in an inability to activate the classical pathway while C3 deficient individuals fail to execute the final common pathway. We tested the first serum sample from two patients, MM8 and MM28. In the presence of complement we observed almost 70% inactivation of input virus, which was completely ablated when the source of complement was heat-inactivated (Fig. 3b). When the source of complement was derived from C1q or C3 deficient individuals, the antiviral effect of both patients' sera was reduced to less than 20% (C1q) and 10% (C3), respectively (Fig. 3b). For C3 deficient serum, the antiviral effect was recovered by addition of recombinant C3, demonstrating the specificity of the antiviral effect (Fig. 3b). The ablation of the antiviral effect in the absence of C1q confirms that the classical complement pathway is largely responsible for the antiviral effects observed.

Following infection with HIV-1, IgG1 and IgG3 are the predominant subtypes induced in plasma and cervicovaginal, seminal and rectal secretions (27), and these are potent activators of the complement pathway (6). We wished to establish if the early C'MI activity observed is mediated by IgG antibodies. This is of interest since vaccine-induced

immunological memory is likely to be mediated through IgG antibodies. Affinity purified IgG from day 13 serum from patient MM19 was tested for C'MI activity and resulted in 50% reduction of input virus (Fig. 3c). This is less than the 90% reduction observed with unfractionated sera (Fig. 1a), possible due to dilution of IgG or the removal of IgM during the purification process. As expected, since whole serum was non-neutralizing, purified IgG either alone (data not shown) or in the presence of heat-inactivated complement had no effect on viral infectivity (Fig. 3c). The C'MI was also mediated largely through IgG antibodies from the same patient in the chronic phase (day 701) (Fig. 3c).

We sought to define the mechanism of this IgG mediated antiviral effect. If direct lysis of virus plays a role, incubation of virus with antibody and complement would enhance the release of virion components normally contained within the viral envelope, for example RT (31). We measured the levels of RT activity in viral supernatants after treatment with serum and complement and compared them to virus supernatants in the presence of complement alone. Virus treated with both anti- HIV-1 serum and complement resulted in approximately 100% more RT release compared to treatment with non-immune serum and complement (Fig. 3d). The level of viral lysis was similar in 293T and PBMC produced viruses. This indicates that viral lysis is at least partially responsible for C'MI.

DISCUSSION

Here we demonstrate that antibodies to the HIV-1 envelope, like CTLs, may control the acute viremia in HIV-1 infection. We detected IgG antibody-mediated complement inactivation of either autologous and/or heterologous HIV-1 strains in all patients as early as 6-28 days post onset of symptomatic PHI. In contrast, autologous Nabs generally developed more than 200 days after symptomatic PHI and heterologous neutralization was even later, if at all detectable. We also demonstrate that activation of the classical complement pathway is the major effector pathway. When serum from uninfected C1q or C3 deficient individuals was used as a complement source no anti-viral activity was observed. Direct lysis of virions is at least in part the mechanism by which virus is inactivated, although coating of virons by complement components may also contribute to the viral inactivation **(**32, 35**)**. Other studies have implicated antibodies in ADCC mediated antiviral activity early in infection, preceding the development of neutralizing activity (9, 10). Although we have not formally demonstrated it here, it is likely that Nabs serve the 'dual role' of both neutralizing and complement-activating antiviral activity. Since IgG antibodies mediated the C'MI we suggest that it should be possible to induce an effective C'MI memory B-cell response with candidate vaccines. It may also be advisable to ensure that complement-activating antibody-subtypes are induced through the choice of suitable adjuvants (8, 23).

The susceptibility of a virus to C'MI has been linked to the level of complement regulatory proteins such as CD55, CD46 and CD59 expressed on the producer cells and incorporated into virions (18, 29, 30, 32). The viruses used in this study were produced

in 293T cells, which express all three regulatory proteins (data not shown). In agreement with others (30, 32), we observed that PBMC-derived virus is less susceptible to C'MI compared to 293T-derived virus. Indeed, the *in vivo* interactions between HIV-1 and complement are controversial and may in certain circumstances enhance infection, for example infection of dendritic cells (3, 33). Nonetheless, Spear and colleagues have shown that uncultured HIV-1 purified directly from patient plasma is highly susceptible to C'MI (34), supporting the *in vivo* relevance of our findings. Furthermore, data from Gauduin *et al.* support a role for complement in passive antibody-mediated protection against HIV-1 infection in a (human-PBL-SCID) mouse model (11).

In conclusion we have demonstrated a previously overlooked potential role for antibodies in the control of viremia in acute HIV-1 infection. We recommend that envelope antigens capable of inducing both neutralizing and 'C'MI-neutralizing' antibodies should be included in addition to CTL inducing antigens in candidate HIV-1 vaccines. Further studies will be needed to determine whether specific epitopes of the HIV-1 envelope will need to be targeted.

ACKNOWLEGMENTS

We thank Robin Weiss and Stuart Neil for helpful discussions and reading the manuscript, Srinika Ranasinghe for technical assistance and Marina Botto for advice and sera from complement deficient patients. We also thank Diana Aldam for her help with the clinical side of the study.

The Wellcome Trust, AVERT-UK, the MRC-UK, NIH grant AI 41530 and core funding from the Edward Jenner Institute for Vaccine Research (EJIVR, publication number 90), supported this work.

REFERENCES

1. **Aasa-Chapman, M. M. I., A. Hayman, P. Newton, D. Cornforth, I. Williams, P. Borrow, P. Balfe, and A. McKnight.** 2004. Development of the antibody response in acute HIV-1 infection. AIDS **18:**371-381.

2. **Albert, J., B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nystrom, and E. M. Fenyo.** 1990. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. AIDS **4:**107-12.

3. **Bajtay, Z., C. Speth, A. Erdei, and M. P. Dierich.** 2004. Cutting Edge: Productive HIV-1 Infection of Dendritic Cells via Complement Receptor Type 3 (CR3, CD11b/CD18). J Immunol **173:**4775-8.

4. **Blue, C. E., O. B. Spiller, and D. J. Blackbourn.** 2004. The relevance of complement to virus biology. Virology **319:**176-84.

5. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol **68:**6103-10.

6. **Burton, D. R.** 1985. Immunoglobulin G: functional sites. Mol Immunol **22:**161-206.

7. **Casadevall, A., and L. A. Pirofski.** 2003. Antibody-mediated regulation of cellular immunity and the inflammatory response. Trends Immunol **24:**474-8.

8. **Cohen, A. D., J. D. Boyer, and D. B. Weiner.** 1998. Modulating the immune response to genetic immunization. Faseb J **12:**1611-26.

9. **Connick, E., D. G. Marr, X. Q. Zhang, S. J. Clark, M. S. Saag, R. T. Schooley, and T. J. Curiel.** 1996. HIV-specific cellular and humoral immune responses in primary HIV infection. AIDS Res Hum Retroviruses **12:**1129-40.

10. **Forthal, D. N., G. Landucci, and E. S. Daar.** 2001. Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells. J Virol **75:**6953-61.

11. **Gauduin, M. C., R. Weir, M. S. Fung, and R. A. Koup.** 1998. Involvement of the complement system in antibody-mediated post-exposure protection against human immunodeficiency virus type 1. AIDS Res Hum Retroviruses **14:**205-11.

12. **Katzenstein, T. L., C. Pedersen, C. Nielsen, J. D. Lundgren, P. H. Jakobsen, and J. Gerstoft.** 1996. Longitudinal serum HIV RNA quantification: correlation to viral phenotype at seroconversion and clinical outcome. AIDS **10:**167-73.

13. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol **68:**4650-5.

14. **Larsson, M., X. Jin, B. Ramratnam, G. S. Ogg, J. Engelmayer, M. A. Demoitie, A. J. McMichael, W. I. Cox, R. M. Steinman, D. Nixon, and N. Bhardwaj.** 1999. A recombinant vaccinia virus based ELISPOT assay detects high frequencies of Pol-specific CD8 T cells in HIV-1-positive individuals. AIDS **13:**767-77.

15. **Lindback, S., A. C. Karlsson, J. Mittler, A. Blaxhult, M. Carlsson, G. Briheim, A. Sonnerborg, and H. Gaines.** 2000. Viral dynamics in primary HIV-

1 infection. Karolinska Institutet Primary HIV Infection Study Group. AIDS **14:**2283-91.

16. **Lindback, S., R. Thorstensson, A. C. Karlsson, M. von Sydow, L. Flamholc, A. Blaxhult, A. Sonnerborg, G. Biberfeld, and H. Gaines.** 2000. Diagnosis of primary HIV-1 infection and duration of follow-up after HIV exposure. Karolinska Institute Primary HIV Infection Study Group. AIDS **14:**2333-9.

17. **Lyles, R. H., A. Munoz, T. E. Yamashita, H. Bazmi, R. Detels, C. R. Rinaldo, J. B. Margolick, J. P. Phair, and J. W. Mellors.** 2000. Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study. J Infect Dis **181:**872-80.

18. **Magre, S., Y. Takeuchi, G. Langford, A. Richards, C. Patience, and R. Weiss.** 2004. Reduced Sensitivity to Human Serum Inactivation of Enveloped Viruses Produced by Pig cells Transgenic for Human CD55 or Deficient for the Galactosyl-alpha(1-3) Galactosyl Epitope. Journal of Virology **78:**5812-19.

19. **McKeating, J. A., Y. J. Zhang, C. Arnold, R. Frederiksson, E. M. Fenyo, and P. Balfe.** 1996. Chimeric viruses expressing primary envelope glycoproteins of human immunodeficiency virus type I show increased sensitivity to neutralization by human sera. Virology **220:**450-60.

20. **McKnight, A., P. R. Clapham, J. Goudsmit, R. Cheingsong-Popov, J. N. Weber, and R. A. Weiss.** 1992. Development of HIV-1 group-specific neutralizing antibodies after seroconversion. AIDS **6:**799-802.

21. **McMichael, A. J., and T. Hanke.** 2003. HIV vaccines 1983-2003. Nat Med **9:**874-80.

22. **Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd, and L. A. Kingsley.** 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science **272:**1167-70.

23. **O'Hagan, D. T., and N. M. Valiante.** 2003. Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov **2:**727-35.

24. **Pantaleo, G., J. F. Demarest, H. Soudeyns, C. Graziosi, F. Denis, J. W. Adelsberger, P. Borrow, M. S. Saag, G. M. Shaw, R. P. Sekaly, and et al.** 1994. Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. Nature **370:**463-7.

25. **Pellegrin, I., E. Legrand, D. Neau, P. Bonot, B. Masquelier, J. L. Pellegrin, J. M. Ragnaud, N. Bernard, and H. J. Fleury.** 1996. Kinetics of appearance of neutralizing antibodies in 12 patients with primary or recent HIV-1 infection and relationship with plasma and cellular viral loads. J Acquir Immune Defic Syndr Hum Retrovirol **11:**438-47.

26. **Pilgrim, A. K., G. Pantaleo, O. J. Cohen, L. M. Fink, J. Y. Zhou, J. T. Zhou, D. P. Bolognesi, A. S. Fauci, and D. C. Montefiori.** 1997. Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. J Infect Dis **176:**924-32.

27. **Raux, M., L. Finkielsztejn, D. Salmon-Ceron, H. Bouchez, J. L. Excler, E. Dulioust, J. M. Grouin, D. Sicard, and C. Blondeau.** 2000. IgG subclass

distribution in serum and various mucosal fluids of HIV type 1-infected subjects. AIDS Res Hum Retroviruses **16:**583-94.

28. **Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A **100:**4144-9.

29. **Saifuddin, M., T. Hedayati, J. P. Atkinson, M. H. Holguin, C. J. Parker, and G. T. Spear.** 1997. Human immunodeficiency virus type 1 incorporates both glycosyl phosphatidylinositol-anchored CD55 and CD59 and integral membrane CD46 at levels that protect from complement-mediated destruction. J Gen Virol **78 (Pt 8):**1907-11.

30. **Saifuddin, M., C. J. Parker, M. E. Peeples, M. K. Gorny, S. Zolla-Pazner, M. Ghassemi, I. A. Rooney, J. P. Atkinson, and G. T. Spear.** 1995. Role of virion-associated glycosylphosphatidylinositol-linked proteins CD55 and CD59 in complement resistance of cell line-derived and primary isolates of HIV-1. J Exp Med **182:**501-9.

31. **Spear, G. T., B. L. Sullivan, A. L. Landay, and T. F. Lint.** 1990. Neutralization of human immunodeficiency virus type 1 by complement occurs by viral lysis. J Virol **64:**5869-73.

32. **Stoiber, H., C. Pinter, A. G. Siccardi, A. Clivio, and M. P. Dierich.** 1996. Efficient destruction of human immunodeficiency virus in human serum by inhibiting the protective action of complement factor H and decay accelerating factor (DAF, CD55). J Exp Med **183:**307-10.

33. **Stoiber, H., C. Speth, and M. P. Dierich.** 2003. Role of complement in the control of HIV dynamics and pathogenesis. Vaccine **21 Suppl 2:**S77-82.

34. **Sullivan, B. L., E. J. Knopoff, M. Saifuddin, D. M. Takefman, M. N. Saarloos, B. E. Sha, and G. T. Spear.** 1996. Susceptibility of HIV-1 plasma virus to complement-mediated lysis. Evidence for a role in clerance of virus in vivo. J Immunol **157:**1791-8.

35. **Sullivan, B. L., D. M. Takefman, and G. T. Spear.** 1998. Complement can neutralize HIV-1 plasma virus by a C5-independent mechanism. Virology **248:**173-81.

36. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307-12.

37. **Wilson, J. D., G. S. Ogg, R. L. Allen, C. Davis, S. Shaunak, J. Downie, W. Dyer, C. Workman, S. Sullivan, A. J. McMichael, and S. L. Rowland-Jones.** 2000. Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. AIDS **14:**225-33.

FIGURE LEGENDS

FIG. 1. Development of C'MI and neutralization activity in HIV-1 infected patients. (a) The percentage reduction in infection of 100 FFU of autologous virus in the presence of sequential patient sera (at a dilution of 1:10), assayed in the presence (closed data points) or absence (i.e. neutralization, open data points) of complement is shown. HIV-1 seronegative sera served as complement source (added at a final concentration of 10% (v/v)), and heat-inactivated aliquots as negative controls. The error bars refer to the standard deviation between two or three independent experiments. (b) Typical C'MI and neutralization with increasing dilution of patient-serum from an early and a later infection time-point. The error bars refer to the standard deviation between two independent experiments.

FIG. 2. C'MI and neutralization of heterologous viruses. For experimental details see Fig. 1. As before closed symbols represents C'MI and open symbols neutralization. The error bars refer to the standard deviation between two or three independent experiments. (a) C'MI and neutralization of heterologous viruses produced in 293T cells. Data for HIV-1_{YU2} is indicated with squares, and HIV-$1_{4.1.33}$ data with triangles. (b) Data for HIV-1_{YU2} passaged through PBMC.

FIG. 3. Characterization of the anti-HIV activity in patient sera. (a) Guinea pig complement can substitute for human complement in C'MI assays using day 49 serum from patient MM8 (closed squares). C'MI activity is lost following heat-inactivation

(open squares). (b) Serum form C1q or C3 deficient individuals cannot mediate the antiviral activity observed with normal human serum as complement source. Reconstitution of C3 restores the antiviral activity. (c) Affinity purified IgG antibodies mediate the complement-dependent virus inactivation. Black fill represents activity in the presence of complement and white is in the presence of heat-inactivated complement. (d) Release of RT from virions was measured by Lenti-RT Activity Assay (Cavidi Tech AB, Sweden), under non-lysing detergent-free conditions, after treatment with serum and complement. HIV-1_{YU2} derived from either 293T cells or PBMC was assayed for susceptibility to complement mediated lysis. Non-immune serum had no effect on RT release compared to complement alone (open squares). Immune serum, however, resulted in 100% increase in RT activity (closed symbols). Data presented show activity of MM28 sera, from day 20 for the 293T produced HIV-1_{YU2} and from day 34 (squares) and day 93 (diamonds) for PBMC produced HIV-$1_{YU2.}$

TABLE 1. Neutralising activity of sequential autologous sera

Patient	Virus[b]	Isolation day[c]	IC_{90} at days from onset of symptomatic primary HIV-1 infection[a]												
			<14	15-28	30-40	49-66	80-110	185-206	269-300	310-340	350-410	456-466	490-520	570-631	>690
MM4[d]	4.1.33	17	-	<10	<10	<10	<10	20	10	20	20	-	20	20	40
MM8[d]	8.2.50	12	<10	<10	-	<10	10	20	-	20	-	-	-	20	40
	8.2.51	12	<10	<10	-	<10	10	20	-	80	-	-	-	80	80
MM19	19.1.A	13	<10	<10	<10	<10	<10	<10	<10	-	<10	<10	<10	-	<10
MM22	22.2.D	14	<10	<10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
MM23	23.2.E	15	<10	<10	<10	<10	-	80	-	160	-	-	320	160	-
MM27	27.1.J	28	-	<10	<10	<10	<10	<10	<10	-	-	10	-	20	-
MM28	28.1.5	6	<10	<10	<10	<10	<10	<10	-	-	<10	-	<10	-	-
	28.1.6	6	<10	<10	<10	<10	<10	<10	-	-	<10	-	<10	-	-

[a] Neutralization titers are expressed as the reciprocal dilution of serum required to reduce infectivity ≥90% (IC_{90}) compaired to HIV-1 seronegative sera (at a dilution of 1:10), as measured by immunostaining of infected NP2/CD4/CCR5 cells.

[b] The sequential sera were tested against chimeric viruses with patient-derived viral envelopes.

[c] Time-point from which patients' viral envelopes were cloned, numbers refering to DFOSx.

[d] The neutralization profiles for these patients have been described previously (14).

Symbols: -, no samples obtained; <10, 90% neutralization was not observed at the lowest (1:10) serum dilution tested; nd, not determined, as MM22 commenced antiretroviral therapy 26 days after onset of symptoms.

TABLE 2. Virus-specific T-cell activity during the acute-phase primary viremic burst

Patient	Time-point (DFOSx)	T cell response[a]				
		gp160	Gag	Pol	Nef	Tat
MM8	12	473 ± 7	0	528 ± 130	0	0
MM19	13	45 ± 20	92 ± 20	47 ± 27	5 ± 20	0
MM22	14	78 ± 20	35 ± 55	247 ± 21	0	0
MM23	9	265 ± 221	345 ± 154	87 ± 42	38 ± 59	85 ± 74
MM27	28	4 ± 13	17 ± 21	28 ± 5	0	28 ± 26
MM28	6	73 ± 0	58 ± 7	105 ± 32	55 ± 32	858 ± 148

[a] T-cell responses to five HIV proteins (gp160, Gag. Pol, Nef and Tat) were assessed at the indicated time-points (days from onset of symptoms) during acute (MM8, 19, 22, 23, and 28) or very early (MM27) infection using recombinant vaccinia virus-based IFN-γ ELISPOT assays. The values shown indicate the magnitude of the response observed to each HIV protein, expressed as the mean number of protein-specific IFN-γ spot forming cells per 10^6 PBMCs (± 1 standard deviation).

TABLE 3. First detection time-points for T-cell and antibody mediated antiviral activity following infection

Patient	Time-point (DFOSx)	Viral load (RNA copies/ml)	Anti-HIV Abs[a]	Anti-gp120 Abs (reciprocal end-point titer)[b]	T-cell activity[c]	C'MI[d]	Nab[e]
MM4	17	160,000	+	100	nd	+	-
	206	30,200		12,800			+
MM8	12	5,927,000	+	<100	+	+	-
	81	41,900		3,200			+
MM19	13	5,678,900	+	<100	+	+	-
	519	371,700		12,800			-
MM22	14	8,311,000	+	100	+	nd	nd
MM23	9	11,105,300	+	<100	+	+	-
	204	117,600		51,200			+
MM27	28	353,200	+	800	+	+	-
	466	10,600		12,800			+
MM28	6	4,337,100	+	100	+	+	-
	503	38,600		102,400			-

[a] All patients had detectable levels of anti-HIV Abs from the first study sample in diagnostic tests (Murex HIV-1.2.0, Abbott, Germany; Wellcozyme HIV Recombinant, Abbott, Germany; Serodia HIV-1/2, Fujirebio INC, Japan and VIDAS HIV Duo, bioMérieux, France).

[b] The titer of anti-gp120 Abs in patient sera was determined by an in-house ELISA, as described in Material and Methods, the lowest serum dilution tested was 1:100.

[c] HIV-specific T-cell responses were detected in recombinant vaccinia virus-based IFN-γ ELISPOT assays (Table 2).

[d] The antibody dependent complement mediated virus inactivation (C'MI) was intermediate (approximately 50% inactivation of input virus) in early serum from patients

MM23, MM27 and MM28, and strong (approximately 90% inactivation of input virus) in early serum from patients MM4, MM8 and MM19.

[e] Neutralization defined as ≥90% reduction of infection of input virus (Table 1).

605 Symbols: +, detectable response; -, undetectable responses; nd, not determined, as no (suitable) samples were available for analysis.

Figure 1

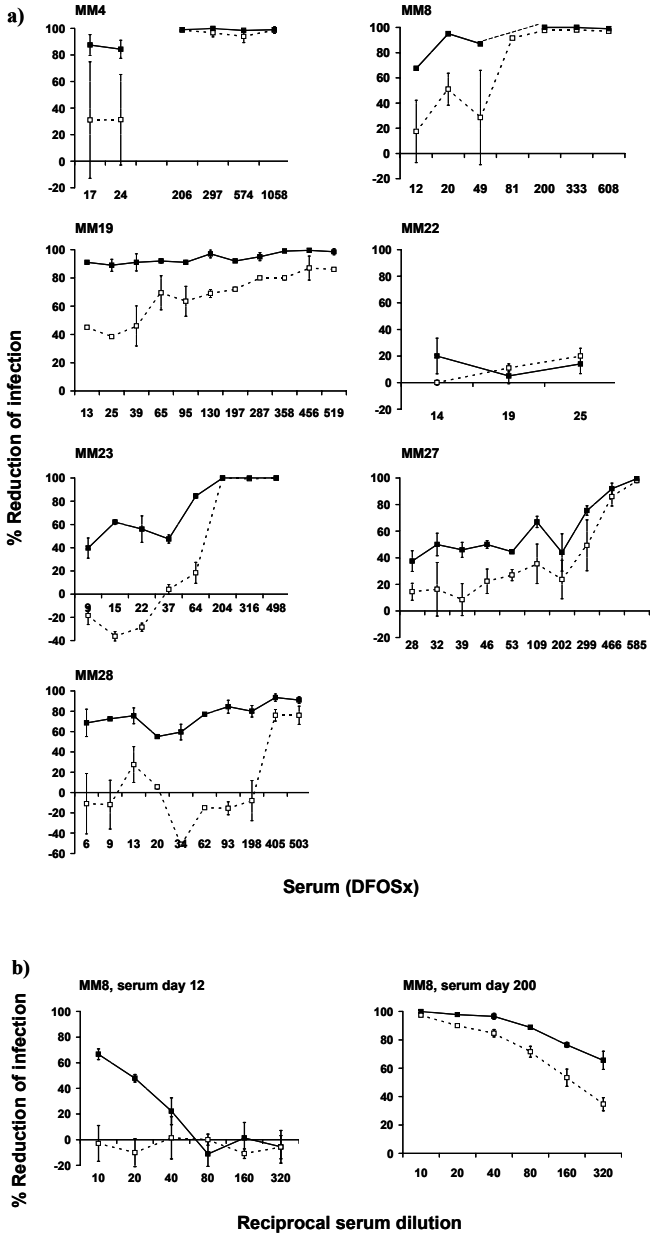

a)
MM4
MM8
MM19
MM22
MM23
MM27
MM28
% Reduction of infection
Serum (DFOSx)
b)
MM8, serum day 12
MM8, serum day 200
% Reduction of infection
Reciprocal serum dilution

Figure 2

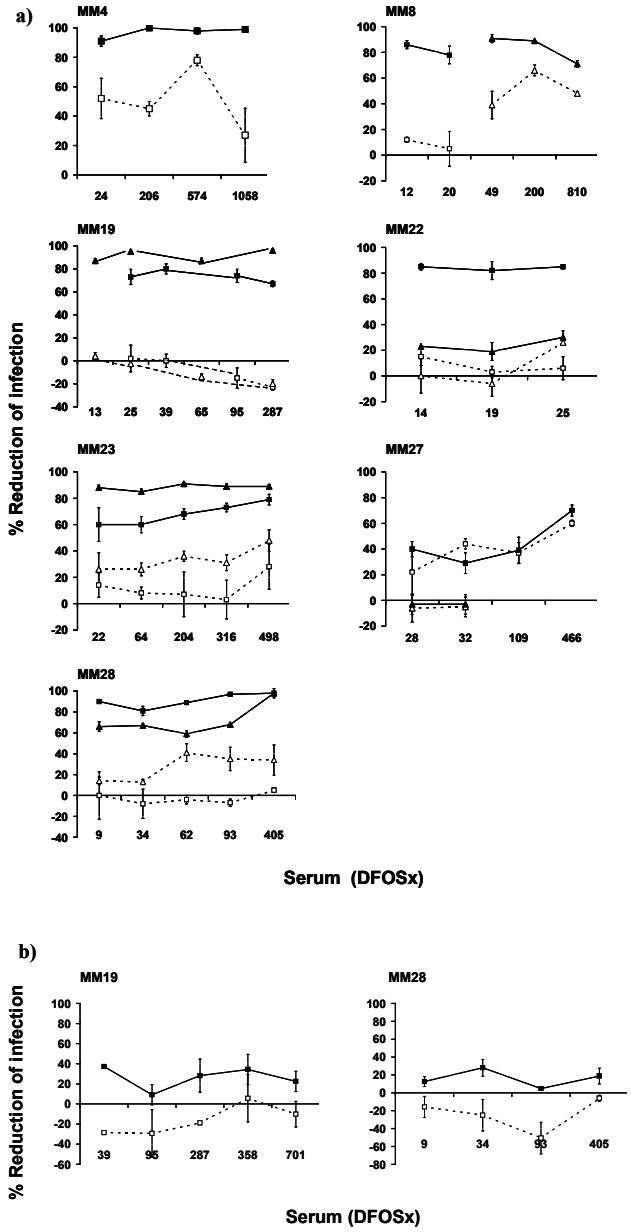
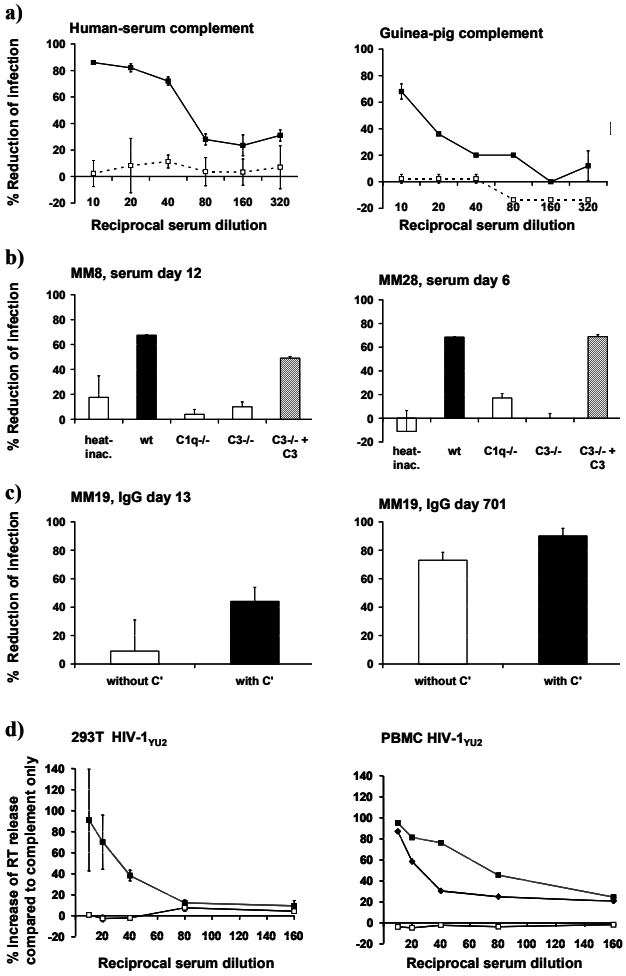

a)
MM4
MM8
MM19
MM22
MM23
MM27
MM28
% Reduction of Infection
Serum (DFOSx)
b)
MM19
MM28
% Reduction of Infection
Serum (DFOSx)

Figure 3


a)
Human-serum complement
Guinea-pig complement
% Reduction of infection
Reciprocal serum dilution
b)
MM8, serum day 12
MM28, serum day 6
heat-inac.
wt
C1q-/-
C3-/-
C3-/- + C3
c)
MM19, IgG day 13
MM19, IgG day 701
without C'
with C'
d)
293T HIV-1YU2
PBMC HIV-1YU2
% increase of RT release compared to complement only