JOURNAL OF VIROLOGY, Mar. 2005, p. 2823–2830 Vol. 79, No. 5
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.5.2823–2830.2005

Detection of Antibody-Dependent Complement-Mediated Inactivation of both Autologous and Heterologous Virus in Primary Human Immunodeficiency Virus Type 1 Infection†

Marlén M. I. Aasa-Chapman,[1]‡ Sophie Holuigue,[1]‡ Keith Aubin,[1] MaiLee Wong,[2] Nicola A. Jones,[2] David Cornforth,[3] Pierre Pellegrino,[3] Philippa Newton,[3] Ian Williams,[3] Persephone Borrow,[2] and Áine McKnight[1]*

Wohl Virion Centre, Windeyer Institute of Medical Sciences,[1] and Centre for Sexual Health and HIV Research,[3] University College London, London, and The Edward Jenner Institute for Vaccine Research, Compton, Newbury, Berkshire,[2] United Kingdom

Received 28 July 2004/Accepted 15 October 2004

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 antienvelope antibodies develop simultaneously with, or even before, peak viremia. We determined whether such antibodies might control viremia by complement-mediated inactivation (CMI). In each of seven patients studied, antibodies capable of CMI appeared at or shortly after the peak in viremia, concomitantly with detection of virus-specific T-cell responses. The CMI 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 immunoglobulin G (IgG)-antibodies triggered the CMI, specific memory B cells could also be induced by vaccination. Thus, consideration should be given to vaccination strategies that induce IgG antibodies capable of CMI.

To design a successful vaccine against human immunodeficiency virus (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 type 1 (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 concurrently 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 the 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). Antienvelope 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, and complement activation (6, 7). This led us to question whether early antienvelope 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 G (IgG) 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 NP2 cells, stably transfected with CD4 and CCR5, and 293T cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, United Kingdom) with 5% fetal calf serum (Invitrogen). Chimeric viruses and the molecular clone HIV-1_{YU2} were produced by transfecting 293T cells. HIV-1_{YU2} was also grown in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC), which were obtained from blood donors. The PBMC were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum and 20 U of interleukin-2 (Roche, Lewes, United Kingdom) per ml.

Patients. Seven men who have sex with men (27 to 41 years old), presenting with PHI (5 to 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 (for patient MM4) by a fully positive HIV-1 antibody test within 3 months of a negative HIV-1 antibody test. Blood samples were obtained weekly for the first month, monthly for 3 months and then at 3-month intervals. At each

* Corresponding author. Mailing address: Windeyer Institute of Medical Sciences, Wohl Virion Centre, 46 Cleveland St., London W1T 4JF, United Kingdom. Phone: 44 20 7679 9581. Fax: 44 20 7679 9555. E-mail: a.mcknight@ucl.ac.uk.

† Publication no. 90 from the Edward Jenner Institute for Vaccine Research.

‡ M.M.I.A.-C. and S.H contributed equally to this study.

visit the patient's HIV-1 viral load (Chiron [Emeryville, Calif.] 3.0) was determined. Patient MM22 commenced antiretroviral therapy at day 26 after 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, gp120 was amplified by using the primers 988L+ (5′-GTAGCATTAGCGGCCGCAATAATAATAGCAATAG-3′), 943S+ (5′-CAATAG[CT]AGCATTAGTAGTAG-3′), 609RE− (5′-CCCATAGTGCTTCCGGCCGCTCCCAAG-3′), and 628L− (5′-TCATCTAGAGATTTATTACTCC-3′) for the first round. For the nested PCR, primers 626L+ (5′-GTG**GGTCACC**GTCTATTATGGG-3′) and 125Y− (5′-CACC**ACGCGT**CTCTTTGCCTTGGTGGG-3′), which contain BstEII and MluI sites (boldface), were used. The PCR conditions used were 30 cycles of 92°C for 45 s, 45°C for 45 s, and 68°C for 210 s. The amplified DNA fragment was cloned into pGEM-T Easy (Promega, Southampton, United Kingdom) and transferred into pHxB2-MCS-Δ-*env* by digestion with BstEII and MluI. Plasmid pHxB2-MCS-Δ-*env* allows incorporation of heterologous gp120 sequences from amino acid 38 (seven amino acids after the signal peptide) to 6 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. Tenfold serial dilutions of viral stocks were incubated on semiconfluent NP2/CD4/CCR5 cells, seeded in 48-well plates, for 2 h at 37°C. The cells were then washed once and cultured for 72 h. 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 [National Institute for Biological Standards and Control Potters Bar, United Kingdom]; 1:40 dilution for 1 h), followed by a goat anti-mouse Ig antibody conjugated to β-galactosidase (Southern Biotechnology Associates, Birmingham, Ala.; 2.5 μg/ml for 1 h). After incubation with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate at 37°C, infected cells appeared blue and focus-forming units (FFU) were counted with a light microscope.

T-cell assays. HIV-specific T-cell responses were assessed by using recombinant vaccinia virus-based gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays (14). Responses to a control recombinant vaccinia virus (vSC8) and to vaccinia virus recombinants expressing HIV-1 gp160 (vPE16), Gag (vAbT-141.5.1), Pol (vCF21), Nef (vTFnef2), or Tat (vTat) (all derived from clade B viruses) were measured. Results are expressed as the mean (from duplicate or triplicate wells) number of protein-specific spot-forming cells per 10^6 PBMC, 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 to the control recombinant vaccinia virus.

Detection of anti-gp120 antibodies by enzyme-linked immunosorbent assay. For detection of anti-gp120 antibodies in sera, HIV-1_{IIIB} gp120 (product no. EVA657; National Institute for Biological Standards and Control) was bound to 96-well Maxisorb plates (Nalgene, Nunc International) which had been coated with antibody D7324 (10 μg/ml, overnight) and preblocked with 1% milk powder (Marvel, 1 h). D7324 (Aalto Bio Reagents, Dublin, Ireland) is a polyclonal antibody that recognizes a conserved epitope in the C terminus of HIV-1 gp120. Serial diluted patient sera (from 1:100) in 100 μl of TMT-GS (4% Marvel, 10% goat serum, and 0.05% Tween 20 in Tris-buffered saline) 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-conjugated goat anti-human Ig antibody (Harlan SeraLab, Loughborough, United Kingdom) diluted 1:2,000 in TMT-GS, followed by a substrate solution (Lumi-Phos Plus; Aureon BioSystems GmbH, Vienna, Austria). Relative light units were measured at 405 nm. The cutoff for a positive reaction was set to 100 light units above background. At this cutoff value, none of more than 10 HIV-seronegative control sera tested scored positive.

Complement. Complement was prepared from clotted blood from HIV-seronegative individuals and stored in aliquots at −80°C. Complement was inactivated by incubating the serum at 56°C for 2 h. Guinea pig complement was reconstituted in phosphate-buffered saline (Sigma, Poole, United Kingdom). C3-deficient serum was reconstituted with recombinant human C3 (Sigma) to the physiological concentration (1.2 mg/ml).

Neutralization and CMI assays. Serial diluted heat-inactivated patient sera were incubated with 100 FFU of HIV-1 in a volume of 100 μl, for 1 h at 37°C. To detect complement-mediated inactivation (CMI), a source of complement (HIV-seronegative human serum) was also included and was added at a final concentration of 10% (vol/vol). 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 h of incubation at 37°C, the cells were washed once and then cultured for 72 h. Infection was measured by p24 immunostaining (see "Virus titration" above). The percent reduction of infection in the presence of antibody (i.e., patient serum) and complement was calculated by using the following formula: $100 \times [1 - (\text{average FFU in the presence of patient serum and complement}/\text{average FFU in the presence of complement only})]$. The percent neutralization was calculated by 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, the data presented refer to patient serum activity at a 1:10 dilution, with error bars showing the standard deviations between repeated independent experiments. For determination of serum neutralization end point titers, FFU obtained in the presence of serial dilutions of patient serum were compared with FFU obtained in the presence of HIV-1-seronegative serum at a 1:10 dilution (sera from both sources were 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 by using the MAbTrap kit (Amersham Biosciences, Little Chalfont, United Kingdom).

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 h. 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% (vol/vol) complement in a 100-μl volume. RT activity was measured by 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 poly(A) template (in a microtiter plate) is reverse transcribed by free RT in the samples by using bromodeoxyuridine triphosphate as nucleotide source. Incorporated bromodeoxyuridine monophosphate is quantified in a colorimetric assay with an antibromodeoxyuridine 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 h. The plates was then washed once with detergent-free wash buffer and subsequently processed according to the supplied protocol for RT quantification. RT release is expressed as the percent 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 and 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 days 6 and 28 after 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 after onset of symptomatic PHI (Table 1). Two patients, MM19 and MM28, developed intermediate activity (75 to 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 days 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 (data not shown).

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 de-

TABLE 1. Neutralizing activities 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[e]	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 by ≥90% (IC_{90}) compared to HIV-1-seronegative sera (at a dilution of 1:10), as measured by immunostaining of infected NP2/CD4/CCR5 cells. —, no samples obtained; <10, 90% neutralization was not observed at the lowest (1:10) serum dilution tested.
[b] The sequential sera were tested against chimeric viruses with patient-derived viral envelopes.
[c] Time point (DFOSx) from which patients' viral envelopes were cloned.
[d] The neutralization profile for this patient has been described previously (1).
[e] ND, not determined, as MM22 commenced antiretroviral therapy 26 days after onset of symptoms.

tected 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. For five of the seven patients (MM8, MM19, MM22, MM23, and MM28), T-cell responses were assessed within 2 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 for 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 to at least two of the HIV proteins used at these acute (or very early) time points were detected (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 mediated predominantly by $CD8^+$ T cells.

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

Patient	Time point (DFOSx)	T-cell response[a] to:				
		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, MM19, MM22, MM23, and MM28) or very early (MM27) infection by 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 PBMC (±1 standard deviation).

Antibody-dependent complement-mediated inactivation of virus is also detectable at acute-phase time points. In contrast to the delayed development of NAbs, antienvelope antibodies were detectable in the first serum samples taken 6 to 28 days after onset of symptoms, as expected from our previous study (1). We tested whether the presence of complement could augment the antiviral activity of early sera and thus whether the early antienvelope 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 antiviral activity (Fig. 1a). All activity was eliminated if the source of complement was heat inactivated, with neutralization titers remaining the same as with patient serum alone.

The potency of the CMI response varied from patient to patient, and the profiles fell into three distinct patterns (Fig. 1a). Acute-phase sera from MM4, MM8, and MM19 had weak and varied neutralizing activity in the absence of complement but demonstrated potent viral inactivation (90 to 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 days 204 and >503, respectively), but again substantial CMI activity was evident from the earliest samples at days 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 CMI activity was observed with sera from MM22, but we could analyze sera only

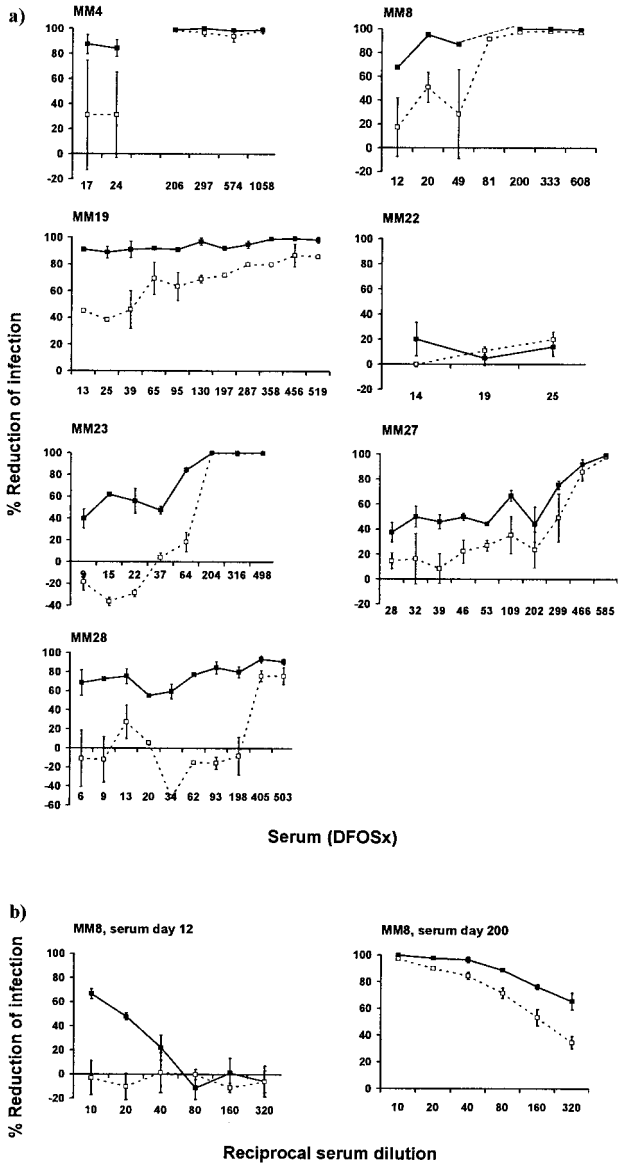

FIG. 1. Development of CMI and neutralization activity in HIV-1-infected patients. (a) Percent reduction in infection of 100 FFU of autologous virus in the presence of sequential patient sera (at a dilution of 1:10), assayed in the presence (closed symbols) or absence (i.e., neutralization) (open symbols) of complement. HIV-1-seronegative sera served as the complement source (added at a final concentration of 10% [vol/vol]), and heat-inactivated aliquots served as negative controls. The error bars show standard deviations between two or three independent experiments. (b) Typical CMI and neutralization with increasing dilution of patient sera from an early and a later infection time point. The error bars show standard deviations between two independent experiments.

between days 14 to 25 since this patient commenced antiretroviral therapy at day 26.

Typical titration curves of CMI activity at early and chronic infection time points are shown in Fig. 1b, illustrating that the CMI activity is detectable at serum dilutions higher (>1:10) than that used for Fig. 1a. At later time points when "conventional" neutralizing activity developed, the CMI effect augmented the neutralizing activity. The 90% inhibitory concentration end point titers were generally enhanced two- to fourfold, irrespective of the CMI potency at early time points (Fig. 1b).

Table 3 summarizes the first time points at which antienvelope antibodies, NAbs, CMI, and T-cell activity were detected for each patient. The values of the patients' virus loads at these time points are also indicated and show that the majority had detectable CMI in the acute viremic phase, characterized by high viral loads (>3,000,000 RNA copies/ml). Autologous CMI and T-cell activity developed at least 2 months earlier, and typically more than 6 months earlier (range, 69 to >503 days), than NAb activity. Apart from MM22, all patients developed antibodies capable of CMI 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 CMI of HIV-1, in contrast to NAbs, arises during acute infection when containment of viral replication is initiated. These results indicate a role for humoral immunity in control of viremia in primary HIV-1 infection.

CMI 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 after 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 CMI activity is by 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 CMI activity in the earliest sample tested between 9 and 28 days after onset of symptomatic PHI (Fig. 2a). In all cases the potency of CMI 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 antiviral activity maintained throughout the study period (Fig. 1a and 2a). The HIV-1_{YU2} virus was often more sensitive to CMI 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 CMI of heterologous viruses by serum from MM27 may be linked to the weak autologous CMI activity in this patient (Fig. 1a).

Previous reports have suggested that PBMC-cultured HIV-1 is relatively resistant to CMI (30, 32). In agreement with those studies, we observed that PBMC-derived HIV-1_{YU2} virus is less susceptible to CMI than is 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 verify that this antiviral response is indeed mediated by complement, rather than by some other, unknown heat-labile component, by using purified guinea pig complement. No neutralization of autologous virus with serum from MM8 (day 49) was observed

TABLE 3. Time points of first detection of T-cell and antibody-mediated antiviral activity following infection

Patient	Time point (DFOSx)	Viral load (RNA copies/ml)	Anti-HIV antibodies[a]	Anti-gp120 antibodies (reciprocal end point titer)[b]	T-cell activity[c,d]	CMI[d,e]	NAb[d,f]
MM4	17	160,000	+	100	ND[g]	+	−
	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 antibodies from the first study sample in diagnostic tests (Murex HIV-1.2.0; Abbott, Wiesbaden, Germany; Wellcozyme HIV Recombinant; Abbott; Serodia HIV-1/2; Fujirebio Inc., Tokyo, Japan; and VIDAS HIV Duo; bioMérieux, Marcy L'Etoile, France).

[b] The titer of anti-gp120 antibodies in patient sera was determined by an in-house enzyme-linked immunosorbent assay 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] +, detectable response; −, undetectable response.

[e] The antibody-dependent complement-mediated virus inactivation was intermediate (approximately 50% inactivation of input virus) in early sera from patients MM23, MM27, and MM28 and was strong (approximately 90% inactivation of input virus) in early sera from patients MM4, MM8, and MM19.

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

[g] ND, not determined, as no (suitable) samples were available for analysis.

in the absence of complement (Fig. 3a). Replacement of human serum as a source of complement with purified guinea pig complement resulted in inactivation profiles similar to those for CMI with human serum as the 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) pathway, an antibody-independent (alternative) pathway, or a lectin pathway (4). In order to determine the pathway involved in the observed antiviral effect, we replaced 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 samples from two patients, MM8 and MM28. In the presence of complement, we observed almost 70% inactivation of input virus, which was completely eliminated 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 elimination 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 whether the early CMI 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 CMI activity and resulted in a 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 removal of IgM during the purification process. As expected, since whole serum was nonneutralizing, purified IgG either alone (data not shown) or in the presence of heat-inactivated complement had no effect on viral infectivity (Fig. 3c). The CMI 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 those in virus supernatants in the presence of complement alone. Treatment of virus with both anti-HIV-1 serum and complement resulted in approximately 100% more RT release than treatment with nonimmune serum and complement (Fig. 3d). The level of viral lysis was similar in 293T- and PBMC-produced viruses.

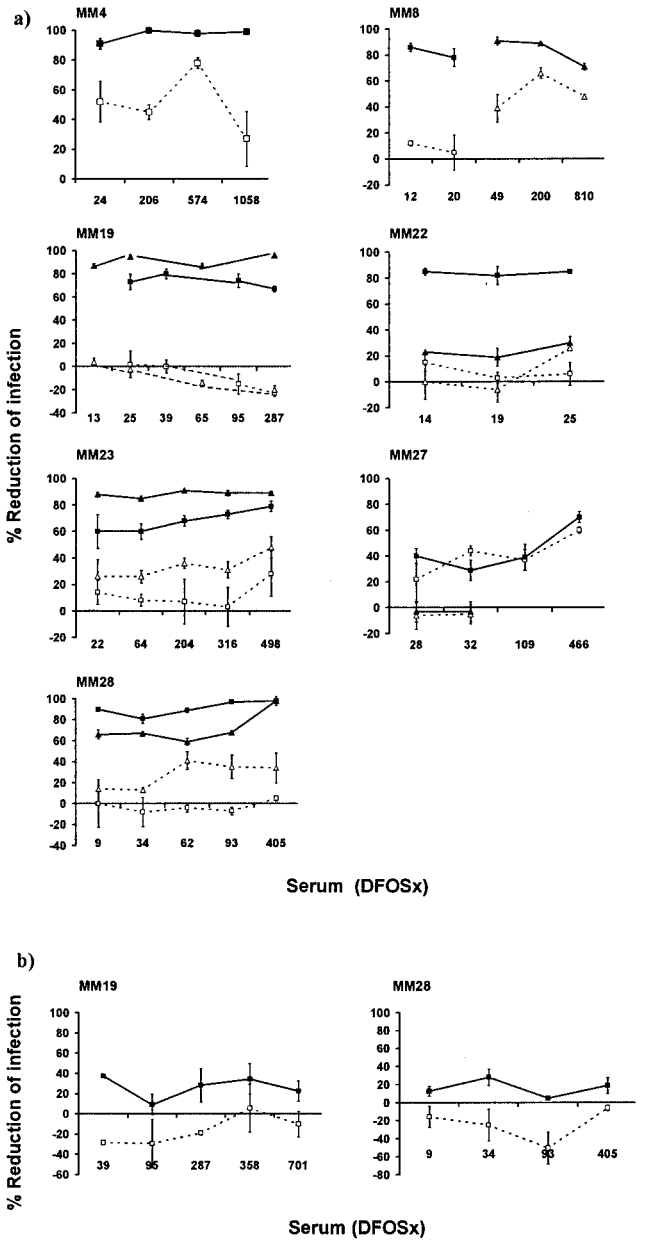

FIG. 2. CMI and neutralization of heterologous viruses. For experimental details, see the legend to Fig. 1. Closed symbols represent CMI, and open symbols represent neutralization. The error bars show standard deviations between two or three independent experiments. (a) CMI and neutralization of heterologous viruses produced in 293T cells. Data for HIV-1_{YU2} are indicated with squares, and those for HIV-$1_{4.1.33}$ are indicated with triangles. (b) Data for HIV-1_{YU2} passaged through PBMC.

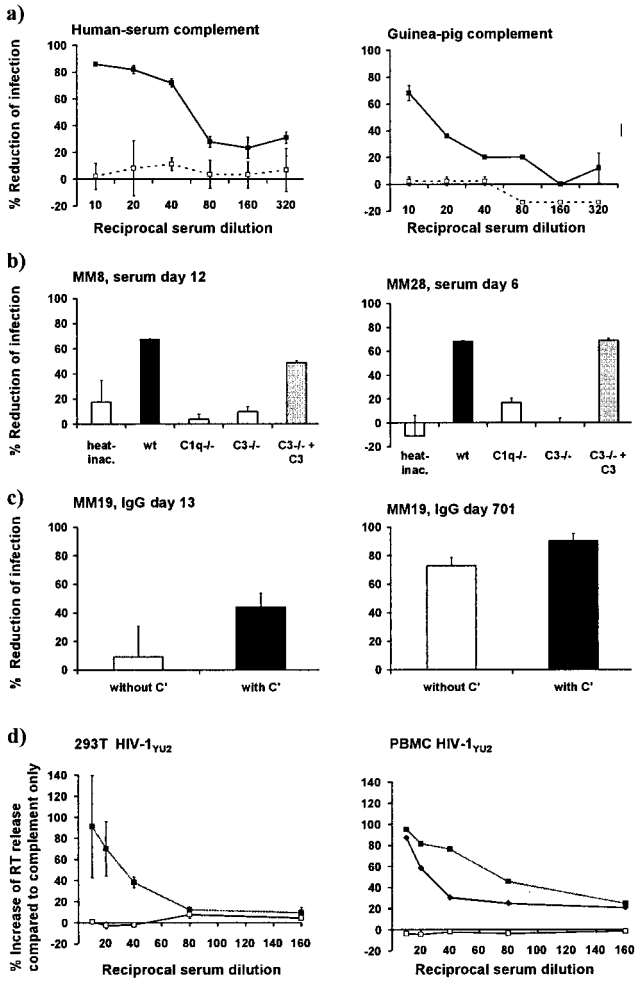

FIG. 3. Characterization of the anti-HIV activity in patient sera. (a) Guinea pig complement can substitute for human complement in CMI assays with day 49 serum from patient MM8 (closed squares). CMI activity is lost following heat inactivation (open squares). (b) Serum from C1q- or C3-deficient individuals cannot mediate the antiviral activity observed with normal human serum as a complement source. Reconstitution of C3 restores the antiviral activity. (c) Affinity-purified IgG antibodies mediate the complement-dependent virus inactivation. Black and white bars represent activity in the presence of complement and in the presence of heat-inactivated complement, respectively. (d) Release of RT from virions was measured by the Lenti-RT activity assay (Cavidi Tech AB) under nonlysing 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. Nonimmune serum had no effect on RT release compared to complement alone (open squares). Immune serum, however, resulted in a 100% increase in RT activity (closed symbols). The data presented show the 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}.

This indicates that viral lysis is at least partially responsible for CMI.

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 autologous and/or heterologous HIV-1 strains in all patients as early as 6 to 28 days after the 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 an

uninfected C1q- or C3-deficient individual was used as a complement source, no antiviral 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 antibody-dependent cellular cytotoxicity-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 CMI, we suggest that it should be possible to induce an effective CMI 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 CMI 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 CMI than is 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, Sullivan et al. have shown that uncultured HIV-1 purified directly from patient plasma is highly susceptible to CMI (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-peripheral blood lymphocyte-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 "CMI-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.

ACKNOWLEDGMENTS

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 for 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 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:**107–112.
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:**4775–4778.
4. **Blue, C. E., O. B. Spiller, and D. J. Blackbourn.** 2004. The relevance of complement to virus biology. Virology **319:**176–184.
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:**6103–6110.
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:**474–478.
8. **Cohen, A. D., J. D. Boyer, and D. B. Weiner.** 1998. Modulating the immune response to genetic immunization. FASEB J. **12:**1611–1626.
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:**1129–1140.
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:**6953–6961.
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:**205–211.
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:**167–173.
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:**4650–4655.
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:**767–777.
15. **Lindback, S., A. C. Karlsson, J. Mittler, A. Blaxhult, M. Carlsson, G. Briheim, A. Sonnerborg, H. Gaines, et al.** 2000. Viral dynamics in primary HIV-1 infection. AIDS **14:**2283–2291.
16. **Lindback, S., R. Thorstensson, A. C. Karlsson, M. von Sydow, L. Flamholc, A. Blaxhult, A. Sonnerborg, G. Biberfeld, H. Gaines, et al.** 2000. Diagnosis of primary HIV-1 infection and duration of follow-up after HIV exposure. AIDS **14:**2333–2339.
17. **Lyles, R. H., A. Munoz, T. E. Yamashita, H. Bazmi, R. Detels, C. R. Rinaldo, J. B. Margolick, J. P. Phair, J. W. Mellors, et al.** 2000. Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. J. Infect. Dis. **181:**872–880.
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. J. Virol. **78:**5812–5819.
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:**450–460.
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:**874–880.
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:**1167–1170.
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:**727–735.
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, et al.** 1994. Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. Nature **370:**463–467.
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:**438–447.
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:**924–932.
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:**583–594.
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. USA **100:**4144–4149.
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:**1907–1911.
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:**501–509.
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:**5869–5873.
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:**307–310.
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–S82.
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 clearance of virus in vivo. J. Immunol. **157:**1791–1798.
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:**173–181.
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:**307–312.
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:**225–233.