Clin Exp Immunol 1995; 99:189–195

Efficacy of HIV-specific and 'antibody-independent' mechanisms for complement activation by HIV-infected cells

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(Accepted for publication 26 October 1994)

SUMMARY

Previous studies in this laboratory have shown that efficient activation of complement (C) on HIV isolates and HIV-infected cells requires the binding of specific anti-HIV antibodies, while other investigators have observed 'antibody-independent' C activation. In an attempt to clarify these disparate findings, we investigated the effect of several variables on C activation by HIV-infected cells using flow cytometric analysis of C3 deposition. Antibody-mediated C activation using pooled sera from infected persons or human MoAbs directed against the V3 region of gp120 was always substantially higher than activation without antibody. Normal human serum (NHS) from a subset of HIV antibody-negative donors did, however, induce low levels of C3 deposition. Differences in C3 activation between the various NHS did not correlate with total haemolytic C levels or mannose-binding protein (MBP) levels. IgM isolated from NHS that induced high levels of C activation was at least partly responsible for the 'antibody-independent' C activation. Although there appeared to be a correlation between NHS that induced C activation and the presence of anti-blood type B IgM, absorption of anti-B did not abrogate the C3 deposition. Additionally, MoAb to the B antigen did not induce C3 deposition. These studies show that IgM in sera from HIV-uninfected donors can induce C3 deposition on HIV-infected cells, but that specific antibody-dependent C activation is substantially more efficient. Therefore, 'antibody-independent' C activation on HIV-infected cells may, in some cases, be more accurately described as HIV-cross-reactive antibody-dependent C activation.

Keywords complement HIV antibody independent activation

INTRODUCTION

The complement (C) system may play a number of important roles in the defence against HIV infection and/or its immunopathogenesis [1,2]. However, the mechanism(s) of C activation by HIV is controversial. Different studies have shown that HIV or HIV-infected cells can activate C in either an antibody-dependent or -independent manner. Studies in this laboratory have shown that activation of C by HIV isolates and HIV-infected cells requires the binding of anti-HIV-specific antibodies [3,4]. By employing a panel of human anti-HIV MoAbs, it was shown that the antibodies capable of inducing high levels of C3 activation recognized the V3 region of gp120 [4]. Antibody-dependent C activation by HIV or HIV-infected cells has been documented by others as well, and involves antibodies specific for gp120/160 and gp41 [5,6]. On the other hand, Ebenbichler *et al.* have reported that isolates of HIV can activate the classical C pathway in sera from uninfected persons independent of antibody [7,8]. The mechanism of activation was reported to be the direct binding of the globular region of C1q to gp41. Peptides representing amino acid region 590–613 of gp160 (an external immunodominant area of gp41) were shown to compete with C1q for binding to solid-phase gp41 [8]. The same group showed that 'antibody-independent' C activation by infected H9 cells was highly variable depending on the HIV isolate [9]. The reason for this variability was attributed to the possible masking of the gp41 molecule by gp120 or the shedding of gp120 after possible CD4 binding. Taken together, these investigations indicate that HIV can activate complement in the absence or presence of specific antibody. Interestingly, no studies have compared the relative efficiency of the two types of C activation by HIV-infected cells or by intact isolates of HIV. Thus, the goals of this investigation were to determine the efficacy of anti-HIV-specific antibody-dependent C activation *versus* 'antibody-independent' C activation on HIV-infected cells, and to identify possible factors in sera which could cause 'antibody-independent' C activation. We showed that HIV-specific antibody-dependent C activation is much more

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effective than 'antibody-independent' C activation, and that the low levels of C activation observed by sera alone were found to be due to non-HIV-specific IgM which showed some cross-reactivity to HIV-infected cells.

MATERIALS AND METHODS

Cell cultures

The H9 T cell line was infected with either the MN, IIIb or RF isolates of HIV-1 (AIDS Research and Reference Program, National Institute of Health, Rockville, MD) and maintained in RPMI 1640 medium containing 1% L-glutamine and 25 mM HEPES (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (FCS; Whittaker Bioproducts). Successful infection of H9 cells with HIV was routinely verified by detection of p24 antigen with an HIV-1 p24 antigen enzyme immunoassay (EIA) kit (Coulter Co., Hialeah, FL). Cells were also regularly tested and shown to be negative for mycoplasma contamination using a commercial detection kit (Gen-Probe, San Diego, CA). The viability of cells immediately before use was $\geqslant$ 95% as determined by trypan blue exclusion. H9 uninfected cells were used as controls.

Sera

Venous blood from healthy HIV$^-$ individuals with various blood types was collected in vacutainer tubes with no additives (Becton Dickinson, Bedford, MA) and allowed to clot for 30 min at room temperature. The clotted blood was centrifuged at 4°C for 15 min at 700 ***g***. The serum was then collected, aliquoted and frozen at −70 °C until needed. Complement CH_{50} levels were determined by standard techniques [10] with modifications in volume as described elsewhere [11]. Mannose binding protein (MBP) levels from eight serum samples were determined by R.A. Ezekowitz and associates (Harvard Medical School, Division of Haematology/Oncology, Boston, MA) as described previously [12].

Isolation and purification of IgM

IgM antibodies from the sera of two individuals, both sero-negative for HIV antibody and possessing blood types AB or O, were isolated by affinity chromatography as described elsewhere [13]. Briefly, serum samples were passed over a column of cyanogen bromide (CNBr)–Sepharose beads (Sigma Chemical Co., St Louis, MO) coupled to μ-specific anti-human antibodies (Organon-Teknika-Cappel Corp., WestChester, PA) and eluted into 0·5-ml fractions. The absorbance of the fractions was determined at 280 nm. Fractions with the largest optical density (OD) were dialysed in PBS and subsequently concentrated with Centricon-30 concentrators (Amicon Inc., Beverly, MA) to approximately 1 mg/ml. The antibodies were then filtered with 0·22-μm Spin-X centrifuge filter units (Costar, Cambridge, MA) and stored at 4°C.

Absorption of anti-B antibody from O donor IgM

Affinity-purified IgM from the type O donor was incubated at 100 μg/ml with an equal volume of packed type B erythrocytes for 1 h on ice. IgM was also incubated with autologous O erythrocytes as a control. The IgM and erythrocyte mixtures were spun down briefly in a microcentrifuge. The absorbed IgM supernatant was then collected.

Flow cytometric detection of cell surface C3 and bound immunoglobulins

Infected or uninfected H9 cells were washed twice with RPMI 1640 medium and re-suspended to 4×10^5 cells/tube. Cells were pelleted and incubated for 30 min on ice with 50 μl of one of the following: (i) 1:10 dilution of pooled heat-inactivated sera from HIV-infected individuals with known HIV protein reactivity [4]; (ii) 10 μg/ml 447–52-D human MoAb (IgG) specific for the V3 region of gp120 (obtained from Dr S. Zolla-Pazner, Laboratory Research Services, New York Veterans Administration Medical Centre, New York, NY); (iii) 25 μg/ml 246-D human MoAb (IgG) specific for gp41 amino acid region 374–604 of gp160 (obtained from Dr S. Zolla-Pazner); (iv) 1·0 μg/ml mouse anti-human B-antigen MoAb (IgM) (Dako Corp., Carpinteria, CA) or 1·0 μg/ml mouse anti-human CD57 (IgM isotype control) (Becton Dickinson). In this last set of experiments, erythrocytes from individuals with B and/or non-B blood types were employed, in addition to infected H-9 cells, to ensure the specificity of the anti-B antibody for the B antigen. Also, in several experiments, infected H9 cells were incubated with 50 μg/ml soluble CD4 (sCD4) at 37°C (obtained from Ray Sweet, Smith, Kline & French Labs, Philadelphia, PA) before incubation with pooled HIV anti-serum or anti-HIV-specific MoAbs.

Following incubation with the above agents, cells were washed with RPMI 1640 medium and resuspended with 100 μl of a 1:20 dilution of C in RPMI 1640 medium for 30 min at 37°C. The C source for these experiments was from an HIV$^-$ AB individual (donor 5) that had low spontaneous C activation on cells. Cells were washed once with PBS containing 0·1% azide (PBS–azide) and stained with 50 μl of FITC-labelled $F(ab')_2$ goat anti-human C3 (Organon-Teknika-Cappel) at a 1:200 dilution in PBS–azide for 30 min on ice. After staining, cells were washed once in PBS–azide and subsequently fixed with 1% paraformaldehyde.

To analyse the effect of affinity-purified IgM on cell surface C3 deposition, infected H9 cells and erythrocytes were incubated with a 1:20 final dilution of C as described earlier plus 30–100 μg/ml affinity-purified IgM isolated from non-HIV-infected healthy donors with blood types AB and O or anti-B absorbed and control absorbed IgM from a healthy O type donor (100 μg/ml before absorption with B or non-B erythrocytes). Cells were then washed and stained as described earlier.

The ability of sera from various other normal individuals to induce C3 deposition on H9-infected or uninfected cells was also examined. In this set of experiments, sera (1:20) were incubated with washed H9 cells and stained as described earlier, but without a preincubation step.

To determine the degree of binding of anti-gp41 and anti-V3 on the cell surface of infected H9 cells, cells were incubated with antibodies as previously described. Cells were then stained with a 1:60 dilution of FITC-conjugated goat anti-human immunoglobulin (Tago Immunochemicals Inc., Burlingame, CA) for 30 min on ice, and subsequently fixed with 1% paraformaldehyde.

The number of cells demonstrating fluorescent staining for C3 and immunoglobulin (anti-V3 and anti-gp41) was determined with an Ortho Cytoron (Ortho Diagnostic Systems, Raritan, NJ) (Figs 2–4, Tables 1 and 2) or EPICS-C flow cytometer (Coulter) (Fig. 1). The fluorescence was presented as the mean channel intensity of fluorescence of 5×10^3 cells,

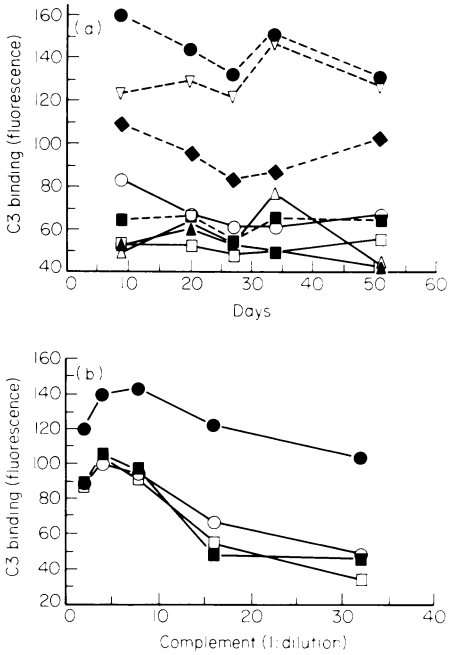

Fig. 1. Effect of cell culture age (a) and dilution of normal human serum (NHS) (b) on anti-HIV antibody-dependent and -independent C activation on H9 cells. Uninfected or HIV-infected cells were incubated with RPMI or a 1:10 dilution of heat-inactivated pooled sera from HIV individuals, before addition of 1:20 dilution of NHS as a C source in a, or 1:2–1:32 dilution of NHS in b. Levels of cell surface C3 were then determined by flow cytometry after staining with FITC-labelled $F(ab')_2$ goat anti-human C3. Data represent the mean logarithmic fluorescence of duplicate values. □, H9; ■, H9 + antibody; ○, H9/MN; ●, H9/MN + antibody; ▲, H9/3B; ◆, H9/3B + antibody; △, H9/RF; ▽, H9/RF + antibody.

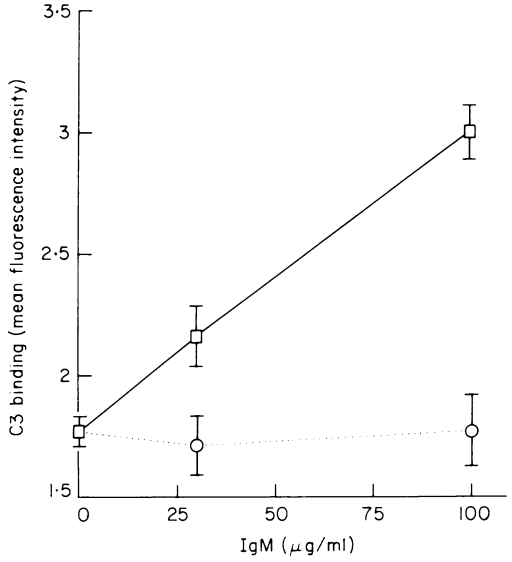

Fig. 2. Differential effects of affinity-purified IgM from normal human serum (NHS) on C activation by HIV_{MN}-infected H9 cells. Cells were co-incubated with 30 or 100 μg/ml IgM from donor 1a (□) or 5a (○) plus a 1:20 dilution of NHS as a C source. Cells were stained with FITC-labelled $F(ab')_2$ goat anti-human C3 and analysed by flow cytometry. Data represent the mean linear fluorescence (± s.d.) of triplicate samples and are representative of two experiments. Anti-V3 antibody-dependent C activation on infected cells yielded a mean fluorescence intensity of 68·03 ± 4·46 (not shown).

measured logarithmically, where a doubling of fluorescence was observed after a 25 channel increase with the EPICS-C flow cytometer and a 22·5 channel increase with the Ortho Cytoron. The fluorescence of cells stained with C3 was sometimes presented in graphic form as the linear fluorescence.

RESULTS

Effect of culture age, HIV strain, and complement dilution on deposition of C3 on HIV-infected cells

Since several studies have reported apparently differing levels of 'antibody-independent' C activation by HIV-infected cells, we wished to evaluate the effect of several variables, including HIV strain, cell culture age and C dilution using a C3 deposition assay. Figure 1a shows the time course of C activation by H9 cells cultured for 10–51 days after infection with viral isolates HIV_{MN}, HIV_{IIIB}, or HIV_{RF}. At all times, and with each of the three HIV strains, substantial antibody-dependent C activation was induced by pooled sera obtained from HIV-infected individuals. On average, C3 deposition on HIV_{MN}, HIV_{IIIB}, and HIV_{RF}-infected cells was 9·2, 2·5 and 6·1 times greater, respectively, than on uninfected cells in the presence of pooled sera. In contrast, 'antibody-independent' C activation by HIV_{MN}, HIV_{IIIB}, and HIV_{RF}-infected cells was 1·6, 1·0 and 1·2 times higher, respectively, than by uninfected cells. The age of the culture did not substantially affect the relationship

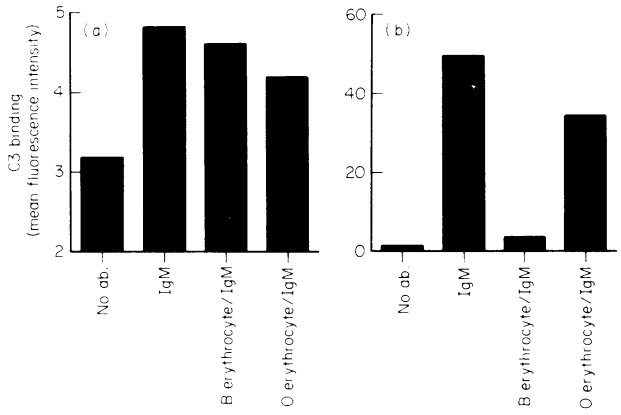

Fig. 3. Effect of anti-B-absorbed IgM on C activation by HIV_{MN}-infected H9 cells (a) or B type erythrocytes (b). Cells were co-incubated with 100 μl non-absorbed or B erythrocyte-absorbed IgM (100 μg/ml before absorption with B erythrocytes) from donor 1a plus C. IgM incubated with O erythrocytes was included as an absorption control. The various IgM groups were indicated as follows: non-absorbed, IgM; anti-B absorbed, B erythrocyte/IgM; or control absorbed, O erythrocyte/IgM. Cells were then analysed by flow cytometry as determined for H9 cells in Fig. 2. Values show the mean linear fluorescence of one sample, which is representative of two experiments. Anti-V3 antibody-dependent C activation on infected H9 cells yielded a mean fluorescence intensity of 91·00 (not shown).

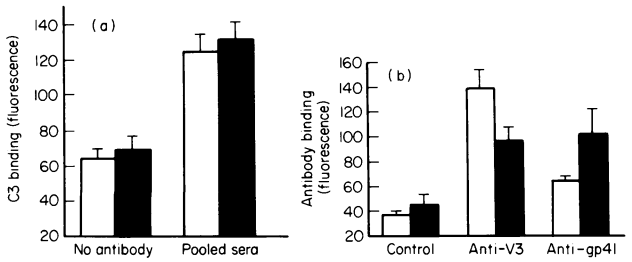

Fig. 4. Amount of C3 (a) or IgG (b) bound to the cell surface of HIV_{IIIB}-infected H9 cells after treatment with sCD4 and incubation with RPMI or HIV-specific antibodies. Soluble CD4-treated (■) and -untreated (□) cells were incubated with RPMI or pooled anti-HIV serum (a), or RPMI, anti-V3 or anti-gp41 (b). Cells were then incubated with C and FITC-labelled anti-C3 or with FITC-labelled goat anti-human immunoglobulin. Levels of cell surface C3 or IgG were then determined by flow cytometry. Values represent the mean logarithmic fluorescence of triplicate (± s.d.) samples.

between antibody-dependent and 'antibody-independent' C activation.

Figure 1b shows the degree of surface C3 deposition on uninfected and HIV_{MN}-infected cells incubated with or without anti-HIV antibody before incubation with normal human serum (NHS) as a C source at dilutions ranging from 1:2 to 1:32. NHS induced similar low levels of C3 activation on both uninfected and infected cells which increased as the concentration of NHS increased, while the addition of anti-HIV antibody resulted in a further increase of C3 activation only on infected cells, which was also dose-dependent.

C3 deposition on HIV-infected cells incubated with NHS from different donors

Since, in a number of experiments, low levels of 'antibody-independent' C activation by HIV-infected cells were observed, the ability of NHS from five different donors (1–5) to induce C3 deposition on the surface of uninfected and HIV_{MN}-infected cells was initially assessed. All NHS induced significant ($P < 0.05$ ANOVA Scheffe F-test) C3 deposition on both uninfected and infected cells compared with no serum (Table 1). However, C3 deposition on infected cells was significantly higher than on uninfected cells (paired t-test, $P < 0.006$). NHS from donors 1–3 induced the greatest degree of C3 deposition on infected cells, while NHS from donors 4 and 5 induced significantly less ($P < 0.05$, ANOVA Scheffe F-test). The variable effects of NHS on C3 activation were not due to differences in total haemolytic C levels or serum MBP levels. The CH_{50} values were similar between all donors (Table 1), and the slight differences in values that were observed did not correspond with C3 deposition. Similarly, the differences in sera MBP levels did not correspond with C3 deposition (Table 1). For example, sera that induced high (donor 2) and low (donor 5a) levels of C3 deposition had MBP levels of 1363 ng/ml and 1704 ng/ml, respectively.

To determine the frequency of NHS samples inducing relatively high or low levels of 'antibody-independent' C activation on infected cells, 15 different NHS samples in addition to several of the samples already tested were compared for their effect on C3 deposition on uninfected and infected H9 cells. All sera induced some degree of C3 deposition on the surface of uninfected and infected cells compared with no serum, with the C3 deposition being higher on infected cells (Table 2). The frequency of NHS samples inducing low values of C3 deposition, ⩽ to and including donors 4 and 5, was 4/20 or 20% (Table 2, donors 4, 5, 18 and 20), while 15/20 or 75% of NHS samples induced notably higher levels of C3 deposition.

The effect of affinity-purified IgM from HIV-uninfected donors and the possible role for anti-B antibodies on the induction of cell surface C3

Analysis of data in Table 1 suggested the possible involvement of antibodies specific for blood group B antigen in inducing C3 deposition on HIV-infected cells. For example, the serum inducing the highest degree of 'antibody-independent' C3 deposition was from donor 1 who had O type blood, and consequently antibodies to A and B blood antigens, whereas the sera inducing the lowest levels of C3 deposition were from donors 4 and 5, who had B and AB type blood and therefore no anti-B antibodies (Table 1). By increasing the number of NHS samples tested for C3 deposition on infected cells, it was

Table 1. 'Antibody-independent' C activation, CH_{50} levels, mannose binding protein (MBP) levels and blood group antigens of sera from several HIV-antibody-negative donors (NHS)

NHS donor	C3 deposition H9 cells‡§	C3 deposition H9/MN cells‡§	$CH_{50}^{\S}$ assay 1/final dilution	Serum MBP levels (ng/ml)¶	Blood group antigen/antibody
1a*	4·07 ± 0·54	8·32 ± 0·4	212 ± 7·21	1827 ± 2·2	O/anti-A, anti-B
1b*	2·97 ± 0·32	6·39 ± 0·26	258 ± 12·1	1917 ± 2·2	O/anti-A, anti-B
2	3·57 ± 0·51	6·87 ± 0·2	301·3 ± 6·8	1363 ± 2·8	A/anti-B
3	2·53 ± 0·35	4·04 ± 0·46	243 ± 7·2	1465 ± 0·68	O/anti-A, anti-B
4	1·24 ± 0·1	2·64 ± 0·16	340·3 ± 11·9	815 ± 1·6	B/anti-A
5a†	1·86 ± 0·46	2·5 ± 0·49	229 ± 7·8	1704 ± 3·2	AB/none

*Donor 1 drawn at two different times within a period of 3 months.
†Donor 5 drawn for serum at a different time interval than indicated in Table 2.
‡As determined by flow cytometry; values represent linear mean channel intensity with background fluorescence subtracted.
§Mean of triplicate values ± s.d.
¶Average of duplicate values ± percentage of value differences.

Table 2. 'Antibody-independent' C activation and blood group antigens of sera from 16 additional HIV⁻ donors in addition to the previously tested donors in Table 1

Serum donor	C3 deposition uninfected cells‡§	C3 deposition H9/MN cells‡§	Blood group antigen/antibody
1c*	2·07	5·50	O
2	3·72	6·51	A
3	2·78	3·61	O
4	1·38	2·58	B
5b*	1·83	2·66	AB
6	6·34	11·86	O
7	3·76	7·64	O
8	2·73	7·15	O
9	2·73	5·47	O
10	3·33	7·03	O
11	3·76	6·60	O
12	2·95	4·41	O
13	5·01	9·36	A
14	6·21	15·56	A
15	3·03	5·40	A
16	3·62	5·63	A
17	1·73	4·72	A
18	3·01	2·56	B
19	6·93	7·74	B
20	1·67	2·49	B
CVID†	1·98	3·43	A

*Donors 1 and 5 drawn for serum at different time intervals than indicated in Table 1.

†Serum from donor with common variable immunodefeciency (CVID), and possessing normal CH_{50} levels (data not shown).

‡As determined by flow cytometry; values represent linear mean channel intensity with background fluorescence subtracted.

§Single values, but representative of two experiments.

confirmed that samples from A and O blood type donors induced significantly higher levels of C3 deposition than AB and B donors ($P = 0{\cdot}026$, Mann–Whitney test) (Table 2). To test the possibility that anti-B IgM caused C3 deposition, 30–100 μg/ml of IgM affinity-purified from the sera of donors 1a and 5a were incubated with infected cells. The amount of IgM used was based on the amount of serum IgM found in normal adults (1250–1600 μg/ml) and the dilution of NHS used in Tables 1 and 2 (1:20). IgM from donor 1 caused a dose-dependent increase of C3 deposition on infected cells, whereas IgM from donor 5 had no effect on C3 deposition (Fig. 2). (IgM from neither donor 1 nor 5 could consistently increase C3 deposition on uninfected cells (data not shown).) Serum from an individual with common variable immunodeficiency (CVID) or acquired hypogammaglobulinaemia and blood type A could induce only low levels of C3 deposition, possibly because of insufficient numbers of anti-B antibody (Table 2), thereby supporting the previous observations of anti-B IgM antibody inducing C3 deposition.

Incubation of IgM from donors 1 and 5 with B erythrocytes (as described in Materials and Methods) confirmed the presence of anti-B antibody in donor 1 IgM and the absence of anti-B in donor 5 IgM (data not shown). However, absorption of anti-B antibody from donor 1 IgM with allogeneic type B erythrocytes did not abrogate C3 deposition on infected H9 cells (Fig. 3a), but did prevent C3 deposition on B erythrocytes (Fig. 3b), indicating that the specificity of the IgM that induced C3 deposition on infected H9 cells was not anti-B. As an additional means for determining whether anti-B antibody was responsible for the C3-inducing effects, an anti-B MoAb was incubated with infected cells. No induction of C3 deposition was induced by the MoAb on HIV-infected cells, while C3 deposition was induced on B erythrocytes (data not shown).

Surface C3 activation by sCD4 or pooled anti-HIV serum

Since previous studies by Ebenbichler et al. have reported that gp41 can bind C1q and thus activate C [7,8], experiments were also performed to determine whether treatment of HIV-infected cells with sCD4 activates C on the cell surface. Cells were treated with sCD4 to permit the binding of CD4 to surface gp120, which in turn results in the dissociation of gp120 from gp41 and the subsequent exposure of the gp41 molecule. The HIV_{IIIB} strain was used in this set of experiments because the dissociation of gp120 from gp41 caused by sCD4 occurs quite readily [14].

Treatment with sCD4 decreased cell surface levels of gp120, as measured by binding of a V3-specific MoAb, and increased exposure of gp41 as measured by binding of a gp41 MoAb (Fig. 4b). However, increased exposure of gp41 sites on infected cells had no effect on C activation (Fig. 4a). High levels of C3 activation were observed when both untreated and sCD4-treated cells were incubated with pooled anti-serum, indicating that both anti-gp120 and anti-gp41 antibodies were present in the pooled serum which were capable of inducing high levels of antibody-dependent C3 activation.

DISCUSSION

This study demonstrated two main features of C activation by HIV-infected cells. First, while HIV-infected cells activated C in the absence of specific antibody to some degree, C deposition on cells in the presence of anti-HIV antibody was significantly more efficacious (10–20-fold). The major specificity of anti-HIV antibodies in the antibody pool which activated C in these assays was for the V3 region of gp120 as determined by peptide blocking studies [15]. Although numerous reports have demonstrated specific anti-HIV antibody-dependent [3–6] or 'antibody-independent' [7–9] C activation on HIV or HIV-infected cells, this is the first report which directly compared the relative efficacy of the two modes of C activation on intact virus-infected cells. The efficacy of antibody-dependent *versus* 'antibody-independent' C activation by HIV proteins, but not by intact isolates of HIV or HIV-infected cells, has been briefly addressed in two other studies. Solid-phase recombinant gp160 and gp120 [16] or recombinant gp41 [17] alone could activate C in whole serum, but when anti-HIV IgG from heat-inactivated seropositive sera or anti-gp41 antibodies were added, C activation was enhanced. However, those studies did not compare the two modes of C activation with intact isolates of HIV or HIV-infected cells.

The second main feature of C activation by HIV-infected cells observed in this study was that the low levels of C activation due to 'antibody-independent' mechanisms were mediated, at least in part, by non-HIV-specific IgM from NHS which apparently showed some cross-reactivity to HIV-infected cells. The ability of NHS (1:20) to induce low levels of C activation in an 'antibody-independent' manner, while attributed in part to IgM, was higher in some donors than in

others. Because sera from O and A *versus* B and AB type HIV$^-$ donors resulted in high and low levels of C3 deposition, respectively, the specificity of the IgM was initially thought to be directed against the blood type B antigen. However, this was ruled out when we observed that IgM absorbed with B erythrocytes could still induce C3 deposition on HIV-infected cells. In addition, it has been shown that H9 cells possess an AO and not a BB/BO/OO genotype [18] which would implicate anti-A antibodies in the induction of C3 on infected cells. Contrary to our results, Arendrup *et al.* have shown that incubation of HTLV-III$_B$ (propagated in a culture with H9 cells) with a purified anti-A antibody resulted in neutralization [18].

It is not known why most serum samples from B and AB individuals induced significantly less C3 deposition on infected cells than A and O individuals. One possibility is that AB and B type donors possess lower levels of IgM against some antigens (i.e. less cross-reactive IgM to HIV antigens) than O and A donors. Higher anti-A serum IgM levels in O, relative to B type donors, have been reported [19].

The current investigation demonstrated that IgM from NHS showed cross-reactivity to HIV-infected cells. The phenomenon of NHS cross-reactivity to HIV antigens, as tested in Western blot assays, has been reported [20,21]. Other studies, as well, have demonstrated that IgG and IgM from NHS were cross-reactive with *Mycobacterium leprae* [22], other bacteria [23,24] and the keyhole limpet haemocyanin antigen [25]. Activation of C3 on *Myco. leprae* was completely antibody-dependent and occurred through the classical pathway with low serum concentrations, and through the alternative pathway with high serum concentrations [22]. Thus, cross-reactivity of NHS to various antigens is commonly observed. In fact, Thorton *et al.* hypothesize that natural antibodies from NHS are present in low concentrations that are reactive to any possible antigen [25].

Previous reports of 'antibody-independent' C activation by HIV and HIV-infected cells were made by Solder *et al.* [26] and Marschang *et al.* [9]. These investigators observed that various laboratory isolates of HIV and, to a varying degree, HIV-infected H9 or Jurkat cells, could activate C as found in NHS. Both groups further showed that C activation by HIV isolates occurred via the classical pathway, whereas C activation by HIV-infected cells occurred mainly through the alternative pathway. Initially, the proposed mechanism for C activation was the direct binding of gp160 to C [26]. Subsequent studies, using a solid-phase binding assay, revealed that an external immunodominant region of gp41 was binding directly to the globular region of C1q, thereby activating the classical pathway [8]. Peptides corresponding to amino acid position 590–613 of gp160 were shown to inhibit the binding of radiolabelled C1q to solid-phase gp41. This mechanism explained C activation by various isolates of HIV, but not by HIV-infected cells, since they activated the alternative pathway. Despite this discrepancy, the investigators attributed the mechanism of C activation by infected cells to sites on gp41 which become exposed, probably during binding of gp120 to CD4 [9].

'Antibody-independent' modes of C activation, even though substantially lower than antibody-mediated C activation by HIV, could have significant biologic effects. For example, 'antibody-independent' C activation could be important at the onset of infection before an immune response has been mounted, or in chronically infected individuals where an immune response is constantly being mounted, but where the anti-HIV antibodies are incapable of neutralizing or binding the virus or virally infected cells due to the emergence of antibody escape mutants [27]. In both cases, cells expressing complement receptors (CR) such as phagocytic cells, natural killer cells or neutrophils could conceivably kill virally infected cells more efficiently by binding to C on the infected cells' surface. Additionally, if cross-reactive IgM binds to the free virus, leading to low levels of C3 deposition, then enhancement of viral infection could result [28].

Although 'antibody-independent' C activation has been described *in vitro* in these studies, an *in vivo* observation suggests that gp41 epitopes may not be exposed for interaction with C1q. Thus, the binding of gp120 from primary isolates or isolates from HIV-infected individuals to CD4 does not appear to result in the shedding of gp120 and the subsequent exposure of gp41 [29]. Additionally, membrane-bound gp41, when it is exposed in its native conformation, may not necessarily activate C in the manner observed with recombinant soluble gp41 or synthetically derived gp41 peptides. Lack of C activation by gp41 in its native state was suggested in the current study, since we could not demonstrate C activation by HIV-1_{IIIB}-infected H9 cells with exposed gp41 (as shown by flow cytometry) after treatment with soluble CD4.

Several additional variables which may affect activation were also studied. Since the acute-phase protein MBP has been implicated both in the binding to HIV gp120 and also in the activation of C [12], we examined whether the low levels of C activation caused by NHS could also be partly due to MBP. Although the sera MBP levels varied between donors, the differences observed did not correspond to C activation by the various sera. The effect of HIV strain, cell culture age, and C dilution on infected H9 cells also had little, if any, effect on the overall efficacy of antibody-dependent *versus* -independent C3 deposition. Although some differences existed in the role of HIV strains on C activation, overall the degree of antibody-dependent C activation was always substantially greater than with NHS alone. Thus observations of 'antibody-independent' as opposed to antibody-dependent C activation by HIV as a result of methodological differences can be ruled out with regard to these variables.

In conclusion, antibody-dependent C activation is substantially more effective than 'antibody-independent' C activation by HIV-infected cells. The low levels of 'antibody-independent' C activation that were observed were due, at least in part, to IgM isolated from NHS. Thus 'antibody-independent' C activation on HIV-infected cells may, in some cases, be more accurately described as HIV cross-reactive antibody-dependent C activation.

ACKNOWLEDGMENTS

The authors wish to thank Brenda L. Sullivan and Daniel M. Takefman for their technical assistance, and R. Alan Ezekowitz and associates for measuring sera MBP levels. This work was supported by the National Institute of Health, grant AI29661.

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