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Failure of ADCC to predict HIV-associated disease progression or outcome in a haemophiliac cohort

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SUMMARY

Antibody-dependent cell-mediated cytotoxicity (ADCC) has been described in a number of virus infections and occurs in HIV infection. In spite of numerous studies on the ability of HIV-positive sera to mediate ADCC, it remains unclear whether ADCC has any functional significance. Here we examine serial serum samples from a cohort of haemophilia patients who became infected from a common source in 1984, and show that there is no significant difference in ADCC values between those who remained asymptomatic and those who progressed to disease. This study does not compare effector cell function and clinical status and does not exclude activity against different target cell lineages *in vivo*.

Keywords ADCC HIV-1 clinical relevance haemophilia prognosis

INTRODUCTION

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a sensitive measure of circulating antibodies directed against target cell-expressed antigens such as occurs in virus infections and neoplasia (Fuson *et al.*, 1983; Fanger *et al.*, 1989). The humoral response against HIV consists of high-titre antibodies against the envelope of HIV, which are readily detectable in the majority of infected patients. Neutralizing antibodies are very variable with dominant strain-specific responses, although group-specific responses increase with time (Weiss *et al.*, 1985, 1986; Robert-Guroff, Brown & Gallo, 1985). Despite many reported studies of ADCC in HIV-associated disease, there is no clear consensus whether ADCC plays a functional role in either protective or pathogenic capacity (Rook *et al.*, 1987; Ljunggren *et al.*, 1987a, 1988; Blumberg *et al.*, 1987; Lyerly *et al.*, 1987; Ojo-Amaize *et al.*, 1987; Sinclair *et al.*, 1988; Goudsmit *et al.*, 1988; Koup *et al.*, 1989).

HIV-1-specific ADCC can be mediated by all sera containing antibodies to gp41 and gp120. HIV-1-specific sera are markedly less active in ADCC than are HTLV-1 sera under identical assay conditions (Sinclair *et al.*, 1988).

In a previous study the CEM cell line was chosen as the target cell line as it is devoid of MHC class II expression and therefore ADCC measurement is not affected by circulating anti-MHC class II antibodies which have been described in HIV infection (de la Barrera *et al.*, 1987), and which have been shown to interfere with HIV-specific ADCC assays (Howell *et al.*, 1985). In addition, CEM gives the lowest spontaneous (background) killing of a range of T cell lines which are infectable with HIV.

Spontaneous killing (natural killer-like activity) is frequently as high as the antibody-mediated cytotoxicity specific for HIV unless careful selection of donor effector cells giving low background killing is employed. Furthermore, HIV antigen expression tends to vary with the strain of virus used (Howell *et al.*, 1985). The prototype isolate HTLV-IIIB is most commonly used in ADCC (Blumberg *et al.*, 1987; *Lyerly et al.*, 1987; Ojo-Amaize *et al.*, 1987; Ljunggren *et al.*, 1987a, 1987b; Goudsmit *et al.*, 1988; Sinclair *et al.*, 1988; Koup *et al.*, 1989) and expression of HTLV-IIIB antigens in CEM target cells is more variable than expression in a cloned isolate. Therefore, both HTLV-IIIB and a clonal isolate Ma12 were used in this study.

Having described an ADCC assay designed to minimize the many inherent variables, the relations between HIV-associated disease and ADCC titres were examined in a group of patients drawn from the well-defined cohort of Edinburgh haemophilia A patients who were HIV negative until Spring 1984 when they were exposed to a locally produced batch of factor VIII which turned out to be contaminated with infectious HIV (Ludlam *et al.*, 1985). Some of these patients have remained asymptomatic and others have progressed to disease, thus giving an ideal opportunity to compare ADCC activity with disease progression.

PATIENTS AND METHODS

Patients

Eighteen of 32 HIV-exposed haemophiliac patients subsequently seroconverted (Simmonds *et al.*, 1988) and their clinical

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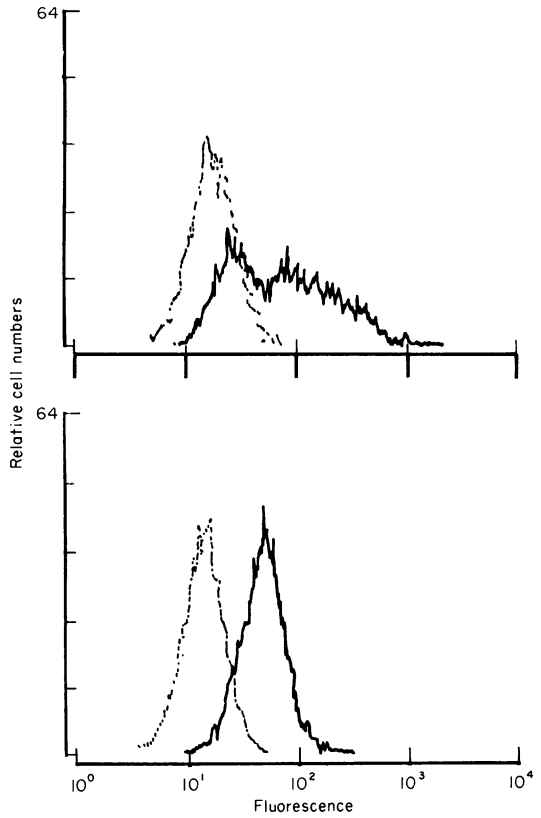

Fig. 1. Cytofluorimetric analysis (FACSTAR Becton Dickinson) of the target cell lines CEM-HTLV/IIIB (top) and CEM Ma12 (bottom). Viral expression was assessed using HIV-IgG prepared from 10 HIV-positive serum donors and 13 labelled with FITC anti-human IgG. Virus expression was also assayed in a 4-h syncytial formation assay using C8166 cells as target cells on the day of each experiment, and by Western blotting as previously described (Spickett *et al.*, 1989). The bold lines represent the virus-infected cells and the faint lines the uninfected cells.

and immunological status has since been documented at intervals (Steel *et al.*, 1988; Cuthbert *et al.*, 1989). In addition to these patients (referred to as group 1), eight haemophiliacs who had become HIV seropositive through the use of commercial factor VIII (referred to as Group 2), one HIV-seronegative haemophiliac and six healthy seronegative laboratory personnel were also monitored for ADCC activity.

Three serum samples per patient (where available) spanning the period 1986–1988 were assayed. Over this time-span, 12 of the seropositive patients remained in Centre for Disease Control (CDC) category II; three had persistent generalized lymphadenopathy (CDC category III) without other clinical progression; and 10 advanced to CDC category IV; of these, six were CDC IVa, two were CDC IVc and two developed lymphomas (CDC IVd), both having had opportunistic infections (IVc) before this stage. (CDC I, acute infection; CDC II, asymptomatic infection; CDC III, persistent generalized lymphadenopathy; CDC IV, other disease). A decline in absolute number of circulating $CD4^+$ T cells was noted for all the seropositive groups but was most marked in those who developed HIV-related symptoms.

Methods of ADCC assay

Details of this assay have been described previously (Sinclair *et al.*, 1988). Briefly, target cells were labelled for 90 min at 37°C with 100 μl of 37Bq/ml ^{51}Cr (New England Nuclear). After washing, 50 μl of suspension containing 1×10^4 cells were incubated with 50 μl of the appropriate serum dilution for 30 min at room temperature. Effector cells at effector-to-target (E/T) ratio of 20/1 in a final volume of 100 μl were added to round-bottomed 96-well plates (Nunc) which were then spun at 200 ***g*** for 5 min prior to incubation for 4 h at 37°C. After spinning, 100 μl of each supernatant were harvested and counted for gamma emission. Maximum release was estimated by addition of 150 μl 1¶ Triton x-100 to 50 μl of target cell suspension. Spontaneous release was calculated from the harvested supernatant of labelled target cells in the absence of sera and effector cells. Control E/T ratios of 10/1 and 5/1 were used to detect natural killer (NK) activity. All determinations were in triplicate.

Specific lysis (%) was calculated according to the formula:

$$\frac{\text{Test ct/min} - \text{Spontaneous ct/min}}{\text{Maximum ct/min} - \text{Spontaneous ct/min}} \times 100$$

The spontaneous ^{51}Cr release was less than 4·5% for these assays.

HIV-I antigen expression on target cell line

Expression of HIV-1 antigens on target cell lines was repeatedly checked over the experimental period by cytofluorimetry and Western blotting. Target cells were also frequently tested throughout the study and shown to be free of mycoplasma infection by the gene probe Mycoplasma rapid detection system (Brook, Rees & Leach, 1979; Hommel-Berrey & Brahmi, 1987). They were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine. Control IgG preparations from HIV-positive and HIV-negative sera from pools of at least 10 donors were employed as standards in preference to xenogeneic HIV antisera. Titration of the ADCC response in each serum sample in comparison with these standard IgG preparations allowed internal comparison and standardization of ADCC titre. Serum samples were coded and analysed without prior knowledge of the clinical status of the donor, and assays were conducted using two different effector cell donors and two different target cell lines for each determination.

Mean specific lysis and lytic titre of antibody mediating specific lysis

Calculation of the mean percentage specific lysis and the mean titre of ADCC mediating antibody obtained using dilutions of 1/100, 1/300 and 1/900 served to indicate any correlation between clinical status and these two ADCC parameters. For this assay, ADCC-specific lysis was defined as that lysis mediated by HIV-positive serum which exceeded the mean lysis mediated by normal pooled IgG (HIV-negative IgG preparation) by $>2{\cdot}4$ s.d.

RESULTS

The persistent expression of HIV-1 antigens on the target cell lines over the experimental period as detected by cytofluorimetry is shown in Fig. 1. The polyclonal nature of HTLV-IIIB isolate and the clonal characteristics of the MA12 isolate were maintained over this period. From the initial grouping of 53 randomly tested samples from the coded pool, ADCC-negative

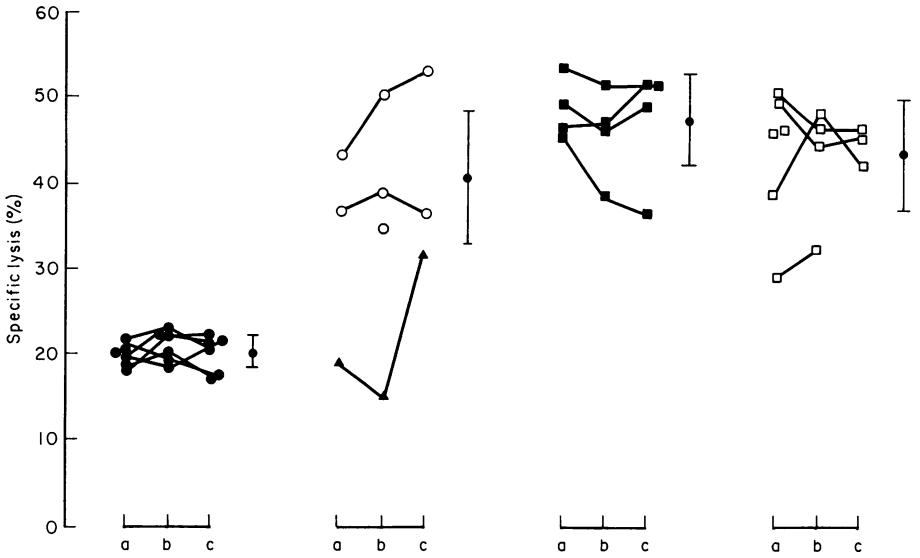

Fig. 2. Mean percentage of HIV-1-specific lysis of CEM, Ma12 target cells with a single effector cell donor, mediated by 20 haemophiliac donor sera. ●, six seronegative donors; ○, three seropositive donors (group I, asymptomatic); ▲, single asymptomatic haemophiliac seronegative by Western blot whose third sample became positive for ADCC and anti-Gp41 antibodies; ■, four seropositive donors (group 1, symptomatic); □, six seropositive donors (group 2). Bars are s.d. Fifty-three samples were tested in a single assay. In 17 patients three serial samples corresponded to (a) early; (b) middle; and (c) late samples. In three patients only one sample was available (spontaneous ^{51}Cr release 4·5% for this assay).

Table 1. Mean HIV-specific lysis comparison of control, asymptomatic and symptomatic HIV serospositive haemophiliacs

Group	*n*	Mean specific lysis(%)+s.d.	samples (*n*)	*P**
Seronegative	6	20·30±1·84	18	
Asymptomatic group 1	3	41·95±7·36	7	
Symptomatic	4	47·44±5·41	12	0·08
Group 2	6	43·58±6·57	13	0·62

* Significance was calculated from mean ct/min by Student's *t*-test. Symptomatic group 1 and group 2 patients were compared with the asymptomatic group of group 1. No significant differences in mean percent specific lysis were detected.

and ADCC-positive sera were separated and found to concur with previous HIV ELISA or Western blot results (Simmonds *et al.*, 1988). There was no significant difference in ADCC titres between the symptomatic and asymptomatic patients of either the original cohort group 1 or of group 2, as shown in Fig. 2.

All HIV-seropositives gave significantly greater mean specific lysis than the seronegative controls. Comparison of the data is shown in Table 1. Assays conducted using two different effector cell donors and two different target cells gave concordant results, with three HIV seropositive donor sera (B, C, F) producing significant ADCC in comparison with three seronegative control groups shown in Table 2. In the absence of a difference in mean percentage specific lysis between groups, differences in the titre of ADCC mediating antibodies were also sought on six serial samples from patient B, four samples from patient C and five samples from patient F. As shown in Table 2, titres of antibody mediating ADCC varied with time in both asymptomatic (B) and symptomatic (C and F) donors.

Sera tested prior to the deaths of patients C and F (both of whom had HIV-related lymphoma) showed a lower geometric mean titre ($P < 0·0075$, $r = 0·027$) when compared with patient B who was asymptomatic. Changes in percentage specific lysis with disease progression are shown graphically in Fig. 3 and no specific trend is detectable. However, the trend of lowering titre paralleling disease progression described in the lymphoma patients C and F could not be confirmed for the groups as a whole, as illustrated in Fig.4. Since the geometric mean of the serum titre mediating ADCC appeared to correlate with disease progression in two symptomatic patients in group 1 the geometric mean ADCC titre was compared with $CD4^+$ T cell number (as measured by Becton Dickinson FACScan) where both ADCC and T cell count data were available for the same sample point (Fig. 5). There was no obvious correlation between ADCC titre and the $CD4^+$ T cell count for the whole cohort.

DISCUSSION

All HIV seropositives assessed here had antibodies to gp120 by Western blot, and to unglycosylated envelope (GP41) as measured by Abbott-ELISA (Simmonds *et al.*, 1988). There was no significant association between anti-gp41 titre in the patient group which remained asymptomatic, and that group with progressive disease, although a trend was seen towards increasing titre with time with large individual variations (Simmonds *et al.*, 1988). This is in accord with another report of HIV-seropositive haemophiliac patients in whom anti-GP41 antibody titre was unrelated to progression of disease (Rollag *et al.*, 1987). Correlation between ADCC titre and other anti-envelope responses is dependent upon the assay used. No correlation between ADCC and anti-envelope antibodies was found in the study of Rook *et al.* (1987) although we and others have found that lack of antibody to gp120 and or gp41 by ELISA correlates

Table 2. Reproducibility of ADCC assay using different effector donors and target cell lines

HIV-1 status	Serum samples	Assay 1 NK1 CEM.IIIB	Assay 2 NK2 CEM.IIIB	Assay 3 NK2 CEM.Ma
Seronegative (Healthy donor)	4	–	–	–
Seronegative (exposed)	4	–	–	–
Seronegative (hypergammaglobulinaemia, hepatitis B positive)	4	–	–	–
Seropositive (patient B)	6	+	+	+
Seropositive (patient C)	6	+	+	+
Seropositive (patient F)	10	+	+	+

NK1 and NK2, two members of low killer donor panel. HIV specific lysis exceeded control values by more than 2.4 s.d. Each determination made in triplicate, at three sample dilutions (1/100, 1/300, 1/900).

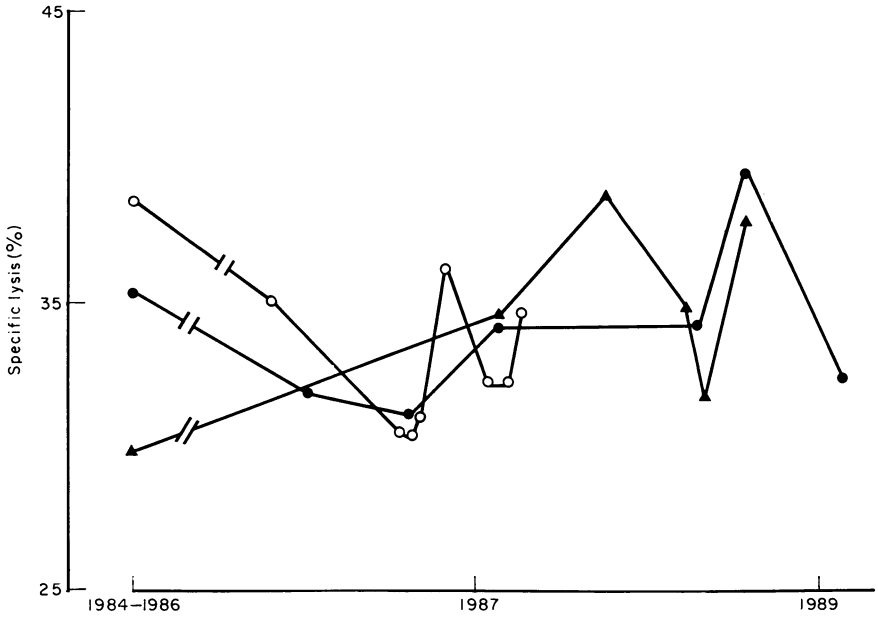

Fig. 3. Sequential changes in percentage specific lysis at a single serum dilution (1/900) between two patients (▲, C and ○, F) with progressive HIV-related disease, and asymptomatic HIV seropositive (patient B, ●). Patients C and F died of HIV-related malignant lymphoma. The geometric mean titre of serum mediating ADCC in patients C and F differed significantly from that seen in patient B. Patient B remained in stage CDC II classification throughout, patient C became 1Vc2 in early 1987 and 1Vd at the end of 1987. Patient F was CDC II in early 1984 and became CDC IVb and IVc, in mid-1986 and IVd in early 1987.

with low ADCC titre (Fuson *et al.*, 1983; Goudsmit *et al.*, 1988). More recently Evans *et al.* (1989) have shown that both anti-gp41 and anti-gp120 antibodies can mediate ADCC. We conclude that ADCC is a highly specific and a highly sensitive method of detecting serum antibody to HIV gp41 and/or gp120. A similar conclusion has been made in other virus systems such as herpes simplex (Subramanian & Rawls, 1977).

Correlation between high ADCC titres and asymptomatic status has been claimed (Goudsmit *et al.*, 1988), although based on very few patients using a different assay system based on the U937 cell line, and standardization with xenogeneic antiserum. This system is less sensitive than the CEM-based assay reported here, since only 60% of HIV seropositives gave positive ADCC with U937, whereas the CEM system shows all HIV-positive sera mediate HIV-specific ADCC (Sinclair *et al.*, 1988).

Our study shows no correlation between the ADCC-mediating activity of serum and the clinical status of patients infected concurrently with presumably the same isolate or isolates of HIV, and therefore ADCC activity would appear to have no prognostic significance. The only possible correlation demonstrated was in two patients who died of lymphoma. This is particularly interesting in view of the reported protective value of ADCC against Epstein–Barr virus (EBV) induced tumours (Pearson, Johanson & Klein, 1987; Pearson *et al.*, 1979). ADCC may only be active against immortalized HIV-positive T cell lines as activity against peripheral blood cells infected with HIV (either *in vivo* or superinfected *in vitro*) has never been reported for HIV. This could suggest that only cells which have unusually high antigen expression are susceptible. Nevertheless, ADCC is a sensitive (if not the most sensitive) measure of serum antibody

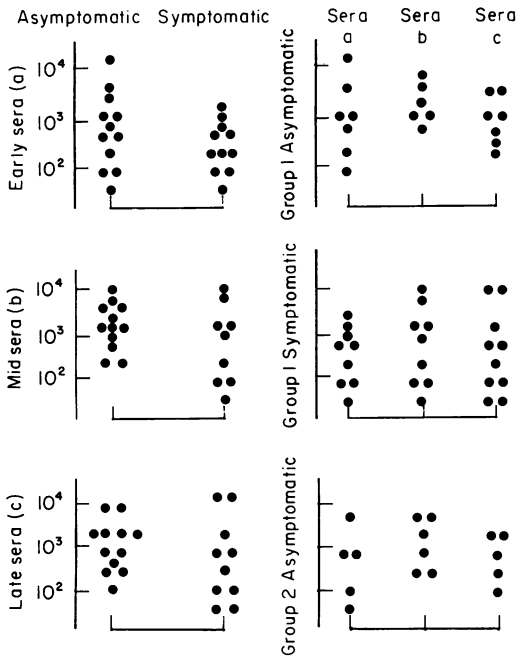

Fig. 4. Mean titre of sera mediating ADCC are shown. In the first (left hand) column the titres are shown for three sequential serum samples (a, b, c) and compared with the groupings of asymptomatic and symptomatic patients of groups 1 and 2. No difference in titre accorded with patient status at any of the three serum sample points (a early; b middle; c late). In the right-hand column titres are shown for patients grouped as asymptomatic or symptomatic in group 1 and the asymptomatic patients of group 2. Within each group there is no significant association between serum titre mediating ADCC, and the corresponding sequence of the serum sample. All the 'early' sera were taken when the patient was asymptomatic shortly after sera conversion. The middle sera was taken in 1986 at least 2 years after seraconversion. Seven of the the nine patients who are grouped symptomatic had progressed by the middle serum sample, and all had progressed by the late serum sample taken at the end of 1987 or early 1988.

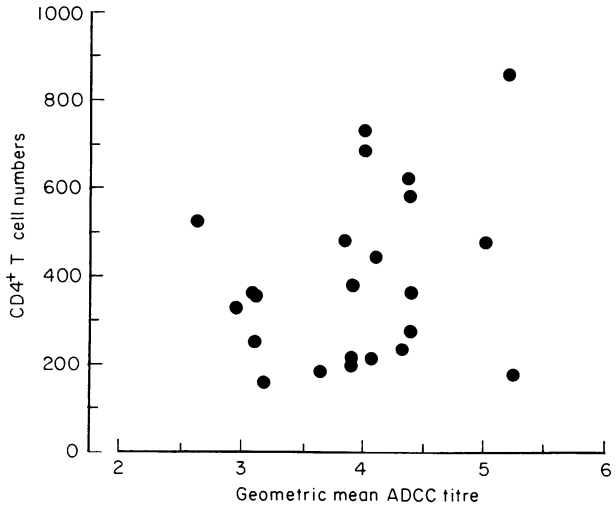

Fig. 5. Plot of geometric mean ADCC titre of late serum samples (c) against CD4 positive T cell number for patients of Group 1 and Group 2 ($n=23$ pairs).

response to HIV, and measures anti-gP41 and anti-gP120 activity in serum.

It is highly likely that most of the asymptomatic group will progress to disease and we are looking for the correlation of ADCC with different rates of disease progression. Indeed, within the Edinburgh haemophilia cohort the rate of decline in the CD4 count predicts the likelihood of a given patient being in CDC category IV by a certain year (Cuthbert *et al.*, submitted for publication). The CD4 counts in those patients in CDC category II have continued to fall and some will inevitably become ill in the near future. The ability of serial CD4 counts (single CD4 counts are relatively meaningless) to predict progression especially in conjunction with core antibodies and antigen (reviewed by Lange, de Wolf & Goudsmit, 1989), suggests that the lack of correlation between ADCC is relevant and concurs with the overall conclusions of the study.

ADCC could also play a pathogenic role by eliminating CD4 cells and thus enhancing the disease. It may be difficult to detect higher titres in those who progress to disease rapidly from those who remain asymptomatic for a long time, without having the opportunity of examining larger groups of patients who were all infected at the same time by the same route and by the same isolates. Nevertheless, the lack of correlation with the CD4 counts argues against a major pathogenic role for sera mediating ADCC. In a recent review of ADCC studies the question whether ADCC is pathogenic or protective is regarded as one of the most important issues to be resolved (Tyler, Lyerly & Weinhold, 1989), and it is suggested that only early ADCC activity could be protective. It therefore follows that ADCC activity could be both protective (early) and contribute to pathogenesis (late).

Although we have shown that measurement of this serum-mediated parameter alone is of no prognostic value or clinical relevance in HIV infection, the possible protective value of IgG-armed NK-like effector cells to mediate ADCC *in vivo* was not assessed here.

The lack of correlation between ADCC activity and progression to disease reported here suggests that reponse towards ADCC-mediating epitopes may not be essential criteria in developing a successful HIV vaccine.

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