Cytofluorometric analysis of anti-lymphocyte antibodies in AIDS

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1. SUMMARY

Anti-lymphocyte antibodies (ALA) have been detected in the plasma of 53.8% of HIV-positive patients tested (CD4/CD8 ratios: mean 0.265; range 0.01 to 0.5) using analytical continuous-flow cytofluorometry. IgG from the AIDS plasma was seen to bind to normal PBL in 53.8% of cases (14/26). In double labelling experiments CD4 + lymphocytes, CD8 + lymphocytes, and B lymphocytes were all bound by the ALA, but monocytes were not bound. Pre-adsorption of the diluted AIDS plasma onto an excess of mouse spleen cells did not remove lymphocyte binding activity. No evidence was found for preferential binding to phytohaemagglutinin-stimulated lymphocytes.

ALA could not be detected in the plasma of normal subjects, patients with acute renal failure undergoing renal dialysis, or patients with high levels of circulating immune complexes.

2. INTRODUCTION

Patients with the acquired immune deficiency syndrome (AIDS) exhibit impaired cellular and humoral immunity manifested clinically by opportunist infections and an increased frequency of malignancies such as Kaposi’s sarcoma or B cell lymphoma [1–3]. The condition is characterised by abnormalities of T cell subpopulations including a decreased ratio of cells expressing the CD4 antigen (helper/inducer T cells) to cells expressing the CD8 antigen (suppressor/cytotoxic T cells) and decreased absolute numbers of cells with the CD4 + phenotype [4].

In vitro, HIV is able to infect and kill CD4 + T cells [5] and this is likely to occur in vivo also. However, initial infection with this virus is known to be followed by a prolonged and variable asymptomatic period until progression to AIDS is triggered by as yet poorly understood mechanisms [6]. Although the human immunodeficiency virus (HIV) preferentially infects lymphocytes expressing the CD4 epitope, HIV is expressed in less than one in ten thousand T cells [7] and it is likely that other factors are involved in the selective destruction of CD4 + T cells.
Murine models which mimic early asymptomatic HIV-1 infection are characterised by hypergammaglobulinaemia and auto-antibody production [8,9] and it is possible that the production of auto-antibodies forms a part of the immune dysfunction associated with HIV infection. Several papers have reported the detection, in AIDS serum, of anti-lymphocyte antibodies (ALA) and these may contribute to the observed immunological abnormalities [10-14]. Other authors, however, have failed to find ALA or have suggested that the apparent detection of these may be an artifact of the experimental methods employed [15].

Methods used to detect ALA have included increases of surface IgG [10] and cytoxicity detected by microscopy [11,13,16], cytoxicity detected by 51chromium release [14], Western blotting [12,17], and flow cytometry [13,15]. Immunofluorescence has been reported to be a more sensitive method for detecting ALA than microtoxicity [11]. In the present study analytical continuous-flow cytofluorimetry was used to measure the interaction of ALA from AIDS plasma with normal peripheral blood lymphocytes (PBL).

3. MATERIALS AND METHODS

3.1. Patients

Control plasma (isolated from heparinised venous blood) was from nine healthy volunteers aged 21 to 48. AIDS plasma was from patients whose blood was taken for routine blood cell enumeration (n = 26). All had inverted CD4/CD8 ratios (mean 0.265; range 0.01–0.5) and a mean immunoglobulin G (IgG) concentration of 18.1 mg ml⁻¹ (range 11.2–34.8). Plasma was also obtained from patients with acute renal failure undergoing renal dialysis (n = 10, mean IgG 5.9 mg ml⁻¹) and bacterial endocarditis patients with high levels of circulating immune complexes (n = 10, mean IgG 20 mg ml⁻¹).

3.2. Cytofluorometric detection of ALA

Normal human peripheral blood lymphocytes (PBL) were isolated over Lymphoprep (Nycomed, Oslo, Norway), washed, aliquots of 2 × 10⁵ cells incubated in 100 μl of 1 in 10 dilutions of plasma or serum samples, in phosphate buffered saline containing 1% w/v bovine serum albumin/0.2% w/v sodium azide/5% v/v normal sheep serum (PBS/A/S), for 30 min on ice. After washing, cell-bound IgG was detected using FITC-conjugated F(AB')2 fraction of goat anti-human IgG (Fc-region sp.) (Bradshaw Biologicals Ltd., Leicestershire, U.K.). Cells were incubated at 4°C for 30 min, washed with PBS/A/S and fluorescence analysed using a Becton Dickinson FACScan (Becton Dickinson Ltd., Oxfordshire, U.K.)

3.3. Analysis of IgG attached in vivo

To detect in vivo bound ALA, blood samples from four AIDS patients and one normal control were diluted with ‘FACS Lysing Solution’ (Becton Dickinson) and, after washing, IgG attached to the cell surfaces of leukocytes was detected with the FITC:anti-human IgG (Fc-region specific) as before. Lymphocytes, polymorphonuclear leukocytes (PMN), and monocytes were separated for analysis during flow cytometry by gating of separate cell populations on forward and side scatter.

3.4. Double staining experiments

PBL were prepared and stained with FITC:anti-human IgG (Fc region) as before. After washing, the cells were incubated with mouse monoclonal antisera to various CD antigens (Dakopatts, Denmark), again for 30 min at 4°C, and, after further washing, detected with R-phycocerythrin conjugated anti-mouse IgG (Sigma, Dorset, U.K.).

3.5. PHA stimulation

Aliquots of 2 × 10⁵ lymphocytes were cultured (under sterile conditions), in 175 μl of RPMI-1640 culture medium (Gibco, Strathclyde, U.K.) containing 14% v/v autologous plasma, in wells of 96-well microtitre plates. Stimulated (1 μg/ml PHA) and unstimulated cultures were incubated for 72 h at 37°C, in 5% CO₂ in air (humidified) after which aliquots were removed and incubated with either autologous plasma or AIDS plasma (7 in number) as detailed in 3.2.
3.6. Effect of anti-CD43

Aliquots of $2 \times 10^5$ cells were given one of three treatments:
1) Pre-incubated for 30 min at room temperature with anti-CD43;
2) Pre-incubated for 30 min at room temperature without anti-CD43, then anti-CD43 added;
3) Kept on ice during the 30-min incubations (no anti-CD43).

All were then incubated at $4^\circ C$ with the inclusion of 10% normal serum (six in number), and any bound IgG detected as detailed in 3.2.

3.7. Pre-adsorption of AIDS plasma with mouse spleen cells

The spleen from a freshly killed mouse was disrupted and red cells lysed with hypotonic, Tris-buffered ammonium chloride solution. $110 \times 10^6$ cells were recovered, after washing, and these were split into two aliquots which were suspended in 1 in 10 dilutions of two AIDS plasmas before rolling at $4^\circ C$ for 40 min. The AIDS plasmas were thereby exposed to 275 times as many cells as used in the ALA assay. The plasma thus treated was then assayed in the usual manner at the same time as autologous plasma and additional plasma from the same patients which had been diluted and rolled in the absence of mouse spleen cells.

4. RESULTS

4.1. ALA detected in vitro by analytical cytofluorimetry

FACScan events were gated on the basis of forward and side scatter to include lymphocytes and exclude other cells and debris. Monocytes were also gated for comparison. Fluorometric analysis of experimental data yielded mean fluorescence levels for all subjects (Lysys Programme, Becton Dickinson) and these are plotted in Fig. 1 for lymphocytes and in Fig. 2 for monocytes. Table 1 shows that mean fluorescence is significantly higher on lymphocytes incubated with AIDS patient plasma [26] but not significantly different when incubated with plasma from renal dialysis [10] or high immune complex/IgG level endocarditis patients [10].

On monocytes, the general level of fluorescence was very much higher but not significantly different between the AIDS patients [11] and the controls [9].

Fig. 1. Normal PBL were incubated with plasma from patients with AIDS, acute renal failure, or bacterial endocarditis and normal controls. Bound IgG antibodies were identified using a fluorescent probe and detected by analytical continuous-flow cytofluorimetry. Lymphocytes were selected by gating. Bars indicate means of means.

Fig. 2. Normal PBL were incubated with plasma from patients with AIDS and normal controls. Bound IgG antibodies were identified using a fluorescent probe and detected by analytical continuous-flow cytofluorimetry. Monocytes were selected by gating. Bars indicate means of means.
Table 1
Mean fluorescences detected by flow cytometry on normal PBL incubated with 10% plasma

<table>
<thead>
<tr>
<th>Plasma Type</th>
<th>n</th>
<th>FL1 mean</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>9</td>
<td>38.7</td>
<td>2.74</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>AIDS</td>
<td>26</td>
<td>56.1</td>
<td>9.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>R. Dial.</td>
<td>10</td>
<td>37.7</td>
<td>10.2</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Endoca.</td>
<td>10</td>
<td>37.6</td>
<td>4.79</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>9</td>
<td>122.2</td>
<td>6.84</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>AIDS</td>
<td>11</td>
<td>125.3</td>
<td>3.51</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

Table 2
Mean fluorescences detected by flow cytometry on normal PBL incubated with normal plasma with/without co-incubation with anti-CD43 monoclonal antibody

<table>
<thead>
<tr>
<th>Serum</th>
<th>Pre-incubated</th>
<th>Not pre-incubated</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>42.68</td>
<td>45.1</td>
<td>38.61</td>
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<td>2</td>
<td>29.34</td>
<td>30.7</td>
<td>26.71</td>
</tr>
<tr>
<td>3</td>
<td>28.88</td>
<td>29.29</td>
<td>27.67</td>
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<tr>
<td>4</td>
<td>30.84</td>
<td>32.51</td>
<td>29.69</td>
</tr>
<tr>
<td>5</td>
<td>32.19</td>
<td>31.0</td>
<td>29.36</td>
</tr>
<tr>
<td>6</td>
<td>30.52</td>
<td>31.35</td>
<td>28.94</td>
</tr>
</tbody>
</table>

4.2. Surface IgG on lymphocytes

IgG was detected on the surface of lymphocytes from patients with AIDS and, again by gating and analysis, mean fluorescence from four subjects was 136.13 (SD 2.035). Mean fluorescence of a normal control was 83.37 which, at 26 standard deviations less, is very significantly different. Thus, in vivo, more surface IgG is associated with the lymphocytes of AIDS patients than of healthy people.

4.3. Lymphocyte sub-set analysis

Double-staining analysis was performed with several AIDS plasmas to determine if ALA were directed against a particular lymphocyte subset. Second markers used were CD2 (pan T cell), CD4, CD8, and CD19 (B cell). An example, for one particular AIDS plasma, is shown in Fig. 3 in which it can be seen that each subset stains for its CD marker and for ALA activity. Other AIDS plasmas gave similar staining to greater and lesser extents.

4.4. PHA stimulation

The mean fluorescence of unstimulated PBL incubated with diluted AIDS plasma, following 72 h in culture and then ALA detected as before, (40.02) was less than that with fresh PBL, indicating a possible loss of an epitope during culture. Mean fluorescence of unstimulated, cultured cells incubated in autologous plasma (33.53) was not significantly different. On stimulated cells autologous plasma gave a mean fluorescence of 74.18 which again was within the 95% confidence range of AIDS plasma on stimulated cells (51.16–76.02, mean 63.59). Thus there was no evidence of an increase in the expression of an epitope recognised by ALA on activated cells.

4.5. Incubation with anti-CD43

Six normal sera were used in this analysis. Normal PBL were: (1) pre-incubated with anti-CD43; (2) not pre-incubated but co-incubated with anti-CD43; or (3) anti-CD43 was not added (control). Aliquots of the treated cells were then incubated with the inclusion of 10% of one of the normal sera and fluorescence detected as before. The mean fluorescence values obtained are given in Table 2. Paired-sample t-tests were performed between treatment 1 (pre-incubated) and the control and between treatment 2 (not pre-incubated) and the control. Values of t = 1.85 and t = 2.014 (with 5 deg. fr.) respectively are not significant for either treatment (P > 0.1).

Table 3
Fluorescence detected by flow cytometry on normal PBL incubated with 10% plasma comparing non-spleen adsorbed and spleen adsorbed plasma

<table>
<thead>
<tr>
<th></th>
<th>Non-spleen adsorbed</th>
<th>Spleen adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>41.45</td>
<td>–</td>
</tr>
<tr>
<td>Patient 1</td>
<td>59.17</td>
<td>59.09</td>
</tr>
<tr>
<td>Patient 2</td>
<td>55.52</td>
<td>56.19</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>67.86</td>
<td>–</td>
</tr>
<tr>
<td>Patient 1</td>
<td>87.86</td>
<td>86.76</td>
</tr>
<tr>
<td>Patient 2</td>
<td>65.12</td>
<td>69.09</td>
</tr>
</tbody>
</table>
Fig. 3. Double-labelling analysis of PBL incubated with plasma (abscissa) and anti-CD markers before detection with fluorescent probes (anti-human IgG on FL1; anti-mouse IgG, to detect anti-CD monoclonals, on FL2). Plasma was normal control in a-e and from an AIDS patient in f-j. CD-antigens detected on FL2 were: a,f none; b,g CD2; c,h CD4; d,i CD8; and e,j CD19.
4.6. Pre-adsorption to mouse spleen cells

Spleen cell-adsorbed AIDS plasmas (incubated with a large excess of mouse spleen cells) gave an increase in mean fluorescence not significantly different to that given by the corresponding plasma which had not been so exposed (Table 3). Had the binding to lymphocytes been completely non-specific one would have expected fluorescence enhancement to have been lessened. Double-staining demonstrated that after spleen adsorption ALA-like activity was still directed against both T cells and B cells, to the same extent as without this treatment.

5. DISCUSSION

The results of this study (Fig. 1) demonstrate that IgG ALA are present in the blood of people with HIV. The IgG binding was not limited to a particular cell population. Other authors have reached differing conclusions on this point with some finding ALA to T and B cells [11,13,18], T cells only [12], or T helper cells only [10]. These differences could stem from different patient groups but care must be taken when comparing different populations of cells as staining brightness does vary between cell types and antisera. Our results show that the observed phenomenon is common to B cells and to both CD4 + and CD8 + T cells.

Beall et al. [15] have suggested that the conjugate used to detect ALA may be binding to Fc or other receptors and that this binding might be promoted by prior incubation with AIDS serum. This possibility cannot absolutely be ruled out, but steps were taken to minimise non-specific interactions. Thus the antibody conjugate used was directed specifically at the Fc-region which is probably exposed to a lesser extent on IgG expressed on cell surfaces, but freely exposed on IgG bound to cells via antibody binding sites. Also the F(Ab')2 fraction was used so that the conjugate should not, itself, bind to Fc-receptors and 5% sheep serum was included in all incubations to minimise general protein-protein interactions.

ALA, if present, are likely to be of low affinity; high affinity antibodies would not be freely circulating but would bind to their targets in vivo. This fact adds to the difficulty of detecting ALA in vivo as it is likely that only a proportion of ALA would interact in the short incubation time used. It was noted during the study that cells which were formalin-fixed after incubation with AIDS plasma and washing, but before incubation with the anti-human IgG conjugate, showed a greater shift in fluorescence than those fixed after all incubations. It was not clear, however, whether the formalin was fixing weakly bound, low affinity antibodies, which were otherwise lost during subsequent incubations, or merely fixing non-specifically bound IgG.

One possibility considered was that the phenomenon was due to immune complexes (IC) in AIDS plasma adhering to cells. Exposure of the AIDS plasma to mouse spleen cells had no effect on the results of the assay which indicates that this is not simply a case of IC or IgG ‘sticking’ to any cell with which they come into contact. For comparison, a number of sera from patients with bacterial endocarditis (with very high levels of circulating IC’s and raised serum IgG concentrations) were tested in the assay. Fluorescence values obtained were not significantly different from the normal controls.

The AIDS patients used in this study were mostly intravenous drug abusers, and thus some of the antibody activity may have related to alloimmunisation. Plasma from patients undergoing renal dialysis and who had had multiple blood transfusions did not contain ALA, and so the ALA detected in AIDS plasma was probably not due to exposure to the blood of other subjects.

No evidence was found from PHA stimulation studies that ALA activity was increased by activation of lymphocytes. Antibodies to the T cell surface antigen CD43 (sialophorin) have been detected in the serum of individuals infected with HIV [19] and it has been suggested that anti-CD43 antibodies can be involved in T lymphocyte proliferation [20]. This was one possible explanation for a phenomenon noticed during the PHA-stimulation experiments which was that stimulated T cells bound IgG.
from normal plasma/serum to a similar extent to that which resting (and stimulated) cells bound IgG from AIDS plasma. Thus, were anti-CD43 antibodies present in the AIDS patients' plasma 'activating' the lymphocytes used in the assay, and did this account for the difference in surface IgG detectable? In this study monoclonal antibodies to CD43 had only a marginal, statistically insignificant effect on surface-bound IgG from normal serum.

In conclusion, the data presented in this paper show that IgG in the plasma of AIDS patients does interact with healthy lymphocytes in vitro and the patients' own lymphocytes are found to be associated with excessive amounts of IgG in vivo.

No evidence was found for preferential binding to activated cells or to cells of the helper/inducer phenotype, which are the targets of HIV and the numbers of which are selectively reduced in AIDS.

ACKNOWLEDGEMENTS

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REFERENCES


