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# Circulating autoantibodies directed against conjugated fatty acids in sera of HIV-1-infected patients

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## SUMMARY

Several reports have demonstrated that major changes occur in the fatty acid content of HIV-infected cells. In order to evaluate if these changes are recognized by the immune system, we have attempted to assay the possible presence of autoantibodies (autoAb) directed against conjugated fatty acids (CFA). Using an adapted ELISA, anti-CFA autoAb were assayed in sera of 150 HIV-1-infected patients and 116 controls (healthy donors and patients suffering from other diseases). Significantly increased anti-CFA autoAb of IgG class were found in HIV-1-infected patients ($\alpha < 0{\cdot}001$). Using our ELISA method and CFA differing in their length and their degree of unsaturation (lauric, myristic, palmitic, palmitoleic, stearic, oleic, linolenic, linoleic, lignoceric, arachidonic, eicosapentaenoic and docosahexaenoic acids), it was demonstrated that the acyl chain of CFA is the immunodominant part recognized by these autoAb. Anti-CFA autoAb were present in 15/52 asymptomatic carriers, 14/36 symptomatic carriers, 16/39 ARC patients, but only 3/23 AIDS patients. Anti-CFA activity seemed to be linked with the $CD4^+$ T cell count, and was not related to the total IgG amounts. Anti-CFA autoAb could result from self-antigen presentation to immunological cells, and may reflect lipid membrane modifications occurring in HIV-infected cells.

**Keywords** fatty acid conjugates HIV infection ELISA autoantibodies autoimmunity

## INTRODUCTION

The striking similarities between HIV infection and autoimmune disorders [1,2] have prompted several authors to postulate that AIDS might be a typical viral-induced autoimmune disease [3–5]. HIV-infected patients are known to have a high incidence of autoantibodies (autoAb) with different specificities, including anti-cardiolipin antibodies [6], anti-histone antibodies [7] and autoAb directed against structural proteins of T cell membranes [8,9]. Moreover, HIV infection has been associated with an elevated oleic acid content in host plasma membranes, as assayed by analysing the fatty acid composition of membranes from leucocytes of AIDS patients and controls [10], and by *in vitro* infection of $CD4^+$ cultured cell lines [11]. Significant changes in fatty acid composition of $CD4^+$ cells from symptomatic patients with a large drop in the concentrations of arachidonic and linoleic acids have been demonstrated [12]. All these membrane lipid modifications in fatty acid composition associated with HIV infection can lead to membrane disturbances. Thus, a variety of lipidic neoantigens or self-antigens can be exposed to immune cells. AutoAb may be produced by autoreactive B cells, and could be an indicator of lipid membrane modifications expressed in infected cells. In the present study, we report the identification of autoAb directed against conjugated fatty acids (CFA) in a large group of HIV-1-infected patients.

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## PATIENTS AND METHODS

*Patient population*

Sera were collected from: (i) 150 HIV-1-infected individuals (supplied by Professor J.M. Ragnaud, Clinique Médicale et Maladies Infectieuses, Hôpital Pellegrin Tripode, CHU Bordeaux, France), distributed according to the CDC classification [13] into 52 asymptomatic carriers (group II), 36 symptomatic carriers (group III), 39 ARC subjects (group IV-A, IV-C2 or IV-E) and 23 AIDS patients (group IV-C1, IV-B or IV-D); (ii) 116 control subjects including 30 healthy blood donors (obtained from the Centre de Transfusion Sanguine de Bordeaux), 20 patients with multiple sclerosis (supplied by the Service de Neurologie, Hôpital Pellegrin Tripode, CHU Bordeaux), six patients with systemic lupus erythematosus (SLE) (Hôpital Saint-André, Bordeaux), 20 patients with

thrombosis, 20 patients with venous diseases, and 20 patients with graft rejection (obtained from the Hôpital Haut-Lévêque, Bordeaux). All sera were aliquoted and stored at −20°C until assayed.

*Chemicals*

Lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, lignoceric, retinoic and glycocholic, kynurenic and quinolinic acids, and succinyl α-tocopherol were purchased from Sigma (St Louis, MO). Linolenic, arachidonic, eicosapentanoic and docosahexaenoic acids were purchased from Aldrich (Strasbourg, France).

*Lipid-free bovine serum albumin*

One gram of bovine serum albumin (BSA; Sigma) was diluted in 10 ml of distilled water. Then, 10 ml of 1.1.2 trichlorofluorethan (Merck, Nogent sur Marnes, France) were added. The mixture was vigorously stirred for 3 min and centrifuged for 10 min at 2000 **g**. The aqueous fraction of the mixture was then lyophilized. The efficacy of delipidation was evaluated by addition of tritiated fatty acids before trichlorofluoroethan treatment. Delipidation yield was more than 90%.

*Synthesis of CFA*

Twelve fatty acids (lauric, myristic, palmitic, palmitoleic, stearic, oleic, linolenic, linoleic, lignoceric, arachidonic, eicosapentaenoic and docosahexaenoic) were conjugated to previously delipidated BSA (dBSA) by an acylation reaction according to Maneta-Peyret *et al.* [14]. Hapten (2·5 mg) was dissolved in 1 ml of anhydrous methanol (Merck) containing 10 μl of triethylamine (TEA; Merck) and 1 μl of (C14) fatty acid (NEN products, specific activity 1·85 Mbq). Activation of the carboxylic group was initiated by quick addition of 200 μl of anhydrous dimethylformamide (DMF; Merck) solution containing ethylchloroformate (Fluka, Buchs, Switzerland) diluted at 1 : 16 in DMF. The mixture was left for 3 min at 4°C. Then, dBSA solution (20 mg were dissolved in 1 ml 0·01 M $CaCl_2$ phosphate buffer containing 10 μl of TEA) was added to the previous mixture. The conjugates were purified by dialysis first against a mixture of dimethylformamide, methanol and distilled water (1 v/1 v/1 v); then against 0·01 M $CaCl_2$ phosphate buffer for 24 h at 4°C. Beta counting was performed on 100-μl aliquots, both before and after dialysis in order to calculate the concentration of CFA in each conjugate. Then the concentration of dBSA carrier was evaluated by measuring optical density (OD) at 280 nm. Molar coupling ratios (moles of fatty acid per mole dBSA) were calculated and ranged between 15 and 20, whatever the CFA.

*Synthesis of control conjugates*

Other molecules presenting a free carboxylic group (retinoic, kynurenic, quinolinic and glycocholic acids and succinyl α-tocopherol) were conjugated to dBSA according to the same procedure. The final purified conjugates gave molar coupling ratios similar to those found for CFA.

*Detection of anti-CFA autoAb in human sera*

The detection of anti-CFA autoAb in human sera was performed by an indirect ELISA test. First, polystyrene well-plates (Nunc, Polysorp) were coated with either CFA (50 μg/ml) or dBSA (50 μg/ml) in 0·05 M carbonate 0·001 M $CaCl_2$ buffer pH 9·6 for 16 h at 4°C. The well-plates were then rinsed twice with 0·01 M PBS (NaCl 0·15 M) pH 7·2 containing 0·001 M $CaCl_2$ (buffer A) and incubated at 37°C for 2 h with 200 μl of human sera diluted at 1 : 1000 in 0·01 M PBS (NaCl 0·45 M) containing 0·001 M $CaCl_2$ and 0·1% dBSA. The well-plates were rinsed once in buffer A and incubated for 1 h in goat anti-human IgG (γ) or IgM (μ) or IgA (α) labelled with horseradish peroxidase (HRP; Diagnostic Pasteur, Marnes-la-Coquette, France) diluted 1 : 5000 in buffer A containing 0·1% dBSA.

The well-plates were then re-washed three times with PBS 0·05% Tween 20. Peroxidase was assayed by incubating with an orthophenylenediamine (2%) solution in 0·1 M citrate 0·2 M phosphate buffer pH 5, containing 0·1% $H_2O_2$ (Merck) for 10 min in the dark. The reaction was stopped by the addition of 50 μl of a 4 M $H_2SO_4$ solution per well. The OD at 492 nm was read in a multiscan spectrophotometer (MR 610, Dynatech). Experimental values were corrected by subtracting blank values read on well-plates coated with dBSA. Samples were considered positive if their OD reading was more than 5 s.d. above the mean OD of healthy control sera.

*Absolute numbers of $CD4^+$ and $CD8^+$ lymphocytes*

The numbers of $CD4^+$ and $CD8^+$ lymphocytes in whole blood were determined by flow cytometry (FACScan, Becton Dickinson, Grenoble, France) and automated blood cell counts (H2 Technicon). After erythrocyte lysis (Facs Lysing Solution, Becton Dickinson), aliquots of cells were stained with monoclonal antibody (fluorescein-labelled (FITC) anti-CD45 antibody + PE-labelled anti-CD14 antibody) to perform the lymphocyte gate and to determine the lymphocyte purity. Other aliquots of cells were stained with FITC anti-CD4 MoAb + PE anti-CD8 MoAb and with FITC anti-CD19 MoAb (B cells) + PE anti-CD3 MoAb (T cells). All MoAbs were from Becton Dickinson. For each lymphocyte subset positive for both CD3 and CD4 (T helper cells) and for both CD3 and CD8 (T cytotoxic cells), data are recorded as a percentage of the total lymphocytes and corrected for the lymphocyte purity of the gate. The absolute values are obtained by multiplying the lymphocyte subset percentage by the absolute number of lymphocytes.

*Determination of immunoglobulin levels in human sera*

IgG, IgA and IgM levels were evaluated by an immunonephelometric method using a BNA nephelometer (Service d'Immunologie, CHU Pellegrin, Bordeaux, France).

*Anti-p25 and p25 assay*

Antibodies to p25 were measured by using a commercially available enzyme immunoassay (ELAVIA anti-p25 test, Diagnostics Pasteur; code 72254); p25 was assayed by an enzyme immunoassay for the screening of antigens associated with HIV-1 virus (ELAVIA Ag I, Diagnostics Pasteur; code 72236).

*Measurement of serum $\beta_2$-microglobulin*

$\beta_2$-microglobulin was determined by a commercially available antigen capture ELISA (Abott IMX, 2201-20).

*Statistical analysis*

Levels of circulating anti-CFA autoAb were expressed as means of absorbances with the standard deviations resulting from triplicate determinations. Data were checked and found

**Table 1.** Comparison of immunological binding read on different conjugated fatty acids (CFA) and conjugate controls

| Conjugate tested | $R = \frac{\text{OD HIV-1 patient sera} - \text{OD healthy donor sera}}{\text{OD healthy donor sera}}$ |
|---|---|
| Lauric acid | 3·26 |
| Myristic acid | 3·05 |
| Palmitic acid | 3·1 |
| Palmitoleic acid | 2·89 |
| Stearic acid | 2·82 |
| Oleic acid | 2·79 |
| Linolenic acid | 3·09 |
| Linoleic acid | 2·67 |
| Lignoceric acid | 2 |
| Arachidonic acid | 2·65 |
| Eicosapentaenoic acid | 2·65 |
| Docosahexaenoic acid | 3·46 |
| Glycocholic acid | 0·20 |
| Kynurenic acid | 0·20 |
| Quinolinic acid | 0·20 |
| Succinyl α tocopherol | 0·10 |
| Retinoic acid | 0 |

to be normally distributed. Differences between means of groups were evaluated by analysis of variance followed by Student's *t*-test ($\alpha$, $n > 30$) or by the Mann–Whitney test (*U*-test, $\alpha$, $n < 30$). Correlation coefficients between anti-CFA levels and the parameters of disease progression were evaluated by linear regression using the ANOVA test.

## RESULTS

Anti-CFA autoAb were evaluated using an optimized indirect ELISA test by varying: (i) the coating concentrations of CFA, and (ii) human serum dilutions. The best ratio (specific immunological binding read on CFA/non-specific binding read on dBSA) was obtained when CFA were adsorbed on well-plates at 50 μg/ml (data not shown). The ratio *R*: (OD of HIV-1 sera − OD mean of healthy control sera)/OD mean of healthy control sera was optimal when HIV-1 sera were diluted at 1 : 1000 (Fig. 1). These sera gave an OD value < 0·1 on dBSA coated on well-plates, and a value < 0·05 on the buffer alone.

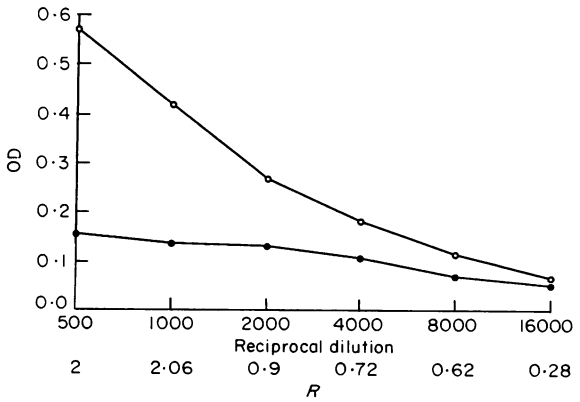


**Fig. 1.** Antibody response curves for anti-conjugated fatty acid (CFA) autoantibodies (autoAb) (expressed as OD at 492 nm) obtained by diluting HIV-1 (○) and helathy control pooled sera (●). The ratio *R* was defined as (mean OD values of HIV-1 patients − mean OD of healthy controls)/mean OD value of healthy controls.

Initially, circulating anti-CFA autoAb were measured in 20 random sera of HIV-1-infected patients and 10 controls using the same adapted ELISA method with the following CFA: lauric, myristic, palmitic, palmitoleic, stearic, oleic, linolenic, linoleic, lignoceric, arachidonic, eicosapentaenoic and docosahexaenoic acids. The immunological binding on CFA was found to be greater in HIV-1 sera than in control sera, and the difference was highly significant ($P = 0{\cdot}01$, *U*-test). The ratio *R* was the same whatever the unsaturation and chain length of CFA ($R = 2{\cdot}0$ to 3·46, Table 1). When control conjugates (retinoic, kynurenic, quinolinic and glycocholic acids and succinyl α-tocopherol, which were all linked to dBSA with an amide bond) were coated on well-plates, the difference of the OD means between HIV-1 and control sera was not significant, and *R* was < 0·2 (Table 1). These data suggest that the anti-CFA autoAb were directed against the fatty acyl chain rather than the carrier or the covalent linkage. Thus, the myristic acid conjugate was chosen for detection of anti-CFA autoAb in subsequent experiments for its ease of handling and better chemical stability.

Anti-CFA autoAb were assayed in a large series of sera (Fig. 2). Mean adsorbance values with s.d. from sera of 150 HIV-1-infected patients (Histogram 1) and 116 control samples including 30 healthy donors (Histogram 2), 20 patients with multiple sclerosis (Histogram 3), 20 patients with graft rejection (Histogram 4), 20 thrombotic patients (Histogram 5), 20 patients with venous diseases (Histogram 6) and six patients with SLE (Histogram 7) were respectively: $0{\cdot}421 \pm 0{\cdot}171$; $0{\cdot}210 \pm 0{\cdot}050$; $0{\cdot}245 \pm 0{\cdot}058$; $0{\cdot}256 \pm 0{\cdot}100$; $0{\cdot}241 \pm 0{\cdot}065$; $0{\cdot}270 \pm 0{\cdot}038$; $0{\cdot}270 \pm 0{\cdot}100$. The difference between the mean absorbance of HIV-1 patients and the healthy donor subgroups was highly significant ($\alpha < 0{\cdot}001$, *t*-test). The ratio *R* defined as above was found to be 1 for the HIV-1 population. Conversely, no significant difference was found between OD from sera of healthy donors and the other control subgroups

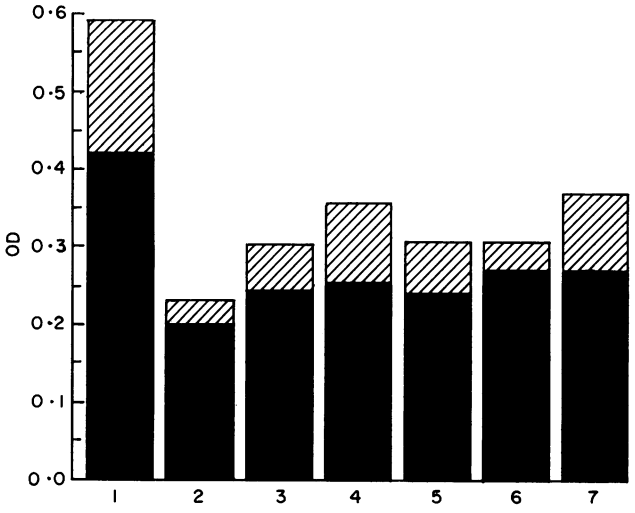


**Fig. 2.** Levels of anti-conjugated fatty acid (CFA) autoantibodies (autoAb) detected in human sera from 150 HIV-1 patients (1) and 116 controls, including 30 healthy donors (2), 20 patients with multiple sclerosis (3), 20 patients with graft rejection (4), 20 patients with thrombotic diseases (5), 20 patients with venous complications (6) and six patients with systemic lupus erythematosus (7). OD represents the binding of human sera (diluted at 1 : 1000) on myristic conjugate coated on well-plates. Histograms show mean OD values (■) with s.d. (▨).

($R = 0{\cdot}15$ to $0{\cdot}28$). According to our cut-off (OD > mean OD of control sera + 5 s.d.), anti-CFA autoAb were found in approximately 32% of HIV-1 individuals. The prevalence of anti-CFA autoAb increased in HIV-1 symptomatic subjects and dropped in AIDS patients. Thus, 15/52 group II patients (28%), 14/36 group III patients (39%), 16/39 ARC patients (41%) and 3/23 AIDS patients (13%) showed anti-CFA activity (Table 2). When HIV-1 subjects were displayed according to their $CD4^+$ T cell count, anti-CFA activity did not reflect the degree of $CD4^+$ T cell depletion (Table 3). Anti-CFA autoAb levels correlated well with the absolute number of $CD4^+$ T cells ($R = 0{\cdot}44$; $P = 0{\cdot}008$) and were not related to levels of $\beta_2$-microglobulin, p25, antibody to p25 or $CD8^+$ T cell count (Table 4). Anti-CFA autoAb were analysed for immunoglobulin class and found to be entirely IgG, with some samples having high amounts of IgM. Conversely, no sera had IgA activity (data not shown). Many HIV-infected patients had hypergammaglobulinaemia with IgG levels between 20 and 30 mg/ml. Since anti-CFA autoAb were defined as IgG, it could have been that elevated anti-CFA levels in such patients simply reflected a non-specific increase in immunoglobulin production. The amounts of IgG found in HIV-1 sera and anti-CFA autoAb levels were then compared. No correlation was found between levels of anti-CFA autoAb and total IgG, suggesting that the increase of anti-CFA activity was not due to non-specific binding (Table 4).

**Table 2.** Anti-conjugated fatty acid (CFA) activity in healthy donors and HIV-1 patients displayed according to the CDC

| Patients | *n* | Anti-CFA activity | | |
|---|---|---|---|---|
| | | OD at 492 nm | Number | Percentage |
| Healthy donors | 30 | 0·210 ± 0·050 | 0 | 0 |
| Group II | 52 | 0·381 ± 0·142 | 15 | 29 |
| Goup III | 36 | 0·462 ± 0·194 | 16 | 39 |
| ARC | 39 | 0·472 ± 0·186 | 19 | 41 |
| AIDS | 23 | 0·357 ± 0·11 | 3 | 13 |

**Table 3.** Anti-conjugated fatty acid (CFA) activity in HIV-1 patients displayed according to their $CD4^+$ T cell count

| $CD4$ count/$mm^3$ | *n* | Anti-CFA activity | |
|---|---|---|---|
| | | Number | Percentage |
| > 250 | 46 | 15 | 33 |
| 250–100 | 54 | 17 | 31 |
| < 100 | 33 | 6 | 18 |

**Table 4.** Linear correlation coefficients. A significant positive correlation with the $CD4^+$ T cell count and anti-conjugated fatty acid (CFA) autoAb levels was observed

| Biological parameters | *R* | *P* |
|---|---|---|
| $CD4^+$ T cells | +0·440 | 0·008 |
| $CD8^+$ T cells | +0·109 (NS) | 0·520 |
| $\beta_2$-microglobulin | +0·164 (NS) | 0·331 |
| p25 | +0·193 (NS) | 0·458 |
| Anti-p25 | +0·026 (NS) | 0·876 |
| IgG | −0·043 (NS) | 0·804 |
| IgA | −0·198 (NS) | 0·130 |
| IgM | −0·079 (NS) | 0·642 |

NS, Not significant; *R*, correlation coefficient; *P*, significance in ANOVA test.

## DISCUSSION

Previous work in our laboratory demonstrated the presence of high levels of circulating autoAb directed against small-sized molecules such as phosphatidyl inositol from cancer patients [15] or conjugated azelaic acid from patients with multiple sclerosis [16]. This autoAb production presumably reflected a cellular breakdown, and the autoAb became a probe for studying autoimmune processes. Since membrane lipid modifications have been reported in HIV infection [10–12], we hypothesized that possible autoAb directed against membrane lipid components might be present in HIV-1 patient sera. To study them, a reproductible ELISA method was developed. Using CFA coated on well-plates, we demonstrated significantly increased anti-CFA IgG levels in HIV-1 sera routinely diluted at 1 : 1000. There was no relationship between anti-CFA IgG titres and total IgG levels, indicating specific IgG binding to CFA. The recognition of anti-CFA autoAb sites was

related to the presence of the fatty acyl chain, whatever its length or degree of unsaturation. Similar results have been found experimentally in our laboratory by raising polyclonal antibodies in mice after administration of myristic acid conjugate. Anti-conjugated myristic acid antibodies bind to different CFA, suggesting that polyclonal recognition sites do not recognize any particular fatty acyl chain (unpublished results). Similar findings using another ELISA assay have been reported [17,18].

We observed that 49 of the 150 HIV-1 subjects tested (32%) had circulating anti-CFA autoAb ($\alpha < 0{\cdot}001$). Our finding supports the view that autoAb activity is present in HIV infection [7], but most autoAb investigated till now appear to be non-specific, since they have also been detected in other autoimmune processes [19] or related to opportunistic infections [20]. The restricted autoAb response in the control subgroups seems to support the hypothesis that anti-CFA autoAb production does not occur in association with non-specific autoimmune reactions occurring in HIV infection. High anti-CFA autoAb levels in group III and ARC patients suggest that autoAb production is not associated with opportunistic infections. The decrease in anti-CFA IgG titres in AIDS patients may be due to high levels of CFA binding these autoAb, and causing their depletion. Therefore, it is possible that anti-CFA autoAb could be a common feature of HIV-1 infection, thus providing further evidence that such antibodies, correlated with the absolute number of $CD4^+$ T lymphocytes, may be involved in the immunopathogenesis of AIDS. Sekigawa *et al.* [21] suggest that a conformational change in C-terminal CD4 domains may be induced by gp120 binding and could lead to anti-CD4 autoAb. Such an antigenic disturbance could disorganize the plasma membrane of the host cells, and exposure of self-fatty acids covalently linked to membrane proteins could occur. Callahan *et al.* [22] propose that HIV-induced abnormal processing of full length CD4 might take place, thereby exposing immunogenic autoepitopes normally hidden from humoral and cellular immune interactions. This cleavage of the anchored CD4 receptor may expose the palmitoyled moieties of CD4 [23] and activate autoreactive B cells, leading to autoimmune disorders. Therefore, it is tempting to speculate on a role of anti-CFA autoAb in the $CD4^+$ cell depletion associated with HIV infection.

Anti-CFA immune responses could be directed against the protein–lipid interface and reflect a membrane disturbance. Therefore, the importance and the role of anti-CFA autoAb following HIV infection must be taken into consideration. Do these autoAb play a role in the pathogenesis of AIDS? Work is under way in our laboratory with a view to understanding their clinical significance and role in HIV infection.

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