Tumour necrosis factor-α increases the sensitivity of human immunodeficiency virus (HIV)-infected monocytic U937 cells to the complement-dependent cytotoxicity of sera from HIV type 1-infected individuals; role of the gp120 protein

B. Szabó,1,3 C. Locardi,1 E. Lo Presti,2 F. Belardelli1* and A. Benedetto2

1 Department of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, 2 Centre of Virology, St Camillo’s Hospital, Circonvallazione Gianicolensce 85, 00100 Rome, Italy and 3 Department of Microbiology, University Medical School, Debrecen, Hungary

Sera of 40 intravenous drug addicts [25 seropositive and 15 seronegative for human immunodeficiency virus (HIV)] were tested for the presence of cytotoxic antibodies against uninfected and HIV-infected monocytic U937 cells. Six of the 25 seropositive samples proved to be cytotoxic for HIV-infected target cells in the presence of complement. The pretreatment of HIV-infected U937 cells with tumour necrosis factor (TNF)-α (which enhances virus production in these cells) increased the detection of serum cytotoxicity and 60% of these sera became cytotoxic. The percentage lysis was also increased after the TNF-α treatment of the target cells (from 16.2±4.5 to 71.2±4.9). The complement-dependent cytotoxic activity of these sera was significantly reduced by pretreatment with recombinant HIV gp120 antigen. This reduction was dose-dependent in the majority of cases. Immunofluorescence studies suggested that the cytotoxic sera mainly interacted with the viral antigens localized on the membrane of HIV-infected TNF-treated U937 cells. Moreover, comparative Western blot analyses using cellular extracts from untreated and TNF-treated U937 cells showed that there was a positive correlation between the cytotoxic phenotype and the capacity of sera to recognize the gp120 protein in extracts from TNF-treated HIV-infected cells. These results suggest that in some circumstances endogenous TNF-α can be a protective factor because it can render persistently infected cells highly sensitive to complement-dependent serum cytotoxicity as a result of increased expression of the relevant viral antigen (gp120) on the cell membrane.

Introduction

The human immunodeficiency virus (HIV) is recognized as the primary aetiological agent of AIDS and related disorders (Barré-Sinoussi et al., 1983; Popovic et al., 1984). Although many studies have been performed on the immune response to HIV (Bolognesi, 1991), the role of anti-HIV antibodies is still the subject of debate (e.g. see Hoffman et al., 1991).

Cytotoxic antibodies may have a role in the pathogenesis of AIDS (Kloster et al., 1984; Stricker et al., 1987) because HIV-infected T cells can serve as targets for complement-dependent cytotoxicity (Toth et al., 1989). These antibodies can be detected more frequently in the late phases of HIV infection (Szabó et al., 1992).

To our knowledge, antibodies reactive against persistently HIV-infected monocytic cells have not yet been detected in sera of patients infected with the virus. The human promonocytic cell line U937 (Sundstrom & Nilsson, 1976) has been used frequently to study aspects of monocyte or macrophage differentiation under defined conditions in vitro. We have recently selected and characterized a chronically HIV-infected U937 cell line (Locardi et al., 1990). In this cell line, tumour necrosis factor (TNF) markedly increased virus production, as evaluated by the measurement of levels of p24, reverse transcriptase activity or infectious virus in cell supernatants (Locardi et al., 1990). It was of interest, therefore, to evaluate whether sera from HIV-infected individuals could exert any complement-mediated cytotoxic effect on untreated or TNF-treated U937 cells and to characterize the relevant viral or cellular molecule(s) involved in the cytotoxic response.

Methods

Sera. Serum samples were obtained from 40 HIV-infected and uninfected drug addicts as well as from healthy control individuals. All were clinically asymptomatic at the time of blood sampling. The sera
were stored at −20 °C until used. The ELISAs were performed using DuPont anti-HTLV-III kits and the results were confirmed by Western blots (Diagnostic Pasteur). All the sera from HIV-infected individuals had similar titres, ranging from 1:320 to 1:640, as evaluated by ELISAs.

**Cell cultures.** The U937 monocyte cell line (Sundstrom & Nilsson, 1976) was infected with the HTLV-III B strain of HIV. The details of HIV-1 infection and cell culture procedures have been described previously (Locardi et al., 1990). HIV-infected and control uninfected U937 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Locardi et al., 1990).

**TNF-α treatment of cell cultures.** Recombinant human TNF-α (specific activity 7 × 10^7 units/mg) was the generous gift of Professor W. Fries and Dr J. Tavernier (Ghent, Belgium). Uninfected and chronically HIV-infected cells (5 × 10^5/ml) were incubated with TNF-α (1 ng/ml) for 24 h before the ^51Cr-release assays.

**Cytotoxic antibody assay.** Complement-dependent antibody cytotoxicity was detected by the ^51Cr-release technique, as described by Szabó et al. (1983). Uninfected and HIV-infected U937 cells (untreated or pretreated with TNF-α) were used as targets. They were resuspended in serum-free RPMI 1640 medium and incubated with ^51Cr in the form of sodium chromate (Amersham) and then 5 × 10^6 ^51Cr-labelled target cells were added to 100 μl of heat-inactivated (56 °C, 30 min) serum samples diluted in PBS. After incubation for 30 min at 37 °C, 100 μl of non-toxic guinea-pig serum was added as a source of complement, and incubation was continued for another 30 min. After centrifugation, the supernatant fluid was assayed for the released radioactivity. Spontaneous release (SR) was determined by incubating the target cells in medium alone. Maximum release (MR) was determined by incubating the cells with 5% Zaponin. Experimental release (ER), SR and MR were determined in triplicate. Results were expressed as: percentage cytotoxicity = [(ER-SR)/(MR-SR)] × 100.

**Inhibition of cytotoxic reactions by HIV gp120 antigen.** Before application to target cells, serum samples were incubated at 37 °C for 30 min with 10 μl of PBS containing increasing amounts of gp120. The recombinant HIV gp120 antigen was produced by the Micro Gene System in a baculovirus vector (Westham).

**Immunofluorescence.** Indirect immunofluorescence assays were performed using TNF-treated and untreated HIV-infected U937 cells, as well as their uninfected counterparts. Briefly, the spotted cells were fixed with cold methanol and samples were treated for 30 min at 37 °C with a 1:50 serum dilution and were then stained with suitable dilutions of fluorescein isothiocyanate-conjugated sheep anti-human IgG serum (Hyland).

**Cell membrane extracts.** The preparation of the membrane extracts of untreated and TNF-treated U937 cells was performed as described previously (Gazzit & Friend, 1981). In brief, the cells were incubated on ice for 10 min with a hypotonic buffer (5 mM-Tris-HCl, 2.5 mM-MgCl₂, pH 7.2). After centrifugation (100 g for 5 min) the resulting pellet was incubated with the same buffer supplemented with NP40 (at a final concentration of 0.1%). After a further centrifugation (700 g for 5 min) the supernatant containing the membrane fraction was collected and stored at −80 °C.

**Western blot assays.** The U937 cell plasma membrane fractions were subjected to electrophoresis in 10% polyacrylamide gels as described by Benedetto et al. (1989). Immunoblotting was performed using a Mini Trans-Blot (Bio-Rad) and with reagents from Diagnostic Pasteur, as previously described (Towbin et al., 1979).

### Results

Sera from 25 HIV-infected intravenous drug addicts were tested for the presence of antibodies to HIV-1 by both conventional ELISA and Western blot techniques and by a complement-dependent cytotoxicity assay against uninfected and HIV-infected U937 cells. Antibody cytotoxicity was also measured against target cells which had been pretreated with TNF-α for 24 h.

All the sera exhibited very similar reactivity to viral proteins when evaluated by ELISAs and Western blot techniques. Six sera (numbers 11 to 16) proved to be cytotoxic against HIV-infected U937 cells (group A). These sera exhibited a higher cytotoxicity against the TNF-treated HIV-infected U937 cells than against the untreated counterparts (Table 1). Notably, nine sera (numbers 17 to 25) which were not cytotoxic against the untreated U937 cells became highly cytotoxic when tested against TNF-treated target cells (group B). Sera of both groups A and B (i.e. samples reaching a cut-off of at least 20% cytotoxicity against untreated and/or TNF-treated U937 cells) were defined as cytotoxic. Some sera (numbers 1 to 10) did not exhibit any significant cytotoxicity against either untreated or TNF-treated cells.

#### Table 1. Cytotoxic activity of drug addicts’ sera on HIV-infected U937 cells

<table>
<thead>
<tr>
<th>Serum number</th>
<th>ELISA Pretreatment of target cells</th>
<th>Cytotoxicity (%)</th>
<th>Comment (group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>None</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>None</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>None</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>None</td>
<td>42</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>None</td>
<td>23</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>None</td>
<td>31</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>None</td>
<td>34</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>None</td>
<td>15</td>
</tr>
<tr>
<td>22</td>
<td>+</td>
<td>None</td>
<td>16</td>
</tr>
<tr>
<td>23</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>26-40</td>
<td>−</td>
<td>−</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*Non-cytotoxic: cytotoxicity < 20%.
† Cytotoxic: cytotoxicity > 20%.
cells, despite their comparable reactivity to viral proteins in ELISAs or Western blot assays. These sera were termed non-cytotoxic.

Fig. 1 illustrates the effect of the TNF-α pretreatment of target cells on the complement-dependent cytotoxicity of sera from both groups A and B tested against uninfected and HIV-infected U937 cells. (No cytotoxic activity could be detected using uninfected target cells, either before or after TNF treatment.) TNF-α treatment of HIV-infected U937 cells markedly increased the cytotoxic effect, compared to that in untreated control cells [from 16.2% (+4.5) to 71.2% (+4.9)].

Sera from 20 healthy control individuals did not demonstrate any cytotoxic activity, against either uninfected or HIV-infected U937 cells. In addition, no cytotoxicity was observed using these control sera against TNF-α-treated target cells.

To determine the virus-specific reactivity of cytotoxic antibodies, antibody cytotoxicity was then tested after incubation of sera with different amounts of recombinant HIV gp120. As shown in Fig. 2(a), preincubation of sera from group A with increasing amounts of HIV gp120 resulted in some reduction of the mean cytotoxic activity against untreated HIV-infected cells [from 29% (+5.9) to 19% (+3.8)]. Dose-dependent inhibition by gp120 was clearly observed when TNF-treated cells were used as target cells [activity was reduced from 80% (+12.5) to 30% (+11.5)]. Fig. 2(b) shows the results obtained in similar experiments with sera from group B. The cytotoxic activity of these sera (observed only when the target cells had been pretreated with TNF) was markedly reduced by preincubation with increasing amounts of competitor [from 56% (+7.2) to 20% (+9.0)].

We then evaluated the reactivity of group A and B sera on untreated and TNF-treated U937 cells as revealed by indirect immunofluorescence techniques. Cytotoxic sera exhibited no immunofluorescence on uninfected cells (Fig. 3a). Fig. 3(b) shows the typical diffuse immunofluorescence observed using cytotoxic sera on untreated HIV-infected U937 cells. Approximately 30% of these cells demonstrated membrane-associated immunofluorescence. TNF-α treatment of the HIV-infected U937 cells resulted in a marked increase in the relative numbers of cells exhibiting a membrane-associated fluorescence (from 30% to 95%).

To characterize further the possible target molecule(s) involved in the cytotoxic antibody response, we then performed Western blot analyses using membrane extracts from untreated or TNF-treated target cells. The separated and blotted cell extracts were tested with the individual sera listed in Table 1. Each serum was also
analysed by commercial Western blot assays. Fig. 4 shows the results of these analyses performed with three representative sera (i.e. one non-cytotoxic serum and two cytotoxic sera, belonging to groups A and B, respectively).

Despite the typical reactivity to the viral gp120 on commercial HIV-1 strips, the non-cytotoxic sera did not recognize the gp120 antigen of the HIV-infected U937 cell extracts, either before or after TNF-α treatment of the target cells. These sera were, however, highly reactive with the viral gp160 protein on U937 cell extracts.

A barely detectable reactivity to the gp120 U937 cell protein was observed using both cytotoxic sera. Notably, TNF-α treatment of HIV-infected U937 cells resulted in a significant change in the Western blot patterns as compared to those of membrane extracts from the control untreated cells. In particular, TNF-α pretreatment of target cells markedly increased the reactivity to the antigen of Mr 120K, especially in the case of the group B cytotoxic serum, which reacted more strongly with the 120K protein than with the 160K antigen. Despite these marked differences in their reactivity to the extracts of TNF-treated cells, these three representative sera uniformly recognized all the major bands of the HIV-1 lysate detectable on the commercial Western blot strips (Fig. 4).

The uninfected U937 cells (treated or not with TNF) did not display any cellular antigen recognized by cytotoxic sera (data not shown). Moreover, sera of
healthy control individuals did not demonstrate any binding to the proteins recognized by cytotoxic sera in the extracts from HIV-infected cells (data not shown).

**Discussion**

Although the presence of cytotoxic antibodies to HIV-infected T cells had been demonstrated previously (Stricker et al., 1987; Kloster et al., 1984; Toth et al., 1989), these results provide the first evidence of the presence of antibodies cytotoxic to persistently HIV-infected monocytic cells. Notably, monocytes and macrophages are considered to be reservoirs for HIV-1 in the infected host because they can be persistently infected, whereas T lymphocytes are generally destroyed by virus infection (Meltzer et al., 1990). It is therefore possible to speculate that cytotoxic anti-HIV antibodies may play some role in vivo in eliminating infected monocytes or macrophages, and so aiding inhibition of the spread of HIV infection. Further studies with human complement would be helpful to evaluate the possible clinical relevance of cytotoxic antibodies.

Approximately 20% of the sera from the HIV-infected individuals tested in our study exhibited cytotoxicity against untreated monocytic cells (Table 1). However, the pretreatment of target cells with TNF-α resulted in a general increase in the extent of antibody cytotoxicity (Table 1 and Fig. 1). For some sera (group B), conversion to the cytotoxic phenotype was observed only after TNF pretreatment of target cells. On evaluation of the data obtained using TNF-treated cells, up to 60% of the sera proved to be cytotoxic (Table 1).

TNF is usually found in the serum of AIDS patients (Reddy et al., 1988) and activation of the TNF system, either by the virus (Merill et al., 1989) or by other infections (Beutler & Cerami, 1987), is thought to be involved in the amplification of HIV infection. It has been reported, in addition, that TNF-α increases HIV expression in different cell types (Rosenberg & Fauci, 1991). In persistently infected U937 cells, it was observed that TNF markedly enhanced the production of infectious HIV particles (Locardi et al., 1990). Moreover, a correlation has been reported between endogenous TNF levels, p24 antigenaemia and some pathological symptoms occurring in AIDS patients (Toshifumi et al., 1991). Our results suggest an additional and beneficial role for TNF. In fact, it is plausible that low levels of endogenous TNF-α enhance the cytotoxic response in vivo of anti-HIV antibodies to virus-infected monocytes or macrophages, thus aiding the elimination of the major source of persistently infected cells.

The importance of membrane-associated gp120 in complement-dependent antibody cytotoxicity is supported by several observations. The preincubation of sera with an excess of exogenous recombinant gp120 resulted in significant inhibition of antibody cytotoxicity (Fig. 2). Moreover, a positive correlation was found between the cytotoxic phenotype and the capacity of sera to recognize the gp120 molecule in Western blots with U937 cell extracts from TNF-treated HIV-infected cells (Fig. 4). In addition, the cytotoxic sera exhibited a membrane-localized reactivity, especially on TNF-treated HIV-infected cells (Fig. 3e), and recognized the gp120 protein on membrane extracts from TNF-treated HIV-infected cells (Fig. 4). In contrast, only a barely detectable reactivity to the corresponding gp120 band was observed using extracts from untreated control cells. These findings suggest that TNF-α considerably enhances the levels of some gp120 epitopes recognizable by the cytotoxic sera in membrane extract. It is possible that the expression of HIV gp120 on the external surface of HIV-infected untreated U937 cells is rather low and, in the majority of cases, is not sufficient to induce a cytotoxic process mediated by the HIV-specific antibodies and complement unless the cells are stimulated by TNF-α.

Increased reactivity to gp120 on TNF-treated cell extracts is often associated with decreased reactivity to gp160 (Fig. 4). This phenomenon may be due to the increased cleavage of the gp160 envelope precursor protein to gp120 and gp41 in TNF-α-treated cells as compared to control cultures.

It is generally accepted that TNF can stimulate HIV production through the NF-κB pathway (Nabel & Baltimore, 1987). The increased cleavage of the gp160 protein in TNF-treated U937 cells might be the result of either the general increase in virus production or the specific effect of some TNF-induced proteases (responsible for the cleavage of the gp160 precursor). Although TNF may affect cellular protease activities (Van Hinsberg et al., 1990) there is, as yet, no direct evidence of the effect of TNF on gp160 cleavage.

Although these data stress the importance of specific gp120 epitopes in cytotoxic antibody response, no correlation was found between cytotoxicity and reactivity of sera to viral gp120 in commercial HIV lysates. In fact, the non-cytotoxic sera recognized the gp120 antigen on commercial Western blot strips, but they did not react with the TNF-induced gp120 protein in the extracts of HIV-infected U937 cells. This finding suggests the existence of some qualitative difference between the non-cytotoxic and the cytotoxic sera (e.g. IgG isotype and/or epitope reactivity). The possible presence of inhibiting factors in the non-cytotoxic sera remains to be evaluated, however, and the characterization of these sera deserves further investigation.

In conclusion, the data discussed herein suggest that, especially in the presence of endogenous TNF, cytotoxic antibodies play a defensive role against HIV infection by
eliminating persistently infected monocytes or macrophages. Further studies on these HIV-infected individuals should allow us to evaluate any possible correlation between antibody cytotoxicity and the progression to full-blown AIDS.

We are indebted to Anna Ferrigno and Maria Cristina Ferrari for secretarial assistance. This study was supported by grants from the Italian Ministry of Health (number 6205.002, IV Progetto di Ricerca sull'AIDS and number 720/0, V Progetto di Ricerca sull'AIDS). Dr Bela Szabò was supported by a fellowship from the International Centre for Genetic Engineering and Biotechnology, UNIDO, Trieste, Italy.

References


MERRILL, J. E., KONAYAGI, Y. & CHEN, I. S. Y. (1989). Interleukin-1 and tumor necrosis factor can be induced from mononuclear phagocytes by human immunodeficiency virus type 1 binding to the CD4 receptor. Journal of Virology 63, 4404-4408.


(Received 4 November 1992; Accepted 9 February 1993)