Overexpression of a Novel Lymphocyte Population, Positive for an Intracellular CD14-Like Antigen, in Patients Positive for Human Immunodeficiency Virus Type 1

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CD14, originally recognized as a lipopolysaccharide (LPS) receptor, has recently been implicated in the process of T-cell suppression and apoptosis. Its soluble form has been shown to bind, in vitro, to human T cells, a process that may carry a negative signal onto these cells. We recently described a novel lymphocyte population in human peripheral blood, a population that expresses an intracellular CD14-like antigen. This novel T-cell population, composed mainly of CD8 cells and of very few CD4 cells, was found to be greatly enhanced in asymptomatic, untreated human immunodeficiency virus (HIV)-positive individuals. In the present study, we further characterized this cell population and found that it differed from other CD8 subpopulations associated with HIV infection such as CD8/CD38. In addition, we followed HIV patients under conditions of highly active antiretroviral therapy (HAART) and observed two groups of patients: patients in whom the CD14-like positive-testing T cells returned to normal within 1 to 3 months, and patients in whom it did not, in spite of a significant plasma HIV-RNA viral load decrease. Thus, this new CD14-like positive-testing lymphocyte population may represent an interesting and important component of the cellular events associated with HIV infection. On the basis of its modulation following HAART, we speculate that it may be used, in the future, as a drug-monitoring cellular marker in antiretroviral treatment.

CD14 is a 55-kDa glycosylphosphatidylinositol (GPI)-linked protein present on the surface membrane of phagocytic leukocytes. It is also present in a soluble form in serum. CD14 is one major molecule responsible for the innate host inflammatory response to microbial infection. As a key receptor for lipopolysaccharide (LPS) on the surface of monocytes and macrophages, the CD14 molecule was thought, until recently, to be involved primarily in nonspecific host defense mechanisms against gram-negative bacteria (2, 6, 10, 19, 20).

A number of recently published results confer to this interesting molecule novel functions that are linked to apoptosis and also to T-cell activation. Thus, CD14 may function as an “apoptotic cell receptor” on the surface of phagocytes, since it seems to bind to phosphatidylinerine which is externally exposed by apoptotic cells (1, 4); it may also be linked to susceptibility of monocytes or other cells to apoptosis, as it has been shown that a high level of expression of CD14 may protect cells from apoptosis and vice versa (11–13). Particularly interesting, CD14 either as a recombimant protein or as a native molecule secreted by monocytes in vitro has been recently shown to bind to the surface of in vitro-activated human T cells (7, 18). Most importantly, this binding was shown to convey a negative signal onto these T cells (16), in the form of interleukin-2 (IL-2), IL-4, and gamma interferon (IFN-γ) inhibition, probably due to the inactivation of NF-κB (18).

These results have been reported from studies of cells cultured in vitro and artificially activated. We have been particularly interested in the implications of these findings and sought to assess the possible contribution of CD14 to T-cell activities and functions, in vivo, in humans.

As a first step, we looked for any evidence for the in vivo presence of CD14 in human lymphocytes and particularly in T cells and found a subpopulation of lymphocytes in which a CD14-like antigen could indeed be visualized by immunofluorescence but only upon fixation and permeabilization of cells, implying that this molecule was located intracellularly and not on the outer membrane of the cells (21). Since human immunodeficiency virus (HIV) disease is characterized by both immunosuppression and apoptosis (8, 14), we sought to explore the possible involvement of these cells in the cellular events that are linked to HIV infection. We found that HIV patients expressed elevated numbers of lymphocytes positive for this intracellular antigen (21). We then looked for evidence of response to drug treatment in patients as well as for the uniqueness of this CD8 subpopulation in HIV disease. The results obtained form the matter of the present contribution.

**MATERIALS AND METHODS**

Mononuclear cells, freshly obtained from peripheral blood of healthy and HIV-infected individuals on a Ficoll-Hypaque gradient (UNI-CEP tubes; Novamed Ltd., Jerusalem, Israel) were washed in phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde in PBS for 30 min at 4°C. Cells were washed in PBS and then permeabilized in PBS supplemented with 0.1% saponin (Sigma) and 1% bovine serum albumin (Sigma) for 30 min at room temperature. Thereafter, cells were divided into staining tubes and processed in the saponin buffer for dual-color fluorescence staining.

Cells (in 50 μl of saponin buffer) were incubated with 5 μl of an anti-CD14 monoclonal antibody (MAb) (MO2 [RD1 conjugated]; Coulter, Hialeah, Fla.) or

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an isotype MAb control (immunoglobulin M [lgM] [RD1 conjugated]: Coulter) and 5 μl of anti-CD3 or -CD4 or -CD8 MAb ([fluorescein isothiocyanate conjugated]; IOP, Groningen, The Netherlands), for 30 min at room temperature followed by one washing in saponin buffer and two washings in PBS.

For the analysis of CD38, live cells were double stained for CD8 and CD38. For the analysis of CD28 and CD57, fixed and permeabilized cells were triple stained for CD8 (FL3), MO2 (FL2), and CD28 or CD57 (FL1).

Cells were resuspended in PBS and immediately analyzed with a FACScalibur apparatus (Becton Dickinson). The analysis of CD8-positive cells was restricted to the CD8 bright cells only and did not include the CD8 dull cells that are NK-like lymphocytes.

Lymphocytes were gated according to forward scatter and side scatter.

RESULTS

Upon fixation and permeabilization of human peripheral blood mononuclear cells, the presence of a subpopulation of lymphocytes that stains with a monoclonal anti-human CD14 MAb (MO2) can be demonstrated. In this present study, we concentrated on the CD3+ subpopulation and looked for the rate of expression of these cells in HIV-positive individuals. Thus, 22 asymptomatic and untreated HIV patients and 35 age- and sex-matched healthy controls were analyzed for MO2-positive T cells. As shown in Fig. 1, a marked difference was observed between these two groups. Thus, HIV-positive individuals as a group displayed a much higher rate of MO2-positive T cells (34.1% ± 12.7% versus 14.2% ± 7.4% in healthy controls; P < 0.0001). In addition, upon double staining of lymphocytes with anti-CD4 or anti-CD8 MAbs and with the MO2 antibody, we observed that the main T-cell population bearing the CD14-like antigen was a CD8 population. A very high percentage of these CD8 cells were found to be MO2 positive in HIV patients (52.5% ± 12.1% versus 19.3% ± 11% in healthy controls; P < 0.0001). Still, in HIV patients a significant percentage of CD4 cells were found to be positive for MO2 (9.2% ± 12.6% versus 2.9% ± 1.2% in healthy controls; P < 0.02).

Next, the issue of follow-up and modulation of this population of cells by antiretroviral treatment was raised. For that purpose, we first tested the reproducibility with time of the expression of this antigen in cells obtained at two different occasions, within a few months. We found that the high and low phenotypes (of HIV and healthy individuals, respectively) of MO2 expression were found to be consistent with time, within reasonable fluctuations (data not shown). Thus, the follow-up of patients after treatment is feasible, as changes, if any, observed under conditions of highly active antiretroviral therapy (HAART) can probably be attributed to the drug treatment itself.

Six patients, whose basic characteristics in terms of CD4-positive cells and plasma viral loads are depicted in Table 1, were tested for the expression of MO2 antigen by T cells before and 1 to 3 months after treatment initiation. For all patients a sharp decrease in HIV RNA plasma viral loads was observed. As clearly demonstrated in Fig. 2, the patients segregated in two distinct groups. In one group the levels of the CD14-like antigen bearing cells (CD3, CD4, and CD8 cells) returned to almost normal values (Fig. 1) compared to their values before treatment, in sharp contrast to the second group of patients, in which the levels remained unchanged or even increased following an apparently successful treatment.

It was interesting to assess whether this segregation into two groups of posttreatment patients was a general phenomenon that was also visible long after HAART initiation. For that purpose, six HIV patients, on HAART for 1 to 3 years, were tested for the MO2 marker. Their characteristics before treatment initiation and at the time of the MO2 staining are detailed in Table 2. As evidenced in Fig. 3, the same dichotomy was observed in these patients also. Three patients displayed a high level of CD14-like positive T cells, whereas the three others demonstrated an almost normal level (Fig. 1) of this population of cells.

Since other CD8 subpopulations of lymphocytes, especially those of the CD8-positive CD8 cells, have been already described as informative for HIV patients, it was of importance to find out whether this CD8+/MO2-positive population of cells indeed represented a novel, separate cell phenotype or whether it was a known, previously described population of CD8 cells that also expressed the MO2 antigen. In attempting to answer to this question, we used two- or three-color flow cytometry to analyze in a group of 13 HIV patients the distrib-

| Patient | Medication | No. of CD4+ cells/μl of blood | Viral load (copies/ml) |
|---------|------------|------------------------------|-----------------------|
|         |            | Before HAART | During HAART | Before HAART | During HAART |
| AP      | NFN, AZT, 3TC | 44 | 42 | 272 | 209,000 | 760 |
| RA      | APV, AZT, 3TC | 100 | 104 | 258 | 300,000 | 18,000 |
| BYY     | IDV, RTV, d4T, 3TC | 55 | 51 | 146 | 220,000 | 1,600 |
| GR      | NVP, AZT, 3TC | 420 | 404 | 435 | 16,000 | 99 |
| VN      | EFV, AZT, 3TC | 286 | 280 | 313 | 17,000 | 9 |
| OD      | NLV, AZT, 3TC | 638 | 591 | 991 | 7,400 | 9 |

* HAART, highly active antiretroviral therapy; NFN, nefiflavir; AZT, zidovudine; 3TC, lamivudine; APV, amprenavir; IDV, indinavir; RTV, ritonavir; d4T, stavudine; NVP, nesinatine; EFV, efavirenz.
* The plasma viral loads were measured by an NASBA amplification system (Organon Teknika; see reference 22), with 50 copies per ml the lower detection limit.
bution of four different CD8 populations: CD38 (10 patients), CD28 (3 patients), CD57 (3 patients), and MO2-positive CD8 cells. The results obtained are found in Fig. 4 and 5. As clearly evidenced in the figures, the different subpopulations of cells seem to represent different cellular compartments. The percentages of CD8 cells expressing the CD38 antigen or the MO2 antigen differ considerably in the vast majority of the patients, being higher in some and lower in others (Fig. 4). It should be noted that the CD38 antigen was found in preliminary studies to be undetectable after fixation and permeabilization of the cells (even when CD38 staining was performed on live cells that were subsequently fixed and permeabilized); thus, the comparison here was based on two separate double staining procedures. In addition, as depicted in Fig. 5, the CD8 MO2- population was found to be composed of both CD28 and CD28- cells. Similar results were obtained with the CD57-positive CD8 cells (data not shown). Thus, the MO2-bearing CD8 population of lymphocytes seems to be unique.

**DISCUSSION**

In a recent study we showed that human peripheral blood lymphocytes expressed an intracellular antigen that cross-re-acted with MO2, an MAb directed against the human CD14 molecule. The presence of this antigen could be visualized only upon fixation and permeabilization of the cell membrane. The staining was obtained with various permeabilizing agents, did not depend on the fluorochrome, and was equally observed in peripheral blood mononuclear cells (PBMCs) and in whole blood. In addition, a fluorescence microscopy examination of the stained T cells revealed that the MO2 antigen was found at the periphery of the cells, close to the outer membrane (21). In this present communication we further elaborate on the MO2-positive lymphocyte population. First, we show that HIV patients, untreated and asymptomatic, display a higher rate of MO2-positive T cells. Than, we demonstrate the modulation of this novel population of cells by HAART, at least in some of these patients. Finally we show that the MO2-positive CD8- subpopulation represents a novel population that is different from those of CD8 CD28, CD8 CD38, or CD8 CD57.

Other recent studies have shown the presence of CD14 in lymphocytes. Indeed, CD14 derived from monocytes or recombinant CD14 was demonstrated to bind in vitro to the outer membrane of activated T cells (7, 18). In one case, it was

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**TABLE 2. Follow-up of HIV-positive individuals on HAART for 1 to 3 years**

| Patient | Medication | No. of CD4+ cells/μl of blood | Viral load (copies/ml) |
|---------|------------|-------------------------------|-----------------------|
|         |            | Before HAART | During HAART | Before HAART | During HAART |
| AA      | NFV, AZT, 3TC | 316 | 656 | 56,000 | <20 |
| YY      | IDV, d4T, 3TC | 96 | 192 | <400 | <20 |
| SC      | EFV, d4T, 3TC | 423 | 902 | 71,000 | <20 |
| GA      | NFV, d4T, 3TC | 42 | 360 | <4,000 | <20 |
| AS      | IDV, AZT, 3TC | 296 | 678 | 56,000 | <20 |
| ABA     | IDV, d4T, 3TC | 22 | 34 | 97,000 | <20 |

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* HAART, highly active antiretroviral therapy; NFV, nelfinavir; AZT, zidovudine; 3TC, lamivudine; IDV, indinavir; d4T, stavudine; EFV, efavirenz.

* The viral loads were measured by an NASBA amplification system (Organon Technika; see reference 22), with 50 copies per ml the lower detection limit.
demonstrated not only that the CD14 bound to the T cells but also that it penetrated the cell membrane and could be visualized by immunofluorescence upon fixation and permeabilization of the cells. Following this interaction a negative signal was delivered, as evidenced by the down regulation of NF-κB and various cytokines (18). Altogether, these data suggested that the interaction of CD14 with T cells might carry an immunosuppressive and possibly an apoptotic signal. We therefore speculate that this is the reason why such a lymphocyte population, expressing a CD14-like antigen, might be elevated in HIV patients. Indeed, recent studies have demonstrated significant elevations in the soluble form of CD14 in sera of HIV patients (15, 17). Elevated CD14 was found both in the serum and on peripheral blood monocytes, in direct correlation to disease development. The elevated level of soluble CD14 was found in all stages of HIV disease and particularly in AIDS patients (15). There is thus a direct correlation between the level of CD14, the level of immune suppression, and the level of HIV RNA in plasma. Our working hypothesis is that soluble CD14 from the plasma or from monocytes indeed binds under certain circumstances to lymphocytes, possibly via a receptor differentially expressed by various lymphocyte subpopulations (24), and is rapidly internalized.

The second aspect of this study relates to the modulation of this new cell population under conditions of HAART. The changes observed are indeed due to the treatment, as we showed that the high or low phenotypes are consistent with time. There seems to be no correlation between the type of drugs administered and the resulting MO2 modulation. It should be emphasized, however, that the number of patients followed up is far too small to allow conclusions to be drawn in this respect. As demonstrated, although the plasma viral load in all treated patients reached an undetectable or very low level, two phenotypes of patients were observed as far as the level of the MO2-positive population of lymphocytes was concerned. In two different groups of patients, tested either a few weeks or 1 to 3 years following treatment initiation, the same two phenotypes were observed, i.e., for patients with whom the CD14-like positive population returned to near normal level and for those with whom it did not.

We know that this difference is not due to minor differences in the kinetics of the drug-induced effect on this new subpopulation, since, in a few patients who have been followed up for more time, no significant changes in their phenotypes could be observed (data not shown). Other possibilities may thus explain this phenotypic dichotomy: in some patients, HIV replication may continue in spite of an undetectable plasma viral load (5). This ongoing replication may result in the continuous stimulation or generation of the MO2-positive lymphocyte population. In this respect, however, it should be noted that our new population of lymphocytes presented here differs considerably from the CD8⁻/CD38⁺ activated population of cells. Indeed, the decrease in the CD8⁺/CD38⁺ population has been repeatedly shown to be directly linked to the drug-induced viral load decreases (3, 9, 23). Our MO2 population of CD8 cells differs as well from the CD57 or the CD28 populations, although, as shown, a large percentage of them seem to be CD28 negative.

Alternatively, the expression of the MO2 marker may reflect, as suggested earlier, the apoptotic status of the cells. Preliminary results in our laboratory suggest that the induction of apoptosis in vitro induces MO2 as well. It is possible that in spite of the low or undetectable viral load, the apoptotic signals on the cells still prevail.

To conclude, we describe a novel and unique lymphocyte subpopulation that expresses an intracellular CD14-like antigen, detected by the MO2 antibody. This population is enhanced in HIV patients and responds to treatment in some of them. We speculate that the MO2 antigen in T cells may correlate with immunodeficiency and/or the apoptotic status of the cells. It is our intention to better characterize this intracellular antigen and its identity and function and to further follow up these subgroups of patients.
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