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Association of Circulating Receptor Fc γ RIII-positive Monocytes in AIDS Patients with Elevated Levels of Transforming Growth Factor- β

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Abstract

Monocytes in the circulation of normal individuals express two receptors for the constant region of immunoglobulin, Fc γ RI and Fc γ RRII. In contrast, we have observed that AIDS monocytes express significant levels of a third Fc γ R, Fc γ RIII (CD16), which is normally associated with activation or maturation of the monocyte population. By dual-fluorescence analysis using a monoclonal antibody specific for Fc γ RIII (MAb 3G8), 38.5 \pm 3.2% of the LeuM3 (CD14)-positive monocytes in AIDS patients were CD16 positive as compared to 10.4 \pm 1.0% for healthy individuals ($n = 29$; $P < 0.005$). Furthermore, AIDS monocytes expressed Fc γ RIII-specific mRNA which is expressed minimally or not at all in control monocytes. As a recently identified inducer of Fc γ RIII expression on blood monocytes, transforming growth factor- β (TGF- β) was found to be elevated in the serum and/or plasma of AIDS patients. Moreover, incubation of normal monocytes with AIDS serum or plasma induced CD16 expression which correlated with serum TGF- β levels ($r = 0.74$, $P < 0.001$) and was inhibited with a neutralizing antibody to TGF- β . Thus, the increased CD16 expression on peripheral blood monocytes in AIDS patients may be the consequence of elevated circulating levels of the polypeptide hormone TGF- β . (*J. Clin. Invest.* 1991. 87:1773–1779.) Key words: CD16, CD32, CD64 • human immunodeficiency virus • immune suppression • maturation • mononuclear phagocytes

Introduction

Receptors for the Fc domain of immunoglobulin (Ig) comprise a group of closely related membrane glycoproteins present on many cells participating in host defense (1). Fc γ RI receptors (CD64) are found primarily on monocytes and macrophages and bind monomeric IgG with high affinity, whereas Fc γ RRII receptors (CDw32) are a polymorphic family of molecules expressed on monocytes, neutrophils, eosinophils, B cells, and platelets and mediate uptake of immune complexes and opsonized particles (2–5). Fc γ RIII receptors (CD16), which are expressed on several cell types (1, 3, 5), are expressed only on a small percentage ($\sim 10\%$) of circulating monocytes (6). However, this receptor appears as monocytes mature and differentiate in culture, in tissues, and at inflammatory sites (7–9). This class of receptor plays an important role in the clearance of immune complexes and within inflammatory sites (7).

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Received for publication 7 May 1990 and in revised form 4 January 1991.

The Journal of Clinical Investigation, Inc.
Volume 87, May 1991, 1773–1779

Recently, Fc γ RIII, but not Fc γ RI or Fc γ RRII, have been implicated as possible sites of HIV-1 entry in mononuclear phagocytes. HIV-1 infection-enhancing antibodies have been identified in the sera of seropositive patients (10–12) and Fc γ RIII antibodies inhibit the enhancement of HIV-infection in vitro (10). Although these results suggest that Fc γ RIII on monocyte/macrophages mediate antibody-dependent enhancement of HIV infectivity by a pathway distinct from the CD4 molecule, it is not clear whether antibody enhancement of HIV infection occurs in vivo (13). The lack of Fc γ RIII would likely preclude this route of infection on circulating monocytes. However, since blood monocytes in AIDS patients have been shown by various criteria to express phenotypic and functional changes characteristic of an activated or differentiated population (14–20), the possibility existed that they might also show increased expression of Fc γ RIII.

In the present study, we show that monocytes freshly isolated from patients with AIDS and AIDS-related complex (ARC)¹ express increased levels of Fc γ RIII on their cell surfaces as compared to normal controls. Moreover, a soluble factor in the serum and/or plasma of HIV-1-infected individuals induced expression of Fc γ RIII in vitro on peripheral blood monocytes obtained from control individuals. This activity could be blocked by neutralizing antibodies to transforming growth factor- β (TGF- β) and is consistent with previous in vitro and in vivo studies demonstrating that TGF- β up-regulates CD16 on human monocytes (8, 9). The identification of elevated circulating levels of TGF- β in AIDS patients and its association with upregulation of Fc γ RIII may provide insight into the immunopathogenesis of AIDS.

Methods

Patients. This study included 22 males with AIDS and 7 asymptomatic, seropositive males. Of the patients with AIDS, 1 had only Kaposi's sarcoma, 20 had one or more opportunistic infections, and 1 had Kaposi's sarcoma, opportunistic infections, and bacterial infections. No patient was on immunomodulatory therapy at the time of study. Control subjects included 22 healthy, seronegative adults.

Peripheral blood samples. Sera were prepared from nonheparinized peripheral blood samples obtained from AIDS patients and control subjects. Plasma and mononuclear leukocytes (MNL) were separated from heparinized blood. The sera and plasma were centrifuged, aliquoted, and stored at -20°C . MNL isolated by Ficoll-Paque sedimentation were washed and suspended in Dulbecco's modified Eagle's medium (DME, Gibco Laboratories, Grand Island, NY) containing 50 $\mu\text{g}/\text{ml}$ gentamicin and 2 mM glutamine. Total MNL and separate monocyte and lymphocyte numbers were determined by size and phe-

1. Abbreviations used in this paper: ARC, AIDS-related complex; FMF, fluorescence microfluorometry; MNL, mononuclear leukocytes; PE, phycoerythrin; TGF- β , transforming growth factor- β .

notypic analysis. To obtain purified populations of normal monocytes ($> 95\%$ LeuM3 $^{+}$), leukapheresis preparations from healthy control subjects (National Institutes of Health Blood Bank) were separated by counterflow centrifugal elutriation (21).

Labeling of cell surface antigens. For analysis using dual-fluorescence microfluorometry (FMF), single-cell suspensions ($50\ \mu\text{l}$) of MNL or monocytes ($10 \times 10^6/\text{ml}$) in phosphate-buffered saline (PBS) supplemented with 0.1% sodium azide and 2% fetal calf serum (FCS) were incubated at 4°C for 30 min with phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies. Human AB serum (10%) was added to the cells 10 min before this incubation to prevent nonspecific binding to Fc receptors. Monoclonal antibodies included those directed against monocyte surface antigens, LeuM3, Mo2, LeuM9, MY9, and HLA-DR; the T cell antigen, Leu1; and the NK antigen, NKG-1 (Becton Dickinson, Mountain View, CA; and Coulter Immunology, Hialeah, FL). Fc γ R-specific monoclonal antibodies were 3G8 (Fc γ RIII, CD16), IV.3 (Fc γ RII, CDw32), and 32.2 (Fc γ RI, CD64) (Medarex Inc., Lebanon, NH) (3). Nonspecific fluorescence was evaluated with the relevant PE/FITC-conjugated IgG $_1$ and IgG $_2$ subclasses (Simultest, Becton Dickinson). After staining, the cells were washed twice in buffer without FCS and immediately resuspended in 0.5 ml 2% paraformaldehyde and stored at 4°C until analysis by FMF using a FACStarPLUS $^{\circ}$ flow cytometer (Becton-Dickinson) with a single excitation source (200 mW argon ion laser) (8). An analysis gate was set to include cells with the forward- and side-scatter characteristics of monocytes.

Northern blot analysis of monocyte mRNA. Total RNA was extracted by a one-step acid guanidine thiocyanate phenol-chloroform extraction method (22) from 5 – 10×10^6 monocytes obtained from peripheral blood MNL of patients or normal individuals that had been adhered for 1.5 h and washed to remove nonadherent cells. RNA ($5\ \mu\text{g}$) was electrophoresed in formaldehyde-containing agarose gels, transferred to nitrocellulose filters, and hybridized 18 h with a ^{32}P -labeled CD16 cDNA probe consisting of a 1-kb Xba I fragment of the pCD16 clone (kindly provided by Dr. Brian Seed, Massachusetts General Hospital, Boston, MA) (23). The filters were then exposed to Kodak XAR-5 film with intensifier screens at -70°C . The ethidium bromide staining pattern of the 28S and 18S ribosomal RNA bands indicated equivalent amounts of RNA loaded in each lane, and hybridization to the constitutively expressed He7 probe (24) served as the internal control.

Incubation of monocytes with serum and plasma. Purified monocyte populations obtained from control subjects ($> 95\%$ LeuM3 positive) were suspended in DME ($5 \times 10^6/\text{ml}$) with 0–20% serum or plasma from patients and controls and cultured for 24 h at 37°C in 5% CO_2 . After the incubation, monocytes were washed, incubated with FITC-conjugated monoclonal antibodies, and analyzed by FMF. In additional experiments, plasma samples ($100\ \mu\text{l}$) were preincubated 0.5 hr with a neutralizing antibody to TGF- β 1 ($140\ \mu\text{g}$; R & D Systems, Minneapolis, MN) or with an equivalent concentration of nonimmune rabbit IgG (Sigma Chemical Co., St. Louis, MO) before addition to the monocyte cultures.

Measurement of TGF- β bioactivity in AIDS plasma. TGF- β was quantitated by its ability to inhibit IL-1-dependent thymocyte proliferation (25, 26). Serial dilutions of plasma in parallel with a purified human platelet-derived TGF- β 1 standard (Collaborative Research, Boston, MA) were added to thymocytes from 4–8-wk-old female C3H/HeJ mice in the presence of suboptimal concentrations of phytohemagglutinin ($1\ \mu\text{g}/\text{ml}$) (Burroughs Wellcome Co., Greenville, NC) and IL-1 at $100\ \text{U}/\text{ml}$ (Genzyme Corp., Boston, MA). Before assay, plasma samples were untreated or adjusted to $\text{pH} \leq 3.0$ with 6 N HCl for 10–30 min, neutralized with 6 N NaOH/1 M Hepes, and diluted fourfold with culture media (26). After 72 h, the thymocyte cultures were pulsed with $0.5\ \mu\text{Ci}$ per well tritiated thymidine (specific activity $1.9\ \text{Ci}/\text{mM}$; Schwarz-Mann, Orangeburg, NY) and harvested, and incorporation of tritiated thymidine was determined. TGF- β concentrations (ng/ml) were determined by comparison to the TGF- β standard curve in which the TGF- β IC_{50} is $\sim 0.05\ \text{ng}/\text{ml}$ (26).

Statistical analysis. Results are presented as mean \pm SE. Statistical significance was determined using Student's t test and $P < 0.05$ was considered statistically significant. Correlations between two variables were determined by Pearson's correlation coefficient.

Results

Fc γ RIII expression on circulating monocytes from AIDS patients. Freshly isolated MNL from individuals with AIDS and normal subjects were incubated with FITC-conjugated MAb 3G8 in parallel with an FITC-IgG control. Fluorescence analysis performed on monocytes electronically gated on the basis of their light scatter patterns revealed a marked difference in the number of MNL staining positive with FITC-3G8 (Fc γ RIII, CD16 $^{+}$). As shown in Fig. 1, cells with the characteristics of monocytes from three patients with AIDS (A1–3) expressed substantial levels of cell surface CD16. In contrast, minimal numbers of monocytes from normal individuals (C1–3) were CD16 $^{+}$. Parallel analysis of CD64 (Fc γ RI) and CDw32 (Fc γ RII) revealed no enhancement in the number of positive cells nor in the density of staining (receptor number) between AIDS and control monocytes. On the contrary, Fc γ RII, and Fc γ RI in particular, often were reduced in number, rather than enhanced (data not shown).

Dual-fluorescence analysis of Fc γ RIII and monocyte antigens. Since Fc γ RIII are also found on neutrophils and natural killer cells (27, 28), CD16 $^{+}$ cells were further characterized by dual-color fluorescence using a battery of monocyte-specific PE-conjugated MAb (red) in combination with FITC-3G8 (green). The majority of cells expressing CD16 in the MNL population co-expressed markers consistent with cells of the monocytic lineage. Shown in Fig. 2 (top) are contour plots of dual-color-labeled MNL from a representative AIDS patient. In these populations, CD16 $^{+}$ cells co-expressed LeuM3, LeuM9, MY9, Mo2, and CD64, all of which are monocyte antigens. In a parallel dual-color analysis of MNL from a representative control subject (Fig. 2, bottom), the number of CD16 $^{+}$ cells which co-stained with any of the monocyte specific antigens never exceeded 10% of the population. For example, all of the cells in the patient population that expressed CD16 $^{+}$ (MAb 3G8) were also positive for CD64 (MAb 32.2), which is found only on monocytes and macrophages, whereas the comparable CD64 $^{+}$ population of the control subject was $< 3\%$ CD16 $^{+}$. A similar pattern was observed for the co-expression of a battery of additional monocyte-specific markers (Fig. 2). Although NKH-1 $^{+}$ cells also express CD16, contamination with these cells was routinely $< 10\%$ and, moreover, these cells did not co-express monocyte-specific antigens. Additionally, the CD16 $^{+}$, CD14 $^{+}$ cells exhibited morphology, phagocytic activity, and superoxide anion production consistent with cells of monocytic lineage.

Using LeuM3 as a representative monocyte-specific antigen, monocytes from 29 individuals with AIDS and ARC and 22 normal controls were subsequently compared for CD16 expression. A significantly higher percentage of LeuM3 $^{+}$ cells from AIDS and ARC patients were CD16 $^{+}$ when compared to healthy donors ($38.5 \pm 3.2\%$ for AIDS vs. $10.4 \pm 1.0\%$ for controls; $P < 0.0005$) (Fig. 3). A similar incidence of CD16 $^{+}$, CD14 $^{+}$ monocytes in the circulation of control subjects has recently been demonstrated in other studies (6). Since both ARC and AIDS patients exhibited CD16 $^{+}$ monocytes, no di-

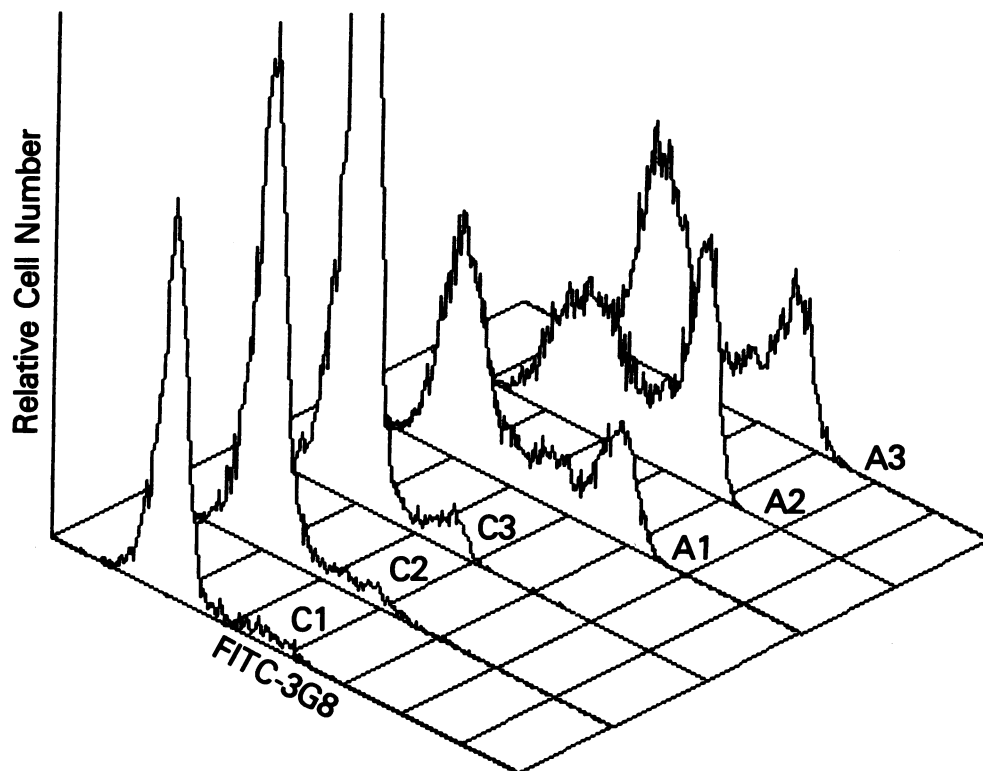


Figure 1. Expression of surface CD16 (Fc γ RIII) on monocytes from AIDS patients. MNL isolated from blood of three control and three AIDS patients were labeled with FITC-3G8. After labeling, cells were washed, fixed in 2% paraformaldehyde and analyzed by FMF with a FACStar Plus[®].

rect correlation was apparent between CD16 levels and clinical status of the corresponding patients.

CD16 gene expression. MNL were further analyzed for the presence of CD16-specific mRNA. Peripheral blood monocytes from four AIDS patients and four control donors were isolated by adherence for 1–2 h. Total cellular RNA was then extracted and analyzed by Northern hybridization with the CD16 cDNA probe (23). As shown in Fig. 4, monocytes from normal subjects (C1–4) expressed little or no CD16-specific mRNA, whereas mRNA from equivalent numbers of AIDS patients' monocytes (A1–4) was CD16 positive. Reprobing the filters for constitutively expressed He7 revealed similar levels in all eight lanes (not shown). These data are consistent with previous observations that monocytes from healthy controls express minimal or no CD16-specific mRNA (8), whereas the monocytes from AIDS patients contain elevated levels of CD16 steady-state mRNA.

Induction of CD16 on normal monocytes by AIDS serum and plasma. To identify potential mechanisms whereby monocytes from HIV-1-infected patients might be induced to express increased CD16 levels, serum and plasma from these patients were evaluated for soluble factors that could influence monocyte CD16 expression. Monocytes from normal individuals were incubated with sera (10–20% in DME) from patients and control subjects and analyzed by FMF for the appearance of CD16. After a 24-h incubation in the presence of serum from AIDS patients, significantly increased levels of CD16 appeared on the control monocytes (Fig. 5). In contrast, sera from control subjects did not significantly augment CD16 staining. Subsequent experiments with plasma (to rule out the potential contribution of factors released during the clotting process) revealed that plasma samples from AIDS patients also induced

monocytes to express CD16 (Fig. 5). Based on these data, it appears that a soluble factor(s) in the blood of AIDS patients can induce monocyte expression of CD16.

Quantitation of TGF- β in plasma of AIDS patients. Since TGF- β has recently been shown to be capable of modulating monocyte CD16 expression in vitro and in vivo (8, 9), additional studies addressed a potential role for this polypeptide hormone in the constitutive expression of CD16 on peripheral blood monocytes in AIDS patients. Plasma samples were assayed for TGF- β bioactivity before and after acid activation (26), since TGF- β in serum is normally bound to α_2 -macroglobulin and biologically inactive (29). Plasma from patients contained detectable TGF- β bioactivity even without acid activation, but upon activation, the levels were dramatically increased (Fig. 6, top). Thus, it appears that patients with AIDS and/or ARC may have circulating levels of TGF- β , a proportion of which is in the active form and furthermore, the levels appear sufficient for induction of CD16 on monocytes (8, 9). After having quantitated TGF- β levels in the sera of patients with AIDS, we attempted to correlate the levels of TGF- β with the ability of the serum samples to induce CD16 on control monocytes and with the incidence of CD16⁺ cells found in the blood of individual subjects. As shown in Fig. 6 (bottom), there was a direct correlation between quantifiable TGF- β levels in control and patient sera and CD16 induction on control monocytes ($r = 0.74$, $P < 0.001$). Furthermore, a positive correlation existed ($r = 0.55$, $P \leq 0.01$) between serum TGF- β levels and the incidence of CD16⁺ cells in the circulation of both patients and controls.

Inhibition of plasma-induced CD16 expression by neutralizing antibody to TGF- β . Because the data suggested that TGF- β in AIDS patients' blood might influence CD16 expression on

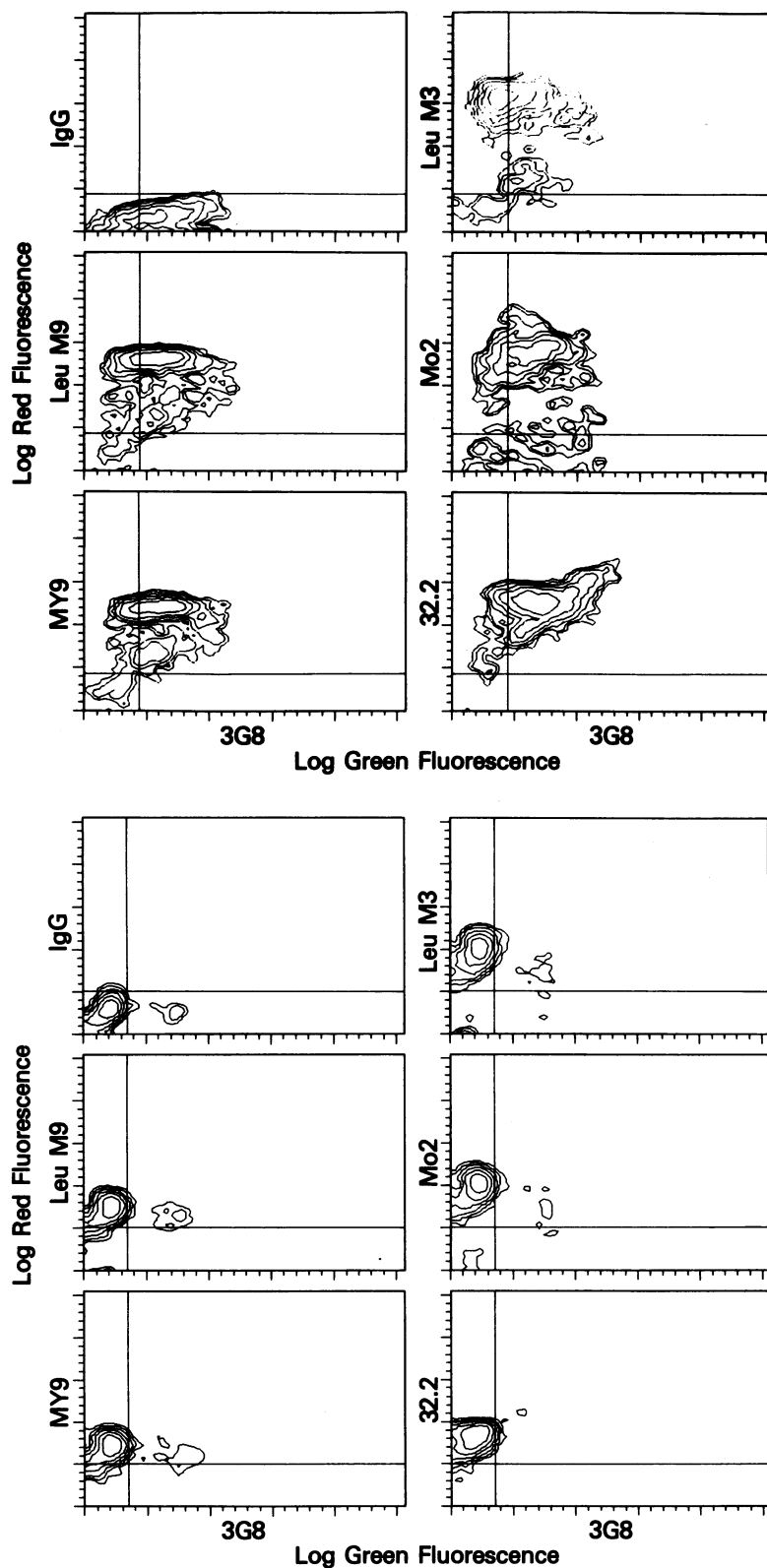


Figure 2. Dual-color fluorescence profiles of CD16 and monocyte antigens. MNL were double-labeled with FITC-3G8 and PE-conjugated antibodies for monocyte antigens or with FITC-IgG₁ and PE-IgG₂ for controls. Shown are contour plots for monocyte populations from a representative AIDS patient (*top*) and control subject (*bottom*).

monocytes, plasma samples were pretreated with a neutralizing antibody to TGF- β before being added to normal monocytes. After 24 h, the cells were analyzed for CD16 expression. As already demonstrated, plasma from AIDS patients induced a

significant increase in CD16⁺ monocytes (40–50%) compared to plasma from healthy controls (< 10%) (Fig. 7). However, if these plasma samples were preincubated with anti-TGF- β 1, the increase in CD16⁺ monocytes was blocked. This did not

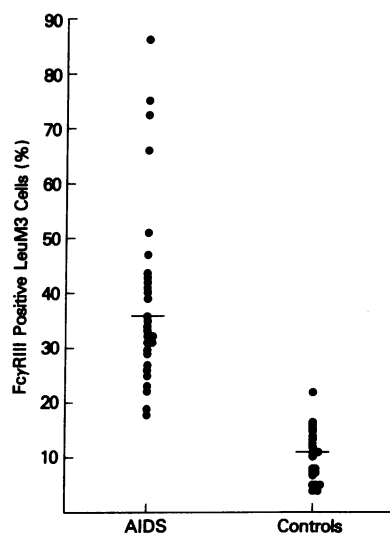


Figure 3. CD16 expression on monocytes from AIDS patients and controls. Cells were labeled and analyzed as in Figs. 1 and 2. Data represent the percent LeuM3⁺ cells that are also CD16 positive for 22 control and 29 AIDS/ARC subjects.

occur when plasma samples were incubated with preimmune IgG. For comparison, induction of monocyte CD16 by recombinant TGF- β was also blocked with the antibody (Fig. 7). Taken together, these data implicate TGF- β in the blood of AIDS patients as a mediator of CD16 expression on circulating monocytes.

Discussion

In this study, we report that freshly isolated peripheral blood monocytes from HIV-1-infected individuals express the gene for FcγRIII (CD16) and exhibit cell surface CD16. Moreover, a soluble factor in the blood of these patients induced CD16 expression on monocytes from seronegative control individuals. Inhibition of the AIDS plasma-derived CD16-inducing

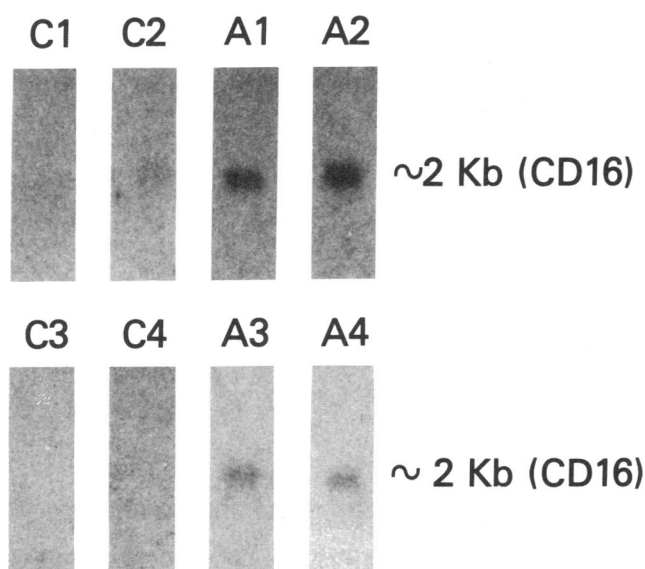


Figure 4. Northern hybridization for monocyte CD16 mRNA. Total RNA was extracted from 10^7 adherent MNL from four AIDS patients (A1, A2, A3, A4) and four controls (C1, C2, C3, C4) and hybridized with a ³²P-labeled CD16 cDNA probe.

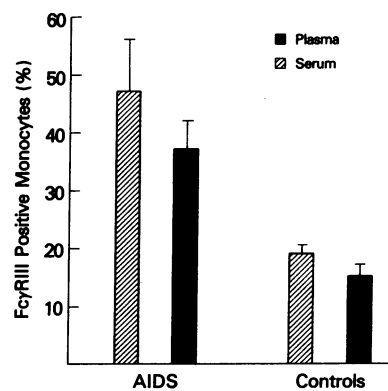


Figure 5. Augmentation of CD16 by a plasma/serum factor from AIDS patients. Purified monocytes (> 95% LeuM3⁺) from normal individuals were incubated with sera ($n = 7$) or plasma ($n = 8$) (20%) from AIDS patients or from control subjects for 24 h. The cells were then labeled with FITC-3G8 and analyzed by FMF for CD16 expression. Control mono-

cytes incubated with DME only were 12.4% FcγRIII positive. Data represent the mean \pm 1 SEM.

activity with a neutralizing antibody to TGF- β implicated this polypeptide factor in the induction of monocyte CD16 expression. As previously shown in vivo and in vitro, TGF- β is a potent mediator of CD16 expression on a significant population, although not all, peripheral blood monocytes (8, 9). Whether a subpopulation of monocytes is particularly susceptible to TGF- β up-regulation of CD16 is unclear, although by

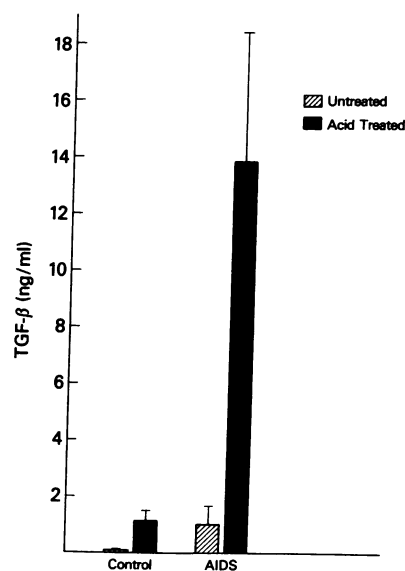
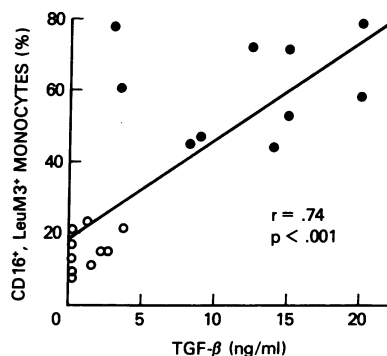


Figure 6. Quantitation of TGF- β bioactivity in serum and correlation with CD16 induction. (Top) Plasma samples from control subjects and AIDS patients were not treated or treated with 6 N HCl and neutralized before assay for TGF- β in the thymocyte proliferation inhibition assay. Data represent mean \pm 1 SEM of plasma samples from 10 controls and 12 AIDS patients. (Bottom) Analysis of correlation between TGF- β levels in serum of control subjects (\circ) and AIDS patients (\bullet) and induction of CD16 on normal monocytes. The correlation coefficient (r) between these two variables equals 0.74 ($P < 0.001$).



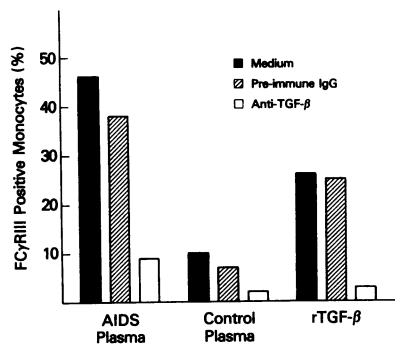


Figure 7. Inhibition of plasma-induced CD16 expression by anti-TGF- β . Plasma and sera samples from control subjects ($n = 4$) and AIDS patients ($n = 4$), as well as rTGF- β (10 ng/ml), were pretreated with a neutralizing antibody to TGF- β . The samples were then added to purified control monocytes. After

24 h, the monocytes were washed, labeled with FITC-3G8, and analyzed by FCM. Data represent the mean of four different samples.

phenotypic analysis (Fig. 2), the CD16⁺ monocytes express common monocyte-specific antigens.

The expression of Fc γ R on the surface of monocytes and other immune cells may play a critical role in the immunopathogenesis of HIV infection. First, several laboratories have recently demonstrated the existence of HIV-specific antibodies that enhance the infectivity of HIV-1 for both T cells and monocytes (10–12). This enhancing effect could be blocked by incubating monocytes with monoclonal antibodies directed at Fc γ RIII but not Fc γ RI, Fc γ RII, or the receptor for complement component 3 (10). Taken together, these data suggest that Fc γ RIII on monocytes may mediate antibody-dependent enhancement of HIV infectivity. Whether this mechanism is independent of the CD4 protein, which is the normal route of HIV internalization, is unknown since both abrogation and no effect have been shown on the enhancement of infectivity after treatment with anti-CD4 (30, 31). Although it is possible that HIV-1 can be internalized directly through Fc γ RIII, it is also possible that IgG-sensitized viral particles binding to Fc γ RIII facilitate the HIV-1–CD4 interaction.

Secondly, additional studies have shown that monocyte functions mediated through Fc γ R are defective or abnormal in patients with AIDS (32–35). However, there appeared to be no correlation between functional defects and expression of these surface receptors. For example, a defect in ADCC as well as in the clearance of IgG-sensitized autologous erythrocytes from the circulation of patients with AIDS has been reported (33). Nevertheless, expression of the relevant Fc γ R was found to be similar in both normal individuals and HIV-infected patients (33–36). Elevated levels of Fc γ RIII (CD16) on AIDS monocyte populations as compared to control monocytes have not previously been reported, likely because the monocyte populations from controls and patients in earlier studies were maintained in culture, a procedure that induces differentiation and CD16 expression (7). Another possible explanation for this discrepancy in phenotype and function is that the defect may occur at the level of signal transduction or at some point later in the activation pathway. However, in a recent study, it has been reported that circulating LeuM3⁺/CD16⁺ monocytes are less, rather than more, phagocytic than LeuM3⁺/CD16[–] cells (6).

Receptor-mediated clearance of IgG-opsonized microorganisms and antigens is important in host defense (37, 38), and its fluctuation may be deleterious in certain disease states. Enhanced Fc γ R expression may, in fact, contribute to the patho-

genesis of certain diseases (36, 37–41). Immune complexes and/or mononuclear cell-derived cytokines reportedly stimulate Fc γ R synthesis by circulating monocytes (42). Among these cytokines, γ -interferon and granulocyte/macrophage colony-stimulating factor stimulate increased expression by human mononuclear phagocytes of Fc γ RI and Fc γ RII, respectively (43, 44). Furthermore, in addition to TGF- β (8, 9) Fc γ RIII expression on monocytic populations has also been shown to be up-regulated by granulocyte/macrophage colony-stimulating factor (45).

In addition to modulating Fc γ RIII expression, TGF- β is a potent inhibitor of immunologic functions in vitro and in vivo (25, 46–49), and it is possible that enhanced levels of TGF- β in patients with AIDS may also contribute to the immunosuppressed state of these individuals. T and B lymphocytes produce TGF- β when stimulated (50, 51), as do cells of monocyte lineage (52–54). Moreover, infection of peripheral blood monocytes with HIV-1 in vitro results in augmented expression of the gene encoding TGF- β (55) implicating this population as at least one potential source of the elevated TGF- β levels seen in these patients. Identification of elevated levels of this potent immunoregulatory cytokine in AIDS patients provides important insight and avenues of further investigation into the mechanisms of immune dysfunction in the acquired immunodeficiency syndrome.

Acknowledgments

The authors are grateful to Dr. L. M. Wahl (National Institutes of Health) for providing elutriated monocytes, Dr. H. M. Shepard (Genentech Inc., South San Francisco, CA) for recombinant TGF- β 1, and Dr. Brian Seed (Massachusetts General Hospital, Boston, MA) for the CD16 cDNA probe. The authors also thank Dr. Phillip Smith (National Institutes of Health) for patient care, Suanne Dougherty for expert assistance, and Mary Campbell for manuscript preparation.

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