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Expression of CD4 by human megakaryocytes*

(acquired immunodeficiency syndrome)

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ABSTRACT **The CD4 antigen, which serves as the receptor for human immunodeficiency virus type 1 (HIV-1) on T cells, has been detected on human megakaryocytes. Recent evidence of impaired thrombopoiesis in HIV-1-related thrombocytopenia suggested that these cells could be directly infected by the virus and prompted a search for a receptor on megakaryocytes of normal subjects that could permit entry of HIV-1. Bone marrow specimens from uninfected normal control subjects were centrifuged over Ficoll-Hypaque (1.077 g/ml) and analyzed by three-color analysis with a flow cytometer utilizing monoclonal antibodies against CD4 and a glycoprotein present on the surface of megakaryocytes and platelets (GPIIb/IIIa; CD41), as well as 7-aminoactinomycin D, a stain for DNA. Cells presumed to be megakaryocytes were identified by having a DNA content greater than tetraploid and staining brightly with anti-CD41. Approximately 0.4% of the nucleated cells of the marrow met these criteria. Twenty-five percent of these megakaryocytes stained as brightly as $CD4^+$ T cells. Several clones of antibody recognizing different epitopes of the CD4 molecule gave similar results. Platelets were $CD4^-$. Staining of megakaryocytes with anti-CD4 was confirmed by direct microscopic examination of Percoll-gradient-enriched megakaryocytes employing two-color (CD4–phycoerythrin and CD41–fluorescein) immunofluorescence analysis and phase-contrast microscopy. The proportion of double-labeled cells among 112 phase-contrast-identifiable megakaryocytes from five bone marrow specimens varied between 20% and 26% with a mean and SD of 22% ± 2.5%. Thus some human megakaryocytes express CD4 on their surface that should be capable of binding the HIV-1 gp120 envelope protein. This could serve as a portal of entry for HIV-1.**

The CD4 antigen is a monomeric cell surface glycoprotein. It was originally described as a differentiation antigen on thymocytes and a subset of peripheral T cells (1, 2) but its expression is not limited to these cells. The antigen has been detected on human monocytes and eosinophils (3–7), and murine CD4 has recently been shown to be present on multipotential hematopoietic stem cells (spleen colony-forming units) and myeloid precursors (8). In human cells CD4 is the receptor for human immunodeficiency virus type 1 (HIV-1) and specifically binds the viral envelope glycoprotein, gp120 (9–11). It is believed to mediate viral entry into T-lineage cells and monocytes (3–6). We have examined human megakaryocytes for the presence of this antigen and found CD4 on a substantial fraction of these cells. The CD4 on megakaryocytes provides a potential portal of entry for HIV-1 and suggests that these cells could be infected directly.

Thrombocytopenia is a common feature of HIV-1 infection (12–14). It can appear before signs of immunologic debilitation (12). Because this thrombocytopenia usually responds to steroid treatment and/or splenectomy (14–16) and is associated with the presence of immunoglobulins on the surface of platelets (12–14, 17), it has been assumed to be an immunologic disorder in which antibody-coated peripheral blood platelets are destroyed by the reticuloendothelial system (18). However, recent platelet kinetic studies in several HIV-1-related thrombocytopenia patients have revealed normal to decreased platelet turnover in addition to shortened platelet survival (19, 20), suggesting impaired thrombopoiesis as well. In addition, we (21) and others (22) have observed that HIV-1-related thrombocytopenia patients respond promptly to treatment with azidodeoxythymidine (AZT) with a clinical response (as early as 1 week) preceding any possible change in antibody level. This suggests either that AZT impairs reticuloendothelial function or that it has a direct effect on megakaryocytes whose function has been impaired by HIV-1 infection. If the latter is true, a mechanism for viral entry into the megakaryocytes should be identifiable.

MATERIALS AND METHODS

Marrow Preparation. Bone marrow specimens were obtained from discarded orthopedic surgical specimens or discarded marrow aspirates collected for routine hematological diagnostic purposes. A single-cell suspension was prepared from surgical specimens by gently pipetting the tissue up and down in CATCH medium (23), using a 30-ml syringe fitted with an 18 gauge needle. The medium consists of calcium- and magnesium-free Hanks' balanced salt solution containing 1 mM adenosine, 2 mM theophylline, 3.8% sodium citrate, bovine serum albumin at 35 μg/ml, DNase at 25 units/ml, and 25 mM Hepes buffer, pH 7.0. All of the reagents were purchased from Sigma. Marrow aspirates were collected in heparinized tubes and diluted 1:3 with CATCH buffer. Only aspirates from patients with normal bone marrow smears were used. These samples were underlaid with an equal volume of Ficoll-Hypaque, 1.077 g/ml (Sigma), and centrifuged at 2000 × *g* for 20 min at room temperature (24). The cells accumulating at the interface were isolated, washed, and resuspended at 2×10^7 per ml in CATCH medium and stained with the appropriate antibodies (see below). For phase-contrast and fluorescence microscopic examination, the preparation was further enriched by using a Percoll (Pharmacia) separation (23). The suspension of stained cells was underlaid with an equal volume of 38% isosmotic Percoll in CATCH medium and centrifuged at 2000 × *g* at 4°C for 20 min. Cells at the interface were again isolated and deposited on a clean glass slide by using a Cytocentrifuge (Shandon Scientific, London). An aqueous mounting medium (Aquapolymount; Polysciences) was added and the slide was immediately covered with a coverslip.

Platelets were stained either in unfractionated whole blood or after isolation from platelet-rich plasma by gel filtration on

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Abbreviations: HIV-1, human immunodeficiency virus type 1; PE, phycoerythrin; FL, fluorescein; 7-AAD, 7-aminoactinomycin D.

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a Sepharose 2B column. They were stained with fluorochrome-conjugated anti-CD4 and/or CD42.

Antibodies. DEK-10 and DEK-16 are IgG1-κ mouse monoclonal antibodies, raised against purified glycoprotein IIIa (GPIIIa), with reported specificity for GPIIIa (25). Flow microfluorometric analysis has revealed additional reactivity of lesser intensity with cells that, on the basis of light-scattering properties, appear to be monocytes or immature myeloid cells (data not shown). Staining of monocytes by monoclonal antibodies to GPIIb/GPIIIa has been reported by others (26–28). A directly fluoresceinated monoclonal antibody with specificity for the GPIIb/IIIa complex (CD41, clone P2, mouse IgG1) was purchased from AMAC (Westbrook, ME). This antibody also appears to react with other bone marrow cells in addition to platelets and megakaryocytes. Anti-CD4 (Leu3a, clone SK3, mouse IgG1) coupled to phycoerythrin (PE) was purchased from Becton Dickinson. Anti-CD4 (clone 13B82, mouse IgG1) coupled to fluorescein (FL) was purchased from AMAC. Another mouse monoclonal anti-CD4 [OKT4 (IgG2b)] was a gift of Gideon Goldstein (Immunobiological Research Institute, Annandale, NJ). Anti-CD42 (GPIb, clone SZ2) coupled to FL was purchased from AMAC. Controls included anti-murine Thy-1.2 (IgG2b), purchased from Caltag (South San Francisco, CA), MOPC21 (IgG1-κ) purchased from Cappel Laboratories and coupled to FL by using dichlorotriazinylaminofluorescein (29), and PE coupled to mouse IgG1, purchased from Becton Dickinson. Goat anti-mouse IgG coupled to PE was purchased from Fisher Biotech (Orangeberg, NY) and absorbed extensively with immobilized normal human immunoglobulin. An FL-labeled $F(ab')_2$ fragment of goat anti-mouse IgG, purchased from Caltag, was also employed after absorption with immobilized normal human immunoglobulin.

Immunofluorescence. Immunofluorescence analysis was performed as described previously (30). All antibodies were used at saturating concentration. For two-color indirect immunofluorescence, the cells were first treated with the unlabeled anti-GPIIIa, washed, and stained with a PE-conjugated goat anti-mouse IgG. When appropriate, control samples were stained with an irrelevant monoclonal antibody (anti-mouse Thy-1.2 or mouse IgG1). The samples were then incubated with normal mouse IgG (1 mg/ml) to block the subsequent binding of the fluoresceinated anti-CD4 or control antibody to unoccupied binding sites of the anti-mouse IgG on the cell surface. The blocked samples were then incubated with either FL-conjugated anti-CD4 antibody or control immunoglobulins, washed, and fixed in 2.0% paraformaldehyde in 0.15 M phosphate-buffered saline (PBS).

For microscopy, the cells were examined in a Leitz Ortholux microscope using epi-illumination with a mercury lamp. The samples were illuminated using a 530- to 560-nm band-pass filter for excitation and a 580-nm long pass filter for the emitted light to detect PE-labeled cells and a 450- to 490-nm band-pass filter for excitation and a 525 ± 20-nm narrow-band-pass filter for the emitted light to detect FL-labeled cells.

DNA Staining. Propidium iodide was used to identify polyploid cells which had been stained for GPIIIa with fluoresceinated antibodies (31). When two-color immunofluorescence was performed, the DNA-binding dye 7-aminoactinomycin D (7-AAD) was used (32). Both dyes were used in a final concentration of 25 μg/ml and were incubated with the cells overnight before analysis. The quality of the DNA histogram obtained with 7-AAD is poorer than that obtained with propidium iodide since the binding of the dye is strongly influenced by chromosomal structure. However, because of the spectral properties of 7-AAD it can be used with two-color (FL/PE) immunofluorescence. The light-scattering properties of the immunofluorescent stained cell were preserved by fixation in 2.0% paraformaldehyde overnight. The cells were permeabilized by incubation with lysolecithin at 10 μg/ml for 20 min and then incubated with the DNA-binding dyes (33).

Flow Cytometry. Cell surface fluorescence was quantified by using a Becton Dickinson FACScan cytometer equipped with a 15-mW argon laser emitting at 488 nm. Viable cells were gated on the basis of both forward and 90° light scatter. PE and 7-AAD fluorescence were detected by sequential dichroic mirrors, reflecting light of wavelengths longer than 560 nm first and then light of wavelengths longer than 640 nm. FL emission was measured by filtering the light passed by the first dichroic mirror through a narrow-band green filter (530 ± 10 nm). Electronic compensation was used to eliminate spectral overlap between FL and PE and between PE and 7-AAD. List mode data were accumulated and analyzed subsequently. To accumulate sufficient events for the analysis of megakaryocytes, each sample was acquired twice. In the first acquisition, data from the entire bone marrow were analyzed, while in the second acquisition the analysis was "live gated" for cells with DNA contents equal to or greater than $4N$ (N = haploid DNA content). To accommodate the broad spectrum of DNA content found among cells of the megakaryocyte lineage, the DNA fluorescence was accumulated in logarithmic mode.

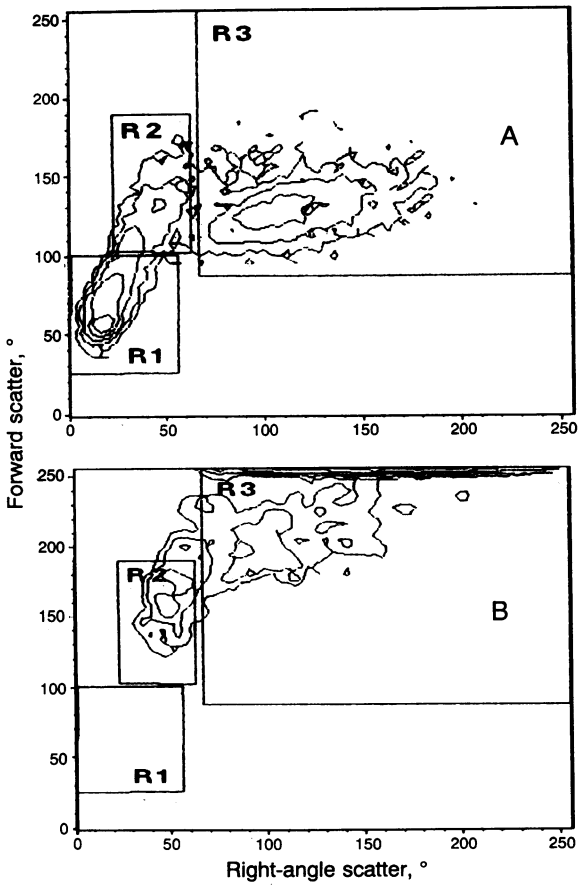

FIG. 1. Correlation between forward and right-angle light scattering of low-density human bone marrow cells. (*A*) Total low-density bone marrow cells. The pattern shown was obtained from all cells recovered from the interface of the Ficoll-Hypaque gradient. The three populations designated R1 (lymphoid), R2 (precursors or blasts), and R3 (myeloid) correspond to those described by Loken *et al.* (34). These were used for reanalysis of the list mode data to correlate immunofluorescence and DNA content with light scattering. (*B*) Hyperdiploid human bone marrow cells. The regions are the same as in *A*. Although a small number of the cells included in this figure may be normal diploid cells in the G_2 phase of the cell cycle, most of the cells are polyploid and very large and have a great deal of right-angle scatter.

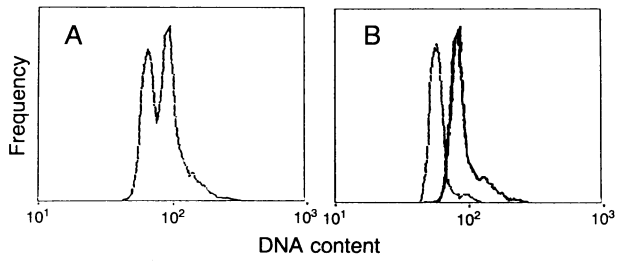

FIG. 2. DNA content (7-AAD binding) of low-density human bone marrow cells. (*A*) Ungated analysis. The pattern of staining obtained by analyzing the total population shown in Fig. 1*A*. (*B*) Gated analysis. Histograms obtained when the analysis is restricted to either the large cells (R2 and R3 of Fig. 1*A*) or the small cells (R1 of Fig. 1*A*). The pattern obtained with the large cells is shown by the darker tracing in the histogram on the right.

RESULTS

Megakaryocytes can be distinguished from other cells of the marrow on the basis of their size, expression of GPIIb/GPIIIa, and DNA content. Fig. 1*A* shows the scatter pattern obtained when normal human bone marrow is examined. Three distinct cell populations are apparent. These have been termed "lymphoid" [small cells with little right-angle scatter (region 1)], "precursors" [intermediate-size cells with little right-angle scatter (region 2)], and "myeloid" [large cells with high right-angle scatter (region 3)] by Loken *et al.* (34). When the DNA of the marrow cells is stained with the DNA fluorochrome 7-AAD, the pattern obtained is shown in Fig. 2*A*. The binding of 7-AAD is not uniform among all cell types and therefore produces a complex staining pattern. However, if the small cells of region 1 are electronically separated, the DNA content of the small and large cells can be analyzed separately, producing the patterns in Fig. 2*B*. Of the "large" cells (regions 2 and 3), 1.55% have a DNA content equal to or greater than 4*N*. The scatter pattern of such a population is shown in Fig. 1*B*. These cells make up 0.55% of the low-density bone marrow cells (density < 1.077 g/ml) and thus make up 0.4% of the total bone marrow, which is within the range reported by others (35–37). When propidium iodide was substituted for 7-AAD, 0.41% of the bone marrow cells recovered from the Ficoll-Hypaque gradient had apparent DNA contents equal to a greater than 4*N*.

Table 1. Proportion of $CD41^+$ cells that are $CD4^+$

DNA content	% positive	
	Bright	Total
>4*N*	19.9	25.3
>8*N*	8.9	18.1
>16*N*	15.5	21.4

Bright $CD4^+$ cells are defined as those whose total fluorescence intensity is equal to or greater than that of peripheral T cells. This is the gate used to define $CD4^+$ cells in Fig. 4. Because of differences in cell size, cells with considerably different concentrations of antigen per unit surface may have the same total antigen content. The total $CD4^+$ population is defined as those cells with a fluorescence intensity greater than that of 95% of the similar cells stained with a an isotype-matched irrelevant control antibody. The difference between the two columns (bright and total) represents dimly $CD4^+$ cells, which have not been further characterized.

By employing monoclonal antibodies to the GPIIb/GPIIIa complex, it is possible to gate on a population of cells which express these glycoproteins on their surface and have a DNA content greater than 4*N*. The two-color immunofluorescent staining of bone marrow cells with PE-labeled antibodies to CD4, and FL-labeled antibodies to CD41 is shown in Fig. 3. Fig. 3*A* shows the two-color staining with the antibodies, while Fig. 3*B* shows the staining with an isotype-matched control reagent. Cells in the region designated I of Fig. 3 *A.1*, *A.2*, and *A.3* are stained only by anti-CD4 and are therefore probably T cells; those in region IV are stained only by anti-CD41, while those in region II stain positive for both antigens. Polyploid cells with these dual-staining properties have not been previously reported, to our knowledge. The staining at various ploidy levels is shown (Fig. 3 *A.1*, *A.2*, *A.3*, etc.). The gate used to identify $CD4^+$ cells was set with peripheral blood T cells. Therefore only brightly staining $CD4^+$ cells are included. Normal bone marrow contains some brightly autofluorescent cells. These cells are spuriously scored as having high 7-AAD fluorescence and emit light at the same wavelengths as do FL and PE. They are obvious in region II of Fig. 3*B*, which shows the staining with a PE-labeled isotype-matched control. The contributions of these cells must be subtracted to calculate the actual number of $CD4^+$ cells in each region. These values are presented in Table 1. Similar results were obtained with both clones of anti-CD4 monoclonal antibodies as well as when the fluorochromes were reversed (FL-labeled anti-CD4 and

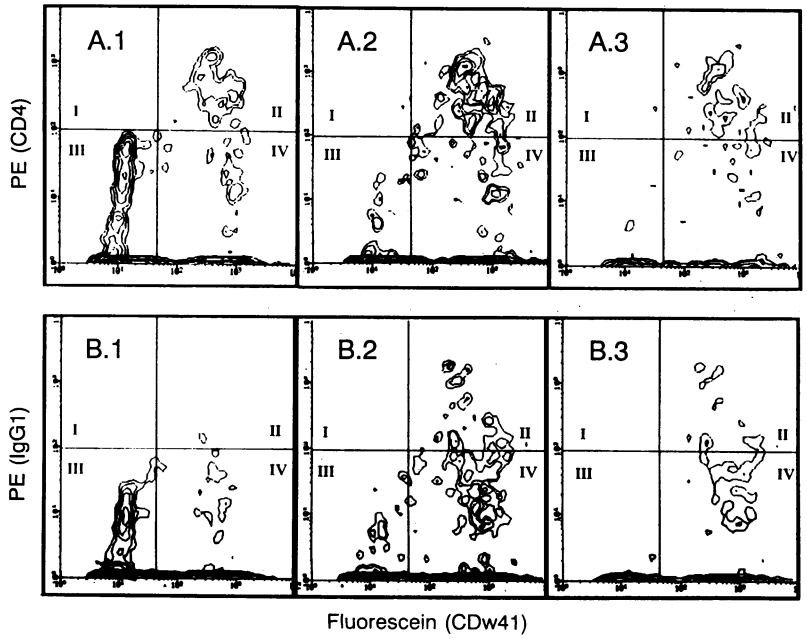

FIG. 3. Immunofluorescence analysis of bone marrow cells stained for CD4 (PE) and CD41 (FL). Both axes are logarithmic. The quadrants used to identify antigen-positive cells (described in text) are identical in all six panels. The gate defining $CD41^+$ cells was set with an isotype-matched (IgG1) fluoresceinated irrelevant immunoglobulin (data not shown). The gate defining $CD4^+$ cells excludes dimly staining cells. (*A*) Staining with anti-CD4 (PE) and anti-CD41 (FL). (*A.1*) Cell DNA content ≥ 4*N* (2829 cells). (*A.2*) Cell DNA content ≥ 8*N* (1040 cells). (*A.3*) Cell DNA content ≥ 16*N* (491 cells). (*B*) Staining with anti-IgG1a (PE) and anti-CD41 (FL). (*B.1*) Cell DNA content ≥ 4*N* (3185 cells). (*B.2*) Cell DNA content ≥ 8*N* (1217 cells). (*B.3*) Cell DNA content ≥ 16*N* (686 cells).

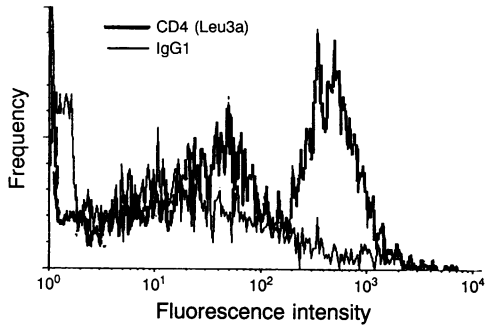

FIG. 4. Histogram of the staining of hyperdiploid large bone marrow cells with anti-CD4. Bone marrow cells stained with PE-labeled Leu3a were fixed with paraformaldehyde, permeabilized with lysolecithin, and stained with 7-AAD. The analysis was gated on regions 2 and 3 of Fig. 1 and on cells with DNA content >4*N*.

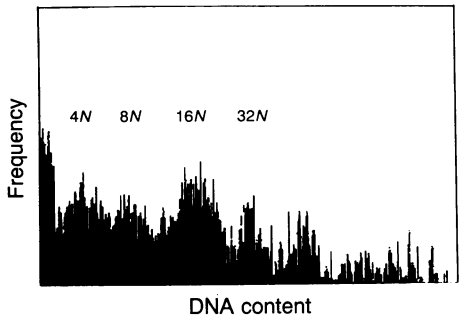

FIG. 5. DNA content (7-AAD binding) of low-density $CD4^+/CD41^+$ hyperdiploid human bone marrow cells. List mode data on 17,000 cells that had a DNA content of 4*N* or more were accumulated. They were then analyzed to show the DNA distribution of cells expressing both CD4 and CD41 (cells which fell into a region equivalent to region II of Fig. 3). The data are again shown on a logarithmic *x*-axis to accommodate the wide range of DNA content found in these cells.

GPIIIa detected with DEK-10/DEK-16 and PE-labeled anti-mouse IgG), as well as when staining with unconjugated OKT4 (detected with PE-labeled anti-mouse IgG and compared with an isotype-matched anti-Thy-1) in combination with directly fluoresceinated monoclonal antibody to GPIIb/IIIa (CD41) was substituted for the indirect procedure (data not shown). The extent and specificity of the staining of hyperdiploid cells is further illustrated in the histogram shown in Fig. 4. The staining of the large cells with >4*N* DNA content by PE-labeled anti-CD4 compared to the staining of an isotype-matched control is shown. The pattern resembles the anti-CD4 staining shown in the *y*-axis of Fig. 3 *A.2* and *B.2*. Approximately 15% of the cells with an apparent DNA content >4*N* are $CD4^+$ (bright). Since two-thirds of the cells with an apparent DNA content >4*N* are $GPIIIa^+$ and essentially all of the $CD4^+$ hyperdiploid cells are also $GPIIIa^+$, it appears at least one-quarter of the megakaryocytes express significant amounts of the receptor for HIV-1 on their surface.

The pattern of DNA staining of the double-positive population, as identified by flow cytometry, is shown in Fig. 5. The majority of these cells appear to have DNA ploidy in the range of 8–16 *N* with discrete clusters visible at 4, 8, 16, and 32 *N*, identifying them as putative megakaryocytes. The percentage of double-positive cells ($CD4^+/CD41^+$) in the higher ploidy megakaryocyte population was not significantly different than in the entire population. Platelets analyzed under the same conditions were uniformly $CDw42^+$ but $CD4^-$.

To confirm the presence of $CD4^+$ megakaryocytes microscopically, bone marrow aspirates were enriched for megakaryocytes as described in *Materials and Methods* and the resultant preparations were examined by phase-contrast and fluorescence microscopy. Large megakaryocytes are clearly recognizable. Fig. 6 shows photomicrographs of a density gradient-enriched megakaryocyte preparation stained for CD4 and GPIIb/IIIa. No staining was obtained with PE-conjugated anti-Thy-1.2. Bone marrow samples from five patients with normal marrows were examined by this procedure. All contained $CD4^+$ megakaryocytes. The proportion of double-labeled cells among 112 phase-contrast-identifiable megakaryocytes varied from 20% to 26% with a mean ± SD of 22% ± 2.5%.

DISCUSSION

CD4 is known to be present on T cells, monocytes, and eosinophils. In this study, we have shown that some megakaryocytes also bear this antigen. Almost a quarter of the cells identifiable as megakaryocytes by either flow cytometry or immunofluorescence microscopy have significant quantities of an antigen recognized by anti-CD4 monoclonal antibodies. Three different anti-CD4 reagents, recognizing different epitopes of the CD4 molecule, gave identical results, suggesting that the molecule being recognized was either CD4 or a closely related material. The latter possibility seems unlikely since multiple genetic or molecular forms of CD4 have not been reported. Direct microscopic examination of the $CD4^+/GPIIb/IIIa^+$ cells eliminated the possibility that the double-positives identified by flow cytometry were actually aggregates of a $CD4^+$ cell with a megakaryocyte. In addition, the ploidy distribution of the double-positives is characteristic of megakaryocytes and not random aggregates.

The function of this molecule in cells of the megakaryocyte lineage is unknown. A monomeric cell surface glycoprotein (38), CD4 was originally identified as a differentiation antigen of T-helper cells. On these cells, it serves both as an acces-

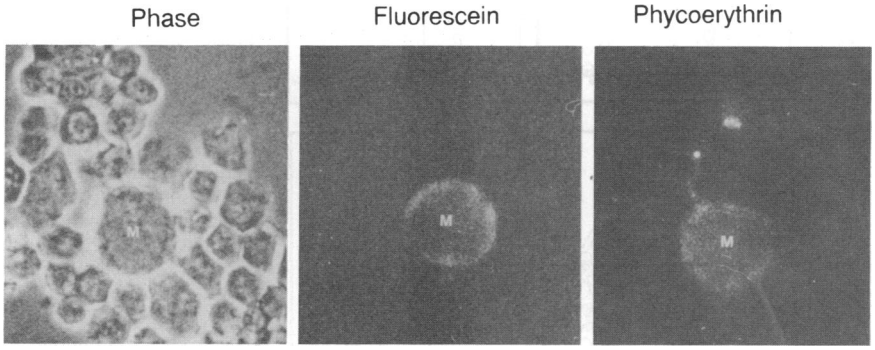

FIG. 6. Double staining of a human megakaryocyte with anti-CD4 and anti-CD41. The three panels show the same megakaryocyte (M). (*Left*) Phase-contrast photomicrograph. (*Center*) Green fluorescent staining of anti-CD41-FL. (*Right*) Red-orange fluorescence of anti-CD4-PE. (×630.)

sory molecule for the T-cell receptor complex, interacting with major histocompatibility complex class II antigen on antigen-presenting cells (39), and as a signal transducer (40). The latter function is mediated by the formation of a noncovalent complex between the cytoplasmic domain of CD4 and a lymphocyte-specific tryosine-protein kinase ($p56^{lck}$) (40). The role of CD4 in signal transduction appears to be limited to cells of the T lineage. The kinase is lymphocyte specific, and tyrosine-protein kinase has not been detected in association with the CD4 expressed by cells outside the T lineage.

The absence of CD4 from platelets, despite its presence on the surface of megakaryocytes, provides further evidence of the precision with which the membranes are segregated during thrombopoiesis. The presence of the antigen on immature, antigen-unresponsive T cells and on immature hematopoietic cells has led to the suggestion that it may play some developmental role (8), perhaps by promoting physical contact between the developing cell and stromal cells producing one or another of the hematopoietic growth factors. A similar role is plausible for megakaryocyte development and maturation.

HIV-1 has been shown to be tropic for T-helper cells (9–11) and monocytes (3–6), both of which bear the CD4 receptor on their surface. The presence of CD4 on the surface of megakaryocytes provides a potential portal of entry for HIV-1 and could explain the impaired thrombopoiesis noted in many HIV-1-related thrombocytopenic patients. It also supports the recent report of megakaryocyte damage noted in HIV-1-infected patients, as well as the presence of HIV-1 mRNA in some of these megakaryocytes (41).

In general, two pathways have been demonstrated for HIV-1 to enter cells. The first and apparently predominant mechanism involves binding of the viral gp120 protein to the CD4 antigen on T cells, followed by membrane fusion (42–46). A second pathway has been proposed for the infection of $CD4^-$ cells. This involves Fc-receptor-mediated endocytosis of antibody-coated virions, which has been reported for several other viruses (45, 46). Since human megakaryocytes express Fc receptors (23), both mechanisms are possible, although Fc-receptor endocytosis has not been demonstrated.

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