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Megakaryocytes of human immunodeficiency virus-infected individuals express viral RNA

(acquired immunodeficiency syndrome marrow/platelets/thrombocytopenia/*in situ* hybridization)

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ABSTRACT **The pathogenesis of thrombocytopenia associated with human immunodeficiency virus (HIV) infection is not fully understood. Immune mechanisms provide a partial explanation but fail to account for a lack of compensatory megakaryocytosis, the rapid reversal after treatment with azidothymidine, and the ultrastructural aberrations seen in the megakaryocytes of patients with acquired immunodeficiency syndrome. Therefore, a direct effect of HIV on megakaryocytes was investigated. The bone marrow of HIV seropositive individuals was analyzed ultrastructurally, and the megakaryocytes of 10 thrombocytopenic patients were subjected to *in situ* hybridization with a HIV RNA probe. The structural aberrations in HIV megakaryocytes were distinct from those in HIV-negative immune thrombocytopenias, and the megakaryocytes of 10 of 10 patients examined by *in situ* hybridization unambiguously expressed viral RNA. Therefore, it is likely that direct infection of megakaryocytes with HIV-1 is one mechanism for the decrease in platelet production.**

Thrombocytopenia is a frequent accompaniment of infection with the human immunodeficiency virus (HIV). It may even occur in the absence of any other clinical manifestations of the acquired immunodeficiency syndrome (AIDS), and considerable time may elapse before other signs of the condition become apparent. Because the majority of such patients have demonstrable platelet antibodies and/or immune complexes that may adsorb onto platelets, it was believed that immune mechanisms were responsible for low platelet counts (1–3). This explanation seems incomplete for several reasons. First, most patients with overt AIDS fail to have a compensatory megakaryocytosis, which is characteristic for other chronic immune thrombocytopenias (ITP). Second, in the course of electron microscopic studies of bone marrows obtained from HIV-infected individuals, we noted ultrastructural alterations in megakaryocytes that we had never seen before in patients with ITP or myeloproliferative diseases (4). These findings included remarkable blebbing of the peripheral zone and an unusually large number of denuded megakaryocyte nuclei (5). It also seemed surprising that treatment of patients with azidothymidine (AZT) improved their platelet counts within a few weeks—i.e., before such treatment could have any effect on the level of circulating antibodies (6, 7)—and that this drug improved platelet production more than platelet survival (8). Although virus particles have never been seen by us in freshly isolated marrow samples, it seemed likely that HIV affects megakaryocytes directly, for all the aforementioned reasons. We report here that the megakaryocytes obtained from 10 of 10 patients who were seropositive for antibody to HIV expressed HIV RNA when tested by *in situ* molecular hybridization with a HIV-1 RNA probe.

MATERIALS AND METHODS

Cell Preparation and *in Situ* Hybridization. Aliquots of bone marrow aspirates that were obtained from patients for diagnostic reasons were directly smeared onto microscope slides that had been coated with poly(L-lysine). All patients had AIDS and were thrombocytopenic at the time the samples were obtained. The specimens were fixed in ethanol/acetic acid, 3:1 (vol/vol) for 10 min and refrigerated for up to 1 month. The slides were scanned by phase microscopy, and 1-cm areas having at least two morphologically identifiable megakaryocytes were demarcated for the *in situ* hybridization experiments. The method used was as recommended by Harper *et al.* (9) with some modification (10). After the slides were dehydrated in 100% ethanol, they were immersed in 0.3% Triton X-100, rinsed twice, and acetylated in 0.1 M triethanolamine (pH 8.0) in acetic anhydride. This was followed by two washes in 50% formamide for 15 min at 37°C. Hybridization was accomplished by using a ^{35}S-labeled HIV RNA hybridization probe (DuPont NEP-200, Biotechnology Systems) in a hybridization mixture containing 1× Denhardt's solution (0.02% polyvinyl pyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 10% dextran sulfate, sperm DNA (1 mg/ml), and *Escherichia coli* tRNA (1 mg/ml) at 50°C for 4 hr. The slides then were dehydrated in graded ethanol. Hybridized preparations were autoradiographed with NTB nuclear track emulsion (Eastman Kodak). After exposure for 10 days at 4°C, the slides were developed with Dektol (Eastman Kodak), dried, and stained with hematoxylin. The bone marrows of five of the patients were subjected to the *in situ* hybridization procedure on two separate occasions. Control slides bearing bone marrow obtained from HIV-1 seronegative individuals without risk factors for AIDS were treated in parallel. In addition, positive controls were prepared with H9 cell cultures heavily infected with replicating HIV isolate "HTLV-IIIB" (provided by David Ho, University of California, Los Angeles).

Electron Microscopy. Aliquots of bone marrow specimens were also placed directly into 3% glutaraldehyde at the bed side. On arrival in the laboratory, they were centrifuged at 400 × *g*, and the buffy coat was aspirated and placed into fresh 3% glutaraldehyde. Dehydration and embedment in Poly/Bed 812 (Polyscience) were done as is routine in our laboratory (11). Thin sections were stained with uranyl acetate and lead citrate and viewed in a Siemens Elmiskop I electron microscope.

RESULTS

Despite a fairly high background, the density of radioisotope grains overlying clearly identifiable megakaryocytes left little

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Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; ITP, immune thrombocytopenias; AZT, azidothymidine.

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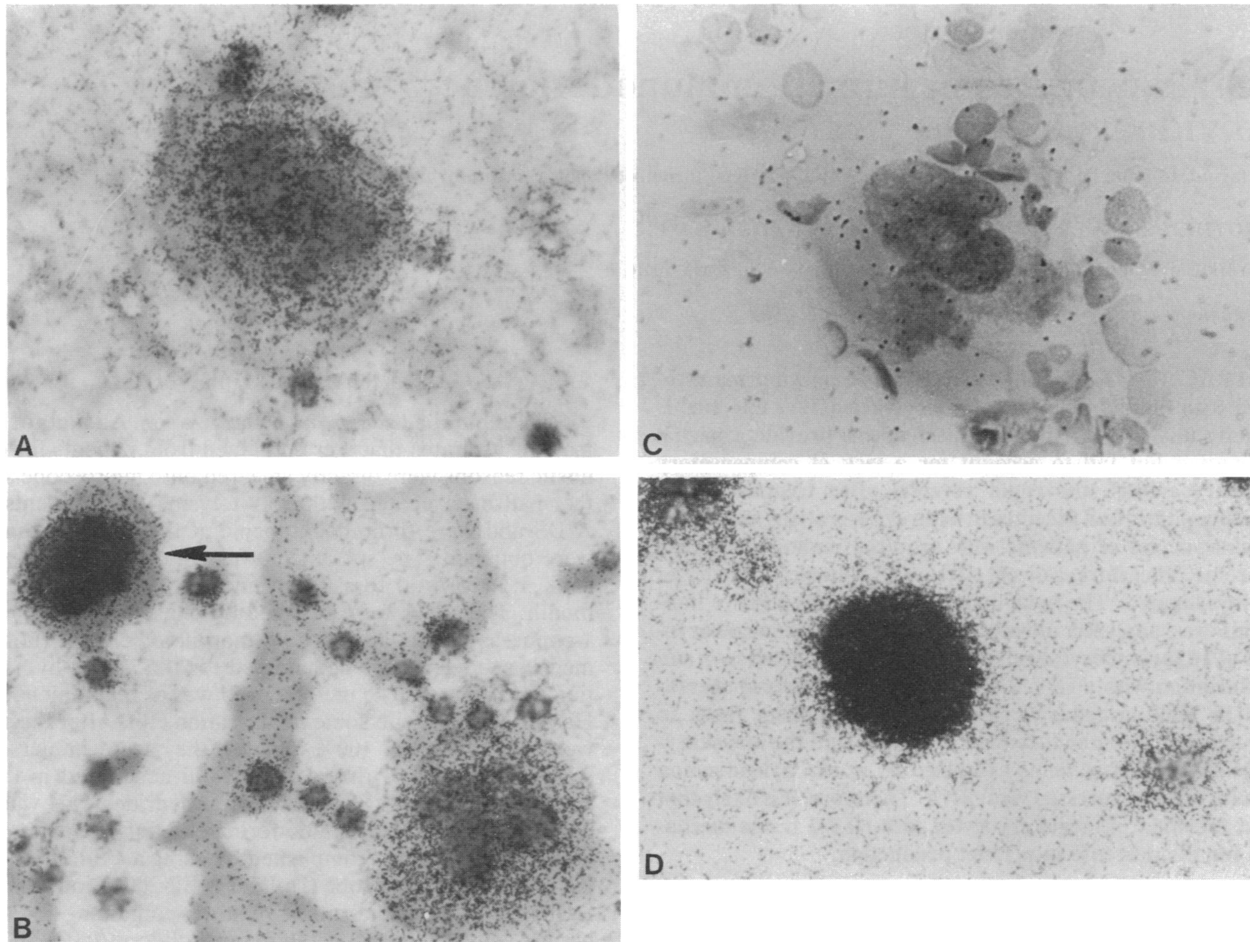

FIG. 1. *In situ* hybridization of marrow with a ^{35}S-labeled HIV RNA probe. (*A* and *B*) Heavily labeled megakaryocytes in the marrow specimens of HIV-seropositive subjects. In *B*, two labeled megakaryocytes are seen. The one at the top (arrow) is almost denuded of cytoplasm. Several smaller cells or fragments are also positive. (*C*) Megakaryocyte in the bone marrow obtained from a HIV-negative subject treated like the specimens in *A* and *B*. Some grains are seen evenly distributed over the entire field. (*D*) A cell taken from an H9 cell culture heavily infected with HIV, treated exactly like the marrow samples, and photographed at the same magnification as *A–C*. Because of the strong signal, the boundary of the cell is obscured.

doubt that these cells expressed viral RNA (Fig. 1 *A* and *B*). This was evident in the megakaryocytes of all 10 patients and in both specimens from 5 of the patients whose marrows were subjected to *in situ* hybridization a second time. It is also noteworthy that some megakaryocytes that were recognizable because of the unambiguous size and morphology of their nuclei appeared to have little cytoplasm (Fig. 1*B*). The number of grains associated with megakaryocytes in the control samples, which were obtained from HIV-seronegative individuals, did not exceed the number of background grains, which were distributed sparsely and evenly over the entire slide (Fig. 1*C*).

In addition to megakaryocytes, there was a variable number of smaller cells that also gave a positive signal. Because of the method of fixation used, the preservation and resolution of such cells (or cell fragments) were inadequate for morphologic identification.

For comparison, Fig. 1*D* shows a H9 cell from a culture heavily infected with replicating HIV (isolate HTLV-IIIB) and subjected to *in situ* hybridization with the ^{35}S-labeled RNA probe. The autoradiograph was exposed for the same length of time as the marrow samples. As expected, the signal was strong and almost obscured the boundary of the cell.

The ultrastructural damage exhibited by megakaryocytes of HIV-infected individuals has been described in detail elsewhere (12). The electron microscopic illustrations shown here were chosen to underscore the type of damage seen in HIV megakaryocytes. The injury appears to involve primarily the surface membrane and the peripheral zone of cytoplasm, where blebbing and vacuolization are the most consistent finding (Fig. 2*A*). Megakaryocyte differentiation does not seem to be impaired. This is shown to good advantage in Fig. 2*B*, which illustrates a thin section of a megakaryocyte that traversed above or below the plane of the nucleus. Individual platelet fields are clearly demarcated. However, the peripheral zone of cytoplasm, which in normal mature megakaryocyte forms a rim of 1 μm or less, here exhibits large blebs measuring 5–10 μm completely devoid of organelles. Although precise quantitation of the incidence of such blebs has not been possible, they were estimated to occur in 30–80% of mature megakaryocytes in all AIDS marrows studied. Similar blebs were not seen by us in the megakaryocytes of seven patients with HIV-negative ITP. The large size and segmentation of the denuded nuclei (Fig. 3) also indicate that AIDS megakaryocytes can attain an advanced stage of ploidy. Some of the cells exhibit vacuoles filled with villous processes, which may have been invaginated surface structures (Fig. 4). These only were encountered in three of the patients.

DISCUSSION

Thrombocytopenia among homosexual men was one of the first phenomena observed during the initial phases of the

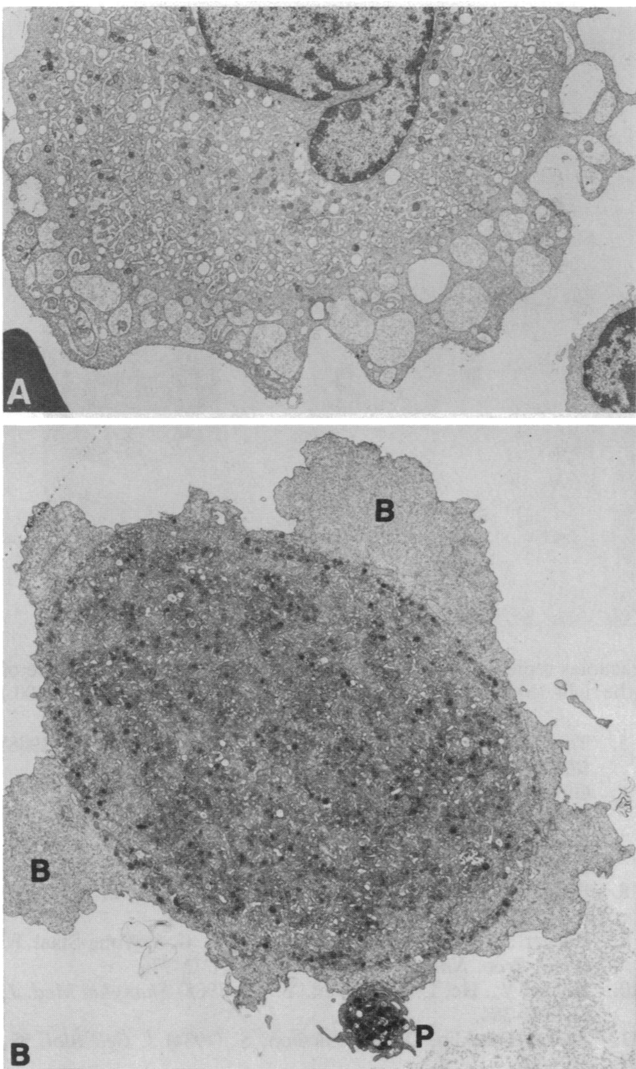

FIG. 2. Details of megakaryocytes from HIV-infected subjects. (*A*) Peripheral blebbing and vacuolization shown to good advantage. (×3500.) (*B*) Megakaryocyte sectioned beyond the plane of the nucleus, illustrating excellent development of platelet fields and huge peripheral blebs (B) that protrude up to 10 μm from the granulated cytoplasm. A large platelet (P) measuring 5 μm is fortuitously located near the megakaryocyte. (×3500.)

AIDS epidemic (1–3). The intriguing aspect was that thrombocytopenia occurred in subjects who were not anemic and whose bone marrows had none of the numerous abnormalities now recognized in patients with full-blown AIDS (13–15). Perhaps for this reason the low platelet counts were attributed primarily to immune mechanisms that, as in chronic ITP, play a major role in accelerated platelet destruction. However, the observations enumerated in the introduction raised doubts that platelet antibodies and/or immune complexes provide a complete explanation for the thrombocytopenia and led to the present studies designed to show that HIV-1 infects megakaryocytes directly. Apart from the ultrastructural features and the large number of denuded nuclei reported in detail elsewhere (12), we have now demonstrated that the megakaryocytes of 10 of 10 patients whose marrows were subjected to *in situ* hybridization with a HIV RNA probe contain viral RNA. This observation raises many questions. At what stage of megakaryocyte differentiation does HIV infect the cells? What is its mode of entry? And why does structural damage appear to take place only relatively late in the development of the megakaryocyte/platelet lineage?

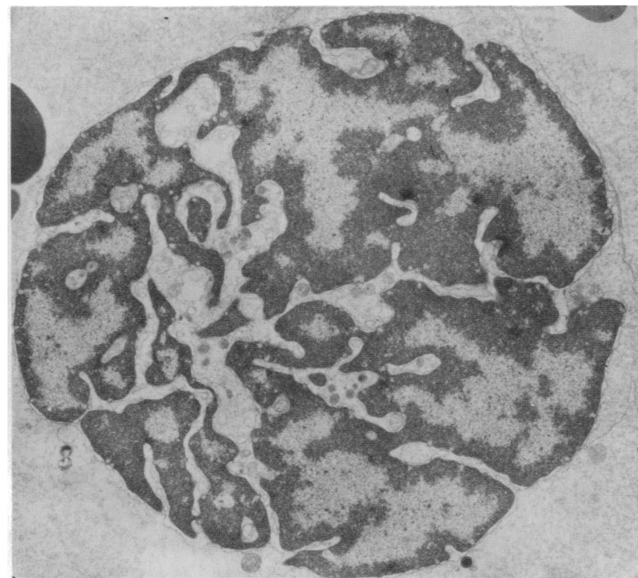

FIG. 3. Denuded megakaryocyte nucleus showing the staghorn appearance characteristic of an advanced stage of ploidy. (×4800.)

It is generally recognized that viruses enter cells by one of two mechanisms (16–18). For HIV-1, the most frequent of these appears to be a process of membrane fusion following attachment to CD4 (19–23). Whether megakaryocytes have CD4 has not been firmly established. Our own investigations have yielded inconsistent results (D.Z.-F. and E. Rabellino, unpublished observations). However, it has been reported by others that CD4 is not a necessary prerequisite for infection (24). The second plausible mechanism of entry is by Fc receptor-mediated endocytosis. Human megakaryocytes possess Fc receptors (25); therefore, antibody-coated virus particles could be interiorized by this means (16). In the case of megakaryocytes, a third pathway is possible—namely, by emperipolesis of virus-infected cells. Emperipolesis is a phenomenon whereby intact migratory cells, such as monocytes, neutrophils, or lymphocytes, invaginate the surface of larger cells, such as monocytes, neutrophils, or lymphocytes, and come to occupy membrane-bound spaces within their cytoplasm. This event is distinct from phagocytosis (26) and is commonly displayed by megakaryocytes, particularly during accelerated thrombocytopoiesis (4). However, even if the incidence of emperipolesis of HIV-infected cells into megakaryocytes were high, it could not account for the observation that almost all megakaryocytes within the specimens examined expressed viral RNA.

On the basis of a report by Folks *et al.* (24), another explanation seems more likely. These investigators demonstrated that HIV can infect bone marrow progenitor cells that are devoid of T-cell markers, including CD4 and of the specific monocyte enzyme α-naphthyl butyrate esterase. Therefore, it is conceivable that HIV also can infect morphologically unidentifiable progenitors of the megakaryocyte lineage. This possibility is supported also by the report of Donahue *et al.* (27), showing that serum obtained from HIV-seropositive individuals caused 50–90% inhibition of colony-forming units in agar cultures when marrow precursors were obtained from AIDS patients, but no inhibition of colony growth was noted when precursors were derived from HIV-seronegative individuals. The authors suggested that HIV may infect progenitors without necessarily perturbing their growth potential *in vivo* (27). Thus, in the case of megakaryocytes, it is possible that progenitors as well as later stages of differentiation and maturation of this cell lineage are susceptible to HIV infection, since several modes of entry are

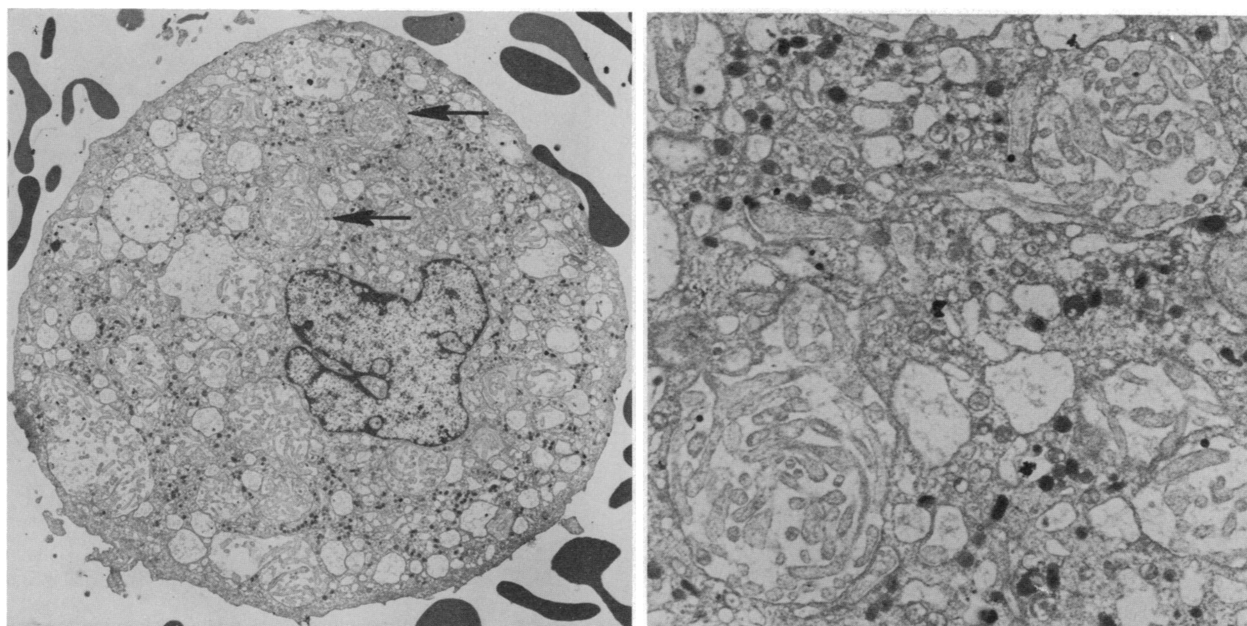

FIG. 4. Megakaryocyte from a patient with AIDS showing numerous vacuoles replete with processes usually seen only on the surface of cells. Arrows indicate two of these vacuoles depicted at higher resolution on the right. (Magnification of the whole cell = ×2100; detail = ×8400.)

theoretically possible. The fact that the megakaryocyte is a polyploid cell that undergoes frequent endoreduplication of its nucleus may also provide a condition favorable for viral replication. Other retroviruses (e.g., mouse leukemia virus) heavily infect megakaryocytes (28). Of additional interest was the observation by Folks *et al.* (24) that the virus seemed to assemble preferentially on internal membranes of the cells derived from the cultured marrow progenitors. If this were also to pertain to megakaryocyte precursors, viral epitopes could be expressed preferentially on developing demarcation membranes before appearing on the surface. Antibodies directed against platelets would not necessarily affect megakaryocytes until they reach the stage of thrombocytopoiesis—i.e., when platelet demarcation membranes become exposed to the extracellular environment. We have reported previously that megakaryocytes and platelet surface membranes are structurally and antigenically distinct and, therefore, that antibodies directed against some platelet epitopes may react with megakaryocytes only when they have become mature enough to release platelets (11, 29, 30). In HIV-related thrombocytopenia, this could account for the advanced ploidy that megakaryocytes are able to attain and for the unique type of structural damage. An immunohistochemical study on the ultrastructural level designed to localize viral antigens subcellularly will be necessary to prove this hypothesis.

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