Monoclonal Antibody–Mediated Tumor Regression by Induction of Apoptosis

BERNHARD C. TRAUTH, CHRISTIANE KLAS, ANKE M. J. PETERS, SIGFRIED MATZKU, PETER MÜLLER, WERNER FALK, KLAUS-MICHAEL DEBATIN, PETER H. KRAMMER

To characterize cell surface molecules involved in control of growth of malignant lymphocytes, monoclonal antibodies were raised against the human B lymphoblast cell line SKW6.4. One monoclonal antibody, anti-APO-1, reacted with a 52-kilodalton antigen (APO-1) on a set of activated human lymphocytes, on malignant human lymphocyte lines, and on some patient-derived leukemic cells. Nanogram quantities of anti–APO-1 completely blocked proliferation of cells bearing APO-1 in vitro in a manner characteristic of a process called programmed cell death or apoptosis. Cell death was preceded by changes in cell morphology and fragmentation of DNA. This process was distinct from antibody- and complement-dependent cell lysis and was mediated by the antibody alone. A single intravenous injection of anti–APO-1 into nu/nu mice carrying a xenotransplant of a human B cell tumor induced regression of this tumor within a few days. Histological thin sections of the regressing tumor showed that anti–APO-1 was able to induce apoptosis in vivo. Thus, induction of apoptosis as a consequence of a signal mediated through cell surface molecules like APO-1 may be a useful therapeutic approach in treatment of malignancy.

CELL SURFACE MOLECULES ARE CRUCIAL IN LYMPHOCYTE GROWTH CONTROL. Such molecules may function as receptors for growth-stimulating cytokines or be associated with receptors and transmit signals essential for growth regulation. Receptor blockade or removal of the stimulating cytokines can lead to decreased lymphocyte growth. Withdrawal of interleukins slow human lymphocyte growth and finally leads to a characteristic form of cell death called “programmed cell death” or apoptosis (1). Apoptosis is the most common form of eukaryotic cell death and occurs in embryogenesis, metamorphosis, tissue atrophy, and tumor regression (2). It is also induced by cytoxic T lymphocytes and natural killer and killer cells; by cytokines like tumor necrosis factor (TNF) and lymphotoxin (LT); and by glucocorticoids (1, 2). The most characteristic signs of apoptosis are segmentation of the nucleus, condensation of the cytoplasm, membrane blebbing, and DNA fragmentation into multimers of about 180 base pairs (called a “DNA ladder”) (1, 2). To analyze mechanisms of lymphocyte growth control and to interfere with the replication of lymphoid tumor cells we raised monoclonal antibodies (MAbs) against cell surface molecules involved in these processes.

We found one MAb (anti–APO-1) that blocks growth and induces apoptosis of SKW6.4 cells (3). Anti–APO-1 (IgG3, \( K_p = 1.9 \times 10^{-9} \)) bound to approximately 4 \( \times 10^4 \) sites on the surface of SKW6.4 cells (4). It specifically immunoprecipitated an endogenously synthesized protein antigen (APO-1) from SKW6.4 cells which, under reducing conditions, was observed on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) as a main band of 52 kD (Fig. 1). Apart from actin (43 kD), which was nonspecifically precipitated with IgG3, anti–APO-1 specifically immunoprecipitated a minor band of 25 kD. This 25-kD protein might either represent a degradation product or be noncovalently associated with the 52-kD protein.

There are two major modes of death in nucleated eukaryotic cells. Necrosis as a result, for example, of complement attack is characterized by swelling of the cells and rupture of the plasma membrane caused by an increase in permeability. Cells that undergo apoptosis, however, show a different biochemical and morphological pattern (2). This pattern corresponds to the one induced by anti–APO-1: condensation of the cytoplasm, membrane blebbing (Fig. 2a), and endonuclease-induced DNA fragmentation (5) into multimers of approximately 180 bp.
were either cells (FII20) labeled with \(^{3}H\)thymidine, additional control MAbs including CD19, CD20, isotype-matched medium (Gibco, 20 mM L-glutamine, streptomycin (100 \(\mu\)g/ml), penicillin (100 U/ml), 20 mM Hepes buffer pH 7.3, and 10% heat-inactivated fetal bovine serum (Conco Lab-Division, Wiesbaden, FRG). For microcultures, 1 \(\times\) 10^4 cells per well were cultured in duplicates in flat-bottom 96-well microtitrater plates (Tecnomara, Fernwald, FRG) (200 \(\mu\)l final volume per well). After 24 hours, the cells were labeled with 0.5 \(\mu\)Ci of \(^{3}H\)thymidine (Amer sham, Braunschweig, FRG) for 4 hours. Before harvesting, the microcultures were examined by microscopic inspection. DNA fragmentation \(1 \times 10^6\) cells were washed with cold phosphate-buffered saline and disrupted with NTE buffer, pH 8 (100 mM NaCl, 10 mM tris, 1 mM EDTA) containing 1% SDS and protease K (0.2 mg/ml). After incubation for 24 hours at 37°C, samples were extracted twice with phenol plus chloroform (1:1, v/v) and precipitated by ethanol. The DNA was dissolved in 38 \(\mu\)l of NTE buffer and digested with ribonuclease (1 mg/ml) for 30 min at 37°C. To each sample 10 \(\mu\)l of loading buffer containing 15% Ficoll 400 (Pharmacia, Uppsala, Sweden), 0.5% SDS, 50 mM EDTA, 0.05% bromomophenol blue, 0.05% xylene cyanol in TBE buffer (2 mM EDTA, 89 mM boric acid, 89 mM tris, pH 8.4) were added. The mixture was loaded onto a 1% agarose gel and stained after electrophoresis with ethidium bromide (0.5 \(\mu\)g/ml). The size marker was Hind III + Eco RI-digested λ DNA.

Fig. 2. Induction of growth inhibition and apoptosis by anti-APO-1. (A) The T cell line CCRF-CEM.S2 (19) was cultured in the presence of purified MAb (1 \(\mu\)g/ml) in a microtitrater plate for 2 hours before photography (left panel control MAb 13B1; right panel anti-APO-1). (B) CCRF-CEM.S2 cells (10^6 per milliliter) were incubated with MAb (1 \(\mu\)g/ml) in culture medium at 37°C. At various times, aliquots of 10^6 were removed and DNA was prepared. M, marker; I, control MAb 13B1 for 2 hours; lanes 3 to 7, anti-APO-1 for the times indicated. (C) SKW6.4 cells were either incubated with the isotype-matched control MAb FII20 (yssey), FII23 (nonbinding MAb) (18), or anti-APO-1 (19) in microcultures for 24 hours before labeling with \(^{3}H\)thymidine for a further 4 hours. The data represent the mean of duplicate cultures with a variation of less than 5%. The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, New York), supplemented with 2 mM l-glutamine, streptomycin (100 \(\mu\)g/ml), penicillin (100 U/ml), 20 mM Hepes buffer pH 7.3, and 10% heat-inactivated fetal bovine serum (Conco Lab-Division, Wiesbaden, FRG). For microcultures, 1 \(\times\) 10^4 cells per well were cultured in duplicates in flat-bottom 96-well microtitrater plates (Tecnomara, Fernwald, FRG) (200 \(\mu\)l final volume per well). After 24 hours, the cells were labeled with 0.5 \(\mu\)Ci of \(^{3}H\)thymidine (Amersham, Braunschweig, FRG) for 4 hours. Before harvesting, the microcultures were examined by microscopic inspection. DNA fragmentation (1 \(\times\) 10^6 cells was washed with cold phosphate-buffered saline and disrupted with NTE buffer, pH 8 (100 mM NaCl, 10 mM tris, 1 mM EDTA) containing 1% SDS and protease K (0.2 mg/ml). After incubation for 24 hours at 37°C, samples were extracted twice with phenol plus chloroform (1:1, v/v) and precipitated by ethanol. The DNA was dissolved in 38 \(\mu\)l of NTE buffer and digested with ribonuclease (1 mg/ml) for 30 min at 37°C. To each sample 10 \(\mu\)l of loading buffer containing 15% Ficoll 400 (Pharmacia, Uppsala, Sweden), 0.5% SDS, 50 mM EDTA, 0.05% bromomophenol blue, 0.05% xylene cyanol in TBE buffer (2 mM EDTA, 89 mM boric acid, 89 mM tris, pH 8.4) were added. The mixture was loaded onto a 1% agarose gel and stained after electrophoresis with ethidium bromide (0.5 \(\mu\)g/ml). The size marker was Hind III + Eco RI-digested λ DNA.

Fig. 3. Anti-APO-1-induced regression of the EBV-negative Burkitt-like lymphoma BJAB in nu/nu mice. BJAB cells (4 \(\times\) 10^5) were injected subcutaneously into the left flanks of nu/nu mice. After 5 weeks (day 0) the mice were injected with 500 \(\mu\)g of MAb into the tail vein. Control MAb FII20 (C), FII23 (O); 13B1 (A); and anti-APO-1 (19). Fourteen days later the size of the tumors was measured at the base of the tumor; the tumors from individual mice are represented by dots.
BJAB cells the nu/nu mice carried tumors with a diameter of approximately 1.0 to 2.5 cm (Fig. 3). These mice were injected intravenously with purified anti-APO-1 (500 μg per mouse) or the same quantities of various isotype-matched control antibodies (FI120, anti-MHC class I antigens, recognizing 5.8 x 10⁵ sites per cell; or one of the two nonbinding MAb FI123 and 13B1). As a control we also injected anti-APO-1 (500 μg per mouse) into three nu/nu mice carrying the APO-1−negative B cell tumor TL-LY1 with tumor diameters of 1.5, 1.8, and 3.4 cm, respectively (11) (see also Table 1). Two days after anti-APO-1 injection, a whitish discoloration of the BJAB tumors was observed that was followed by rapid tumor regression. Macroscopic tumor regression was seen in 10 of 11 treated mice within less than 14 days. The control antibodies had no effect (Fig. 3). In addition, no tumor regression was observed in the mice carrying OCL-LY1, as expected.

To demonstrate proper localization and enrichment of the injected antibodies, labeled MAb s were visualized by autoradiography of sections of the BJAB tumor tissue (Fig. 4a). These autoradiographs showed a pronounced binding of anti-APO-1 in the periphery but only sparse accumulation in the center of the tumor. The binding control MAb FI120 showed a qualitatively similar binding pattern. There was no localization of the nonbinding control MAb FI123 above background. Furthermore, paired label experiments (12) with labeled anti-APO-1 and FI123 revealed that the specific enrichment of anti-APO-1 over FI123 in the tumor was four- and sixfold after 48 and 96 hours, respectively.

The main purpose of our experiments was to assess whether anti-APO-1 can also act in vivo. Therefore, the tumor-bearing mice only received one intravenous injection of anti-APO-1 at a dose in the range used in MAb therapies. In other therapy schedules, however, MAb s are injected repeatedly (13). In our experiments regrowth of the BJAB tumor was observed in three of the ten mice in which tumor regression had been observed (Fig. 3). Regrowth was observed at

### Table 1. Reactivity of anti-APO-1 with different cells.

<table>
<thead>
<tr>
<th>Type</th>
<th>Designation</th>
<th>Cells positive for APO-1 (%)</th>
<th>Relative fluorescence intensity (anti-APO-1 control)</th>
<th>Effects of MAb on [3H]thymidine uptake (10⁶ cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hu B cells</td>
<td>SKW6.4</td>
<td>98</td>
<td>11.1</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>CESS</td>
<td>95</td>
<td>12.0</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>BJAB</td>
<td>80</td>
<td>2.1</td>
<td>70.0</td>
</tr>
<tr>
<td></td>
<td>OCL-LY1</td>
<td>0</td>
<td>1</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>Jurkat</td>
<td>83</td>
<td>2.3</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>Molt</td>
<td>91</td>
<td>2.4</td>
<td>35.7</td>
</tr>
<tr>
<td></td>
<td>CCRF-CEM</td>
<td>64</td>
<td>1.9</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>U937</td>
<td>5</td>
<td>0.97</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td>MLA 144</td>
<td>0</td>
<td>0.96</td>
<td>34.3</td>
</tr>
<tr>
<td>Mouse T cells</td>
<td>EL4</td>
<td>0</td>
<td>1</td>
<td>44.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.3</td>
</tr>
<tr>
<td>Leukemic cells from patients§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Resting</td>
<td>3</td>
<td>1.36</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>Activated</td>
<td>89</td>
<td>7.4</td>
<td>0.23</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Resting</td>
<td>91</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Activated</td>
<td>91</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

*Hu, human; ALL, acute lymphoblastic leukemia. †Aliquots of 10⁶ cells were incubated at 4°C in 100 μl of medium with control MAb (FI123 or 13B1) or anti-APO-1 for 30 min. Then the cells were washed and stained with fluorescein isothiocyanate–coupled goat anti-mouse IgG (Fab′₂) at 70 μg/ml and analyzed by flow cytography (Ortho Diagnostic Systems, Westwood, Massachusetts). ‡Cells (10⁴ per well) were cultured in the presence of MAb (500 ng/ml) for 24 hours and labeled with [3H]thymidine for 2 hours before harvest; the data represent the mean of duplicate cultures with a variation of less than 5%. §Bone marrow cells isolated from the patients were morphologically >95% blasts and showed the following phenotype: pre-T-ALL, cytoplasmic CD3⁺, CD5⁺, CD7⁺, CD19⁺, CD20⁺; T-ALL CD2⁺, cytoplasmic CD3⁺, CD5⁺, CD7⁺ and CD10⁺, surface CD3⁻, CD4⁻, CD8⁻, and CD54⁺; common ALL CD19⁺, CD20⁺, CD24⁺, CD25⁺. The effect of anti-APO-1 on these leukemic cells was not tested, because they died under normal culture conditions. ‖Peripheral blood mononuclear cells (PBMC) from healthy volunteers were isolated by Ficoll Paque (Pharmacia Inc., Upsala, Sweden) density centrifugation. Adherent cells were removed by adherence to plastic culture vessels overnight. T cells were isolated from PBMC by rosetting with 2-amino-ethylisothiouronium bromide (AET)–treated sheep red blood cells as described (20). Freshly prepared resting T cells (2 x 10⁶ per milliliter; 96% OKT11⁺, 1% Tac⁻) were activated with phytohemagglutinin-M (50 μg/ml) and PMA (10 ng/ml) (Sigma Chemical Co., Munich, FRG). Two, 7, and 12 days later the T cells were fed with 20 to 30 U/ml of recombinant human interleukin-2 (20 to 30 U/ml). T cells (5 x 10⁶ per milliliter) activated for 12 days (90% OKT11⁺, 60% Tac⁺) were cultured in the presence of FI123 or anti-APO-1 (1 μg/ml) in triplicates for 24 hours and then labeled with [3H]thymidine for a further 17 hours (see legend to Fig. 2). Resting B cells (35.8% CD19⁺) were isolated by two rounds of rosetting as above, followed by separation via a Sephadex G-10 column as described (21). For activated B cells, PBMC were adjusted to 2 x 10⁶ cells per milliliter and cultured in the presence of pokeweed mitogen at 10 μg/ml (Serva, Heidelberg, FRG) for 6 days. Dead cells and T cells were then eliminated by rosetting with AET-treated sheep red blood cells and subsequent centrifugation over Ficoll Paque. The interphase cells were used as activated B cells (84% IgM⁺).
the margin of the original tumor approximately 3 months after the initial macroscopic tumor regression. One of these tumors was removed and found to express APO-1 by immunofluorescence and to be sensitive to anti-APO-1 in vitro at a MAb concentration similar to the original in vitro BJAB tumor cell line (Table 1).

To determine the histology of the regressing BJAB tumors we prepared thin sections of tumors from MAb-treated mice. Ten days after intravenous injection of FI120, BJAB appeared as a solid tumor composed of densely packed large blasts with numerous mitoses, some tumor giant cells, and rare apoptotic figures (Fig. 4b, left panel). The tumor was penetrated by host vessels. In contrast, almost all remaining BJAB cells of mice treated with anti-APO-1 (Fig. 4b, right panel) showed severe cytopathic changes including nuclear pyknosis and cellular edema most pronounced in perivascular microareas. These morphological changes are characteristic of apoptosis.

Taken together, these data strongly suggest that apoptosis is induced by anti-APO-1 and is the mechanism of death and regression of BJAB tumor cells in vivo. The fact that FI120, which strongly binds to the cell surface of BJAB tumor cells, did not cause regression of BJAB also precludes the possibility that killer cells or complement that might have bound to anti-APO-1 may have been involved in the growth inhibition and tumor regression.

We showed that anti-APO-1 specifically blocked growth and triggered programmed cell death (apoptosis) of a set of activated normal lymphocytes and cells from malignant lymphocyte lines after binding to the cell surface protein antigen APO-1. Recently, it has been shown that anti-CD3 induces apoptosis of immature thymocytes in vitro (14). Therefore, it has been suggested that CD3-triggered apoptosis might be responsible for negative selection of T cells in the thymus. Since APO-1 is expressed on mature activated lymphocytes, additional experiments will be needed to determine whether the antigen might play a similar role in the downregulation of the immune response and to be involved in selection and elimination of lymphocytes. It has previously been shown that LT, TNF, and killer cells with their effector molecules induce apoptotic cell death (15). As anti-APO-1 also induces apoptosis a number of possibilities might be considered for the physiological role of the APO-1 antigen. APO-1 might be a receptor for cytotoxic molecules or for autocrine growth factors. Alternatively, it could be a molecule essential for vertical or lateral growth signal transduction. Thus, anti-APO-1 might trigger receptors for lytic molecules or block receptors for growth signals.

Apoptosis is found in all tissues and also in cells from lower organisms (16). It is conceivable, therefore, that several distinct cell surface antigens with a different tissue distribution are involved in the induction of apoptosis. Elucidation of the structure of APO-1, its possible connection to the cytoskeleton and the molecular events following anti-APO-1 binding might resolve some of these issues.

Our data might also have clinical relevance. APO-1 was found on some lymphoid tumor cells freshly isolated from patients. Thus, anti-APO-1 might be useful as a diagnostic tool to define subsets of normal and malignant lymphocytes. In addition, induction of apoptosis may have implications for anti-tumor therapy. Antibodies have frequently been used as heteroconjugates with toxins or drugs to destroy tumor cells (17). Our data, however, show that MAb alone can be lethal to target cells. Anti-APO-1 and related MABs might, therefore, be considered for ex vivo or in vivo therapy, under conditions where reactivity with vital normal cells can be excluded or tolerated. Finally, the molecular investigation of cell death induced by anti-APO-1 might lead to a general understanding of apoptosis. In this case, the use of modified or normal physiological ligands to the cell surface antigen initiating apoptosis or of chemicals interfering with the apoptotic signal might be envisaged.

REFERENCES AND NOTES

3. BALB/c mice were immunized once per week over a 4-week period by intraperitoneal injection of 1 x 10⁶ SKW6.4 cells. Four days after the last injection, spleen cells from immunized animals were fused with the P3.X63.Ag8.653 myeloma (G. Kohler and C. Milstein, Nature 265, 495 (1975)). Twelve days after fusion culture supernatants from wells positive for growth were tested for their ability to inhibit growth of SKW6.4 cells. Hybridomas that produced blocking MABs were cloned three times by limiting dilution at a concentration of 0.5 cells per well. MABs were purified from serum-free culture supernatants by immunoaffinity chromatography on protein A–Diasorb column (Diagen, Düsselldorf, FRG). Bound MABs were eluted with 0.1M NaCl and 0.1M glycine, pH 2.8, diazylated against phosphate-buffed saline and sterilized. The isotype of the MABs was determined by enzyme-linked immunosorbent assay (S. Kiesel, et al., Leuk. Res. 11, 1119, 1987) with isotype-specific goat anti-mouse Ig that had been conjugated with horseradish peroxidase (Ausbach, FRG).
4. Affinity and number of anti-APO-1 binding sites per cell were determined by Scatchard analysis as described (1). von Hoegen, W. Falk, G. Kojouharoff, P. H. Kramper, Eur. J. Immunol. 19, 329 (1989). Briefly, MABs were iodinated by the IODO-Gen method [P. J. Franken and J. C. Speck, Biochem. Biophys. Res. Commun. 80, 149 (1978)]. Aliquots of 5 x 10⁶ cells were resuspended in 200 μl of culture medium containing 0.1% NaCl and different concentrations of [³H]-labeled MABs. After incubation at 4°C for 4 hours, 25 μl portions were removed and centrifuged as described above by von Hoegen et al.
6. Monoclonal anti-CD19 (HD37) and anti-CD22 (HD39) were kindly provided by B. Dörken (Policlinico di the University of Heidelberg, Germany) and monoclonal anti-CD20 by G. Moldenhauer (IV Leukocyte Typing workshop and conference, Vienna, Austria, 1989), respectively. The 19 nonbinding and 9 binding MABs of the panel were probed by immunofluorescence on SKW6.4 cells and the MABs directed against MHC class II, IgM, and SKW6.4 Ig identities were raised in our own laboratory.
7. The kinetics of membrane blebbing induced by anti-APO-1 (within 30 min; Fig. 2a) was not influenced by the presence of 10 mM EDTA or EGTA. In addition, endonuclease and DNA fragmentation induced by anti-APO-1 was not inhibited by the Ca²⁺ channel blockers Fluridin (50 μM) or Nifedipin (50 μM).
8. When 5 x 10⁶ SFM-cultured SKW6.4 cells were incubated with anti-APO-1 (1μg/ml) for 2, 4, and 24 hours, the specific ⁵¹Cr release [R. C. Duke, R. Chervenak, J. Cohen, Proc. Natl. Acad. Sci. U.S.A. 80, 6361 (1983)] was 2.3, 8.5, 21.3%, and 32.5%, respectively. Trypan blue uptake was measured at the same time points: 2.5%, 4.7%, 10.6%, and 73.6%, respectively, of the cells were trypan blue-positive. After 24 hours after the addition of MABs, plus complement the specific ⁵¹Cr release was 108.7% and 92.7% of the cells stained with trypan blue.
9. Two hours after addition of MABs (1μg/ml) the genomic DNA of each tumor line was isolated and analyzed on agarose gels as described (Fig. 2). Inhibition of [³H]thymidine uptake by anti-APO-1 was paralleled by fragmentation of the genomic DNA. This was not observed after treatment with control MAB (IBSI).
10. Activated B cells (10⁶ per milliliter) were incubated in the presence of MAB FI123 or anti-APO-1 at 1 μg/ml. After 3 days the culture supernatants were collected and the IgM concentration measured with a human IgM-specific ELISA containing HRPO-conjugated goat anti-human IgM (Medac, Hamburg, FRG). IgM secretion after treatment with FI125 or anti-APO-1 was 2100 and 550 ng/ml, respectively.
11. OCI-LY1 was obtained from H. Mesner, Ontario Cancer Institute, Toronto, Canada.
19. The CCRF-CEM-S2 subclone was obtained by cloning cells under limiting dilution conditions from the CCRF-CEM-T cell line at one cell per well in 96-well microtiter plates. CCRF-CEM-S2 was selected because of its high sensitivity to programmed cell death induced by anti-APO-1 (500 μg/ml) as measured by trypan blue uptake.
22. We thank R. H. Hul, J. Molinier, R. Kühnle, M. Mandl, and W. Müller for excellent secretarial assistance; G. Hämmerling and G. Moldenhauer for their criticism; B.
The Reservoir for HIV-1 in Human Peripheral Blood
Is a T Cell That Maintains Expression of CD4

STEVEN M. SCHNITTMAN,* MILTAIDES C. PSALLIDOPoulos,
H. CLIFFORD LANE, LOUIS THOMPSON, MICHAEL BASELER,
FERDINAND MASSARI, CECIL H. FOX, NORMAN P. SALzman,
ANTHONY S. FAuci

Human immunodeficiency virus type 1 (HIV-1) selectively infects cells expressing the CD4 molecule, resulting in substantial quantitative and qualitative defects in CD4+ T lymphocyte function in patients with acquired immunodeficiency syndrome (AIDS). However, only a very small number of cells in the peripheral blood of HIV-1–infected individuals are expressing virus at any given time. Previous studies have demonstrated that in vitro infection of CD4+ T cells with HIV-1 results in downregulation of CD4 expression such that CD4 protein is no longer detectable on the surface of the infected cells. In the present study, highly purified subpopulations of peripheral blood mononuclear cells (PBMCs) from AIDS patients were obtained and purified by fluorescence-automated cell sorting. They were examined with the methodologies of virus isolation by limiting dilution analysis, in situ hybridization, immunofluorescence, and gene amplification. Within PBMCs, HIV-1 was expressed in vivo predominantly in the T cell subpopulation which, in contrast to the in vitro observations, continued to express CD4. The precursor frequency of these HIV-1–expressing cells was about 1/1000 CD4+ T cells. The CD4+ T cell population contained HIV-1 DNA in all HIV-1–infected individuals studied and the frequency in AIDS patients was at least 1/100 cells. This high level of infection may be the primary cause for the progressive decline in number and function of CD4+ T cells in patients with AIDS.

The human immunodeficiency virus type 1 (HIV-1), the etiologic agent of the acquired immunodeficiency syndrome (AIDS), selectively infects cells expressing the CD4 molecule, including T lymphocytes and cells of the monocyte/macrophage lineage (1). In vitro infection of cells with HIV-1 results in a decreased expression of the CD4 molecule on the surface of the infected cells (2).

Patients with AIDS have severe depression of the normal cell-mediated immune mechanisms that is partially attributed to the considerable depletion of CD4 lymphocytes (3). Despite this, examination of cells from lymph nodes and peripheral blood from patients with AIDS and AIDS-related complex (ARC) has revealed a very low frequency of viral RNA synthesis, generally occurring in 1/100,000 to 1/10,000 of total mononuclear cells (4). However, it is possible that a larger proportion of cells may be latently infected (containing proviral DNA but not expressing viral mRNA or protein). Until the development of gene amplification [polymerase chain reaction (PCR)] methodology (5, 6), HIV-1–infected cells not expressing virus were not readily detectable by available techniques.

In the present study, blood was obtained from HIV-1 culture-positive patients with AIDS either directly in heparinized syringes or via apheresis and subjected to Ficoll-Hypaque separation (7). First, peripheral blood mononuclear cells (PBMCs) from patients were stained with fluorescein isothiocyanate (FITC)–conjugated antibody to CD3 and sorted by a fluorescence-activated cell sorter (FACS) into CD3+ and CD3− populations. Sorted cells were cocultivated with an excess of normal phytohemagglutinin (PHA)-stimulated blast cells and we determined the time to peak viral expression, a highly consistent and reproducible parameter of viral expression. A predominance of HIV-1 expression in the >98% enriched CD3+ population, as determined by the time to peak syncytia formation (Fig. 1A) and reverse transcriptase (RT) activity (Fig. 1B), was seen. Similar results were obtained in seven additional AIDS patients. Delayed expression of HIV-1 in cells that were initially 99% CD3− cells (Fig. 1B) was due to outgrowth of the few contaminating CD3+ cells. Phenotypic analysis of noncocultivated enriched CD3+ cells grown under the same conditions revealed that 35 to 65% of the cells were CD3+ by day 10 in culture. In the second series of experiments, PBMCs from AIDS patients were double-stained with FITC-conjugated anti-CD3 and anti-CD4 and sorted by FACS into CD3+/CD4+ and CD3+/CD4− populations. These sorted cells were cocultivated with an excess of normal PHA-stimulated blast cells and showed a predominance of HIV-1 expression in the highly enriched (98 to 99%) CD4+ T cell population as determined by the time to peak syncytia formation (Fig. 1C) and RT activity (Fig. 1D). Similar results were obtained in seven additional AIDS patients. The phenotypic analysis of freshly sorted CD3+/CD4− cells revealed a greater than 98 to 99% CD4+ purity in most experiments when stained with the monoclonal antibody to Leu 3a. Again, the delayed expression of HIV-1 in cells that were initially 99% CD4− (Fig. 1D) was most likely due to outgrowth of a few contaminating CD4+ T cells. Phenotypic analysis of co-cultured enriched CD4+ T cells grown under the same conditions revealed that 30 to 55% of the cells were CD4+ by day 10 in culture. In situ hybridization for HIV-1 viral RNA was then performed at time zero on the highly enriched CD3+/CD4− and CD3+/CD4−–sorted PBMCs. There was a predominance of viral expression in the CD4+ T cell population at a frequency of about 1/1000 cells in the AIDS patients (X ± SEM per 1000 cells was 0.95 ± 0.21) (Fig. 2A). This is in comparison to a level of viral expression in the CD4+ T cell population of <1/100,000 cells (Fig. 2B), which is equivalent to background signal in controls. The frequency of in situ–positive CD4+ T cells remained unchanged in three of the patients reexamined to 6 to 12 months after the initial studies.

Indirect immunofluorescence studies for HIV-1 viral antigens was also performed at time zero on highly enriched CD3+/CD4− and CD3+/CD4−–sorted PBMCs. These demonstrate a predominance of viral expression in the CD4+ T cell population at a frequency of about 1/1000 cells in four AIDS patients (X ± SEM per 1000 cells was 1.10 ± 0.35 (Fig. 2C). This is in comparison to a level of viral expression in the CD4+ T cell population of <1/10,000 cells.

S. M. Schnittman, H. C. Lane, F. Massari, C. H. Fox, A. S. Fauci, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
M. C. Psallidopoulos, L. Thompson, N. P. Salzman, Division of Molecular Virology and Immunology, Georgetown University School of Medicine, Washington, DC 20007.
M. Baseler, Program Resources, Incorporated, Frederick, MD 21701.

*To whom correspondence should be addressed.

Downloaded from www.sciencemag.org on August 4, 2009