Neutrophils from Human Immunodeficiency Virus (HIV)-seronegative Donors Induce HIV Replication from HIV-infected Patients' Mononuclear Cells and Cell Lines: An In Vitro Model of HIV Transmission Facilitated by *Chlamydia trachomatis*

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Summary

Infection with a sexually transmitted disease (STD) increases the risk for human immunodeficiency virus (HIV) infection. Polymorphonuclear leukocytes (PMNs) are recruited into the genital tract by STD pathogens, such as *Chlamydia trachomatis*. Semen of HIV-infected men contains HIV associated with mononuclear cells. This study investigated the interaction among PMNs from HIV-uninfected persons, *C. trachomatis*, and HIV-infected cells and examined the mechanisms for enhanced HIV replication. We demonstrated that PMNs from HIV-seronegative donors induced HIV replication in mononuclear cells from 17 HIV-infected patients in medium without exogenous IL-2. HIV in the cell-free supernatants from cocultures of PMNs and patients' peripheral blood mononuclear cells (PBMCs) was replication competent, as indicated by their capacity to propagate HIV in a second round of culture using PBMCs from HIV-seronegative individuals and by the fact that proviral DNA was found in these cells. PMNs from HIV-seronegative donors increased HIV replication over 100-fold in chronically HIV-infected cell lines of the monocytic, T, and B cell lineages. Moreover, PMNs increased U1 cells' production of p24 antigen by as much as ninefold when compared with U1 cells cocultured with PBMCs. The addition of *C. trachomatis* to PMN and U1 coculture increased HIV replication by an additional ninefold at 24 h, whereas *C. trachomatis* alone had no effect on p24 antigen production by U1 cells. Thus, *C. trachomatis* serves not only to recruit PMNs, but also to interact with PMNs to increase HIV replication. HIV replication is triggered by contact of HIV-infected cells with PMNs, by the generation of reactive oxygen intermediates (ROIs), and by soluble factors such as TNF-α and IL-6. This is based on the findings that production of p24 antigen, IL-6, and TNF-α induced by PMNs is abrogated by disrupting or partitioning PMNs from HIV-infected cells; is inhibited by superoxide dismutase and catalase, enzymes that destroy ROIs; is enhanced by differentiated HL60 cells capable of producing ROIs; and is induced by PMNs tested negative for CMV. Furthermore, the production of ROIs is independent of HIV infection of mononuclear cells, since PMNs cocultured with HIV-uninfected parental monocytic and T cell lines generated ROIs. Therefore, the increased risk for acquiring HIV infection associated with chlamydia cervicitis may be related to the local recruitment of PMNs by *C. trachomatis* and the induction of infectious virus from mononuclear cells present in semen. These observations provide a rationale for strategies to reduce HIV transmission by control of STD.

The World Health Organization and Centers for Disease Control and Prevention (CDC)[1] estimate that as of 1993 over 12 million persons were infected with HIV world- wide, with 1 million in the USA (1). Within the USA, the risk of male-to-female transmission is estimated at less than

[1] *Abbreviations used in this paper:* AE, acridinium ester; ANOVA, analysis of variance; CAT, catalase; CDC, Centers for Disease Control and Prevention; EB, elementary body; EIA, enzyme immunoassay; HEF, human embryonic fibroblast; IEA, immediate early antigen; ROI, reactive oxygen intermediate; SOD, superoxide dismutase; STD, sexually transmitted disease.

1 in 500 episodes of penile–vaginal intercourse with an HIV-infected partner (2–4). Although the risk for each episode of penile–vaginal intercourse is not known for developing countries, the rapidly increasing rates of HIV infection in these countries with similar rates in men and women suggest that the risk during heterosexual activity is significantly higher (1). Since anal intercourse is rarely reported in these regions, cofactors for enhanced transmission during vaginal intercourse have been sought (5, 6).

Epidemiologic studies have implicated sexually transmitted disease (STD) as a cofactor for HIV seroconversion (reviewed in references 5–7). These reports raised the possibility that preexisting STD may alter the host's susceptibility to and/or enhance the efficiency of HIV transmission. Although genital ulcers caused by some STDs may provide a portal for HIV entry, *Chlamydia trachomatis* infection and other nonulcer-producing STDs are also associated with an increased risk for HIV seroconversion (7–10). An intense PMN infiltrate in the genital tract in which neutrophils predominate is a distinguishing feature of symptomatic and asymptomatic chlamydia cervicitis (11, 12).

Transmission of HIV from men to women occurs because semen contains infectious HIV (13–15). Semen of HIV-infected men contains mononuclear leukocytes, and the presence of HIV seems not to be influenced by the stage of HIV disease (13–15). HIV in semen is predominantly cell associated (13, 16), most likely with leukocytes, but may be carried by sperm (17, 18). Cell-free HIV is seldom found without cell-associated virus (13, 16). Although infection of a woman by an HIV-infected man is probably due to the presence of HIV in semen, the mechanism for the increased risk for HIV infection in some women remains undefined.

Because the PMN infiltrate is a feature of many STDs, including chlamydia disease, and because the ability of PMNs to trigger HIV production has not been demonstrated, we examined the effect of PMNs and chlamydia on induction of HIV replication from HIV-infected patients' PBMCs and chronically HIV-infected cell lines. In vitro induction of HIV replication from HIV-infected mononuclear cells has been reported to require coculture with activated mononuclear cells from HIV-seronegative donors and exogenous IL-2. This study reports that PMNs from HIV-seronegative persons trigger HIV replication from HIV-infected persons' mononuclear cells without exogenous IL-2 and that chlamydiae additionally enhance this process. The mechanisms for the interaction among PMNs, chlamydiae, and HIV-infected cells are presented and may point to an additional strategy for decreasing HIV transmission.

Materials and Methods

Materials and Reagents. The materials and reagents used and the respective manufacturers were as follows: RPMI 1640, HBSS, penicillin/streptomycin, L-glutamine, and FBS (GIBCO BRL, Gaithersburg, MD); IL-2 (Boehringer Mannheim, Indianapolis, IN); PHA, catalase (CAT), superoxide dismutase (SOD), dextran, Ficoll, and PMA (Sigma Chemical Co., St. Louis, MO); 76% Hypaque (Winthrop Pharmaceuticals, NY); 25-cm² tissue culture flasks and

24-well culture plates (Corning Glass Works, Corning, NY); HIV-1 antigen kits, p24 antigen standard, 0.9% NaCl solution, sterile water, Quantum II Dual Wavelength Analyzer, and Qwikwash Bead Washing System (Abbott Laboratories, North Chicago, IL); Taq polymerase and gene amplification kit (Perkin-Elmer-Cetus, Norwalk, CT); SK38/SK39 *gag* oligonucleotide primers (gift of Dr. C. Y. Ou, CDC, Atlanta, GA, and Research Genetics, Huntsville, AL); CMV oligonucleotide primers and probe (Research Genetics); hybridization protection assay with acridinium ester (AE)-labeled oligonucleotide *gag* gene probes and Leader-5 luminometer (AccuSearch kit; GenProbe, San Diego, CA); 0.2-μm membrane inserts for tissue culture (Millipore Corp., Bedford, MA); Centicon microconcentrators (Amicon Division, Danvers, MA); CMV IgM- and IgG-specific immunoassays (Diamedix Corp., Miami, FL); and cytokine enzyme immunoassay (EIA, Predicta kits; Genzyme Corp., Cambridge, MA).

Cell Isolation and Culture. Heparinized peripheral blood and citrate leukocyte-rich buffy coat of normal healthy HIV-seronegative, low risk donors were obtained, respectively, from volunteers and from the American Red Cross (Portland, OR). PBMCs separated by Ficoll Hypaque gradient centrifugation (19, 20) were washed twice with 0.9% NaCl and suspended in complete media (RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) at 3–5 × 10⁶ cells per ml and stimulated with PHA (2 μg/ml) for 24–48 h at 37°C and 5% CO₂. PMNs (>99.9% granulocytes, of which ≥95% were neutrophils) were separated from red blood cells by dextran sedimentation (3% dextran in 0.9% NaCl solution, 1 g, 30 min) (21). Residual red blood cells were removed by hypotonic lysis. The purified neutrophils were >95% viable as judged by trypan blue exclusion.

HIV-infected Patients' PBMCs. 17 HIV-seropositive patients from The New York Hospital were recruited, each donating 20 ml of whole blood. 11 of 17 (65%) had AIDS with CD4⁺ T cells <200/μl and AIDS defining illness; 6 (35%) were asymptomatic with CD4⁺ T cell counts ≥500/μl. The patients' PBMCs were isolated by Ficoll Hypaque density centrifugation. The viral culture was performed by a quantitative coculture method as reported by Ho et al. (22). 10-fold dilutions of HIV-seropositive patients' PBMCs (2 × 10⁶ to 2 × 10³ cells per well) were cocultured with heterologous, washed, PHA-stimulated PBMCs (2 × 10⁶ cells per well) or PMNs (2 × 10⁶ cells per well) freshly isolated from HIV-seronegative donors in a total volume of 1.5 ml of complete medium either with or without 10% (vol/vol) IL-2. During the 21-d culture, seven harvests (50% medium change) were performed every 2–4 d. Fresh PMNs (2 × 10⁶ cells per well) were added once weekly, but there were no additional PBMCs. Quantitative cocultures of six asymptomatic HIV-infected patients, using PBMCs from HIV-seronegative donors, ranged from 0.5 to 5,000 tissue culture infective doses (TCID) per 10⁶ PBMCs (*n* = 6).

Chlamydia trachomatis. The L-2 serovar of *C. trachomatis* (from Dr. C. Rothermel or the American Type Culture Collection, [ATCC] VR-902B, Rockville, MD) was propagated in L cells, and elementary bodies (EBs) were purified by renografin gradient centrifugation and stored at −70°C (23, 24). The preparations contained 10¹¹ to 10¹² viable EBs, as determined by titration in L cells (23). Five ID₅₀s were added to PMNs or PBMCs cocultured with HIV-infected patients' PBMCs or cell lines.

HIV-infected Cell Lines. The chronically HIV-infected monocytic cell line U1 and T cell lines ACH-2 and 8E5 cells were obtained from Dr. Tom Folks (CDC) (25, 26). The chronically HIV-infected B cell line B-HIV1 was derived as previously described (27). U1, T, and B cells (from 2 × 10² to 2 × 10⁵ cell per ml) were cocultivated in complete medium (no IL-2) with or without donor

PBMCs, PMNs, or chlamydiae. B-HIV1 cells were grown in 1% FBS. For evaluations of reactive oxygen intermediates (ROIs) stimulated with PMA (50 ng/ml) or PMNs (2 × 10⁶ cells per ml), U1 cells were cultured with medium or medium containing CAT (58 μg/ml) and SOD (300 U/ml), or heat-inactivated (100°C, 1 h) CAT (58 μg/ml) and SOD (300 U/ml). The percent inhibition of the stimulated U1 cells' production of HIV p24 antigen was defined as follows: 100 × [1 − (experimental condition/stimulated U1 cells)].

EIA for the Detection of HIV-1 p24 Antigen in Viral Culture Supernatants. The amount of HIV in culture supernatants was quantitated by commercial p24 antigen capture EIA (Abbott Laboratories). Briefly, culture supernatants diluted 2–200 times in culture medium were tested as described by manufacturer's protocol. Actual amounts of p24 were determined by using standards provided by the manufacturer (Abbott Laboratories). OD values of controls and culture supernatant were determined at 492 nm using an EIA reader (Abbott Laboratories), and OD values of the supernatant from HIV-seronegative donor cells were always below the cutoff for negative controls.

Propagation of HIV by Secondary Culture. Cell-free culture supernatants (200 μl, stored at −70°C) were incubated with PHA-stimulated PBMCs (4 × 10⁶) from HIV-seronegative donors in growth medium for 24 h at 37°C, 5% CO_2. The residual free virus was removed by washing and culturing for 21 d as described for primary HIV coculture. On day 21, cells were pelleted, washed, and dissolved in DNA lysis buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 7.5 mM $MgCl_2$, 0.45% NP-40, 0.45% polysorbate [Tween] 20, 0.1 mg/ml gelatin) (28).

Detection of HIV gag Gene from Secondary Culture Cells by PCR and Oligonucleotide Probes. Cell lysates containing DNA were digested of protein with 100 μg/ml proteinase K at 65°C for 1 h and with proteinase K inactivated by heating at 95°C for 10 min. DNA obtained from the 8E5 T cell line (gift of C. Y. Ou, CDC) has a single copy of integrated HIV-1 proviral DNA per cell. This was diluted so that each PCR contained 0, 1, 2, 4, 8, 16, 31, 62, 125, 250, 500, and 1,000 copies of HIV-1 proviral DNA extracted from the 8E5 T cell line and a constant amount of HIV-seronegative DNA background derived from 250,000 PBMCs (29, 30). Approximately 1.5 μg of DNA was used, representing 250,000 cells from each sample. *gag* gene PCR amplification was performed using oligonucleotide primers (SK38/SK39), reaction mix, and thermal cycler conditions (28, 30). In brief, amplification was performed for 35 cycles in a DNA thermal cycler (Perkin Elmer Cetus) under the following conditions: room temperature to 95°C, 1 min 30 s; 95°C, 1 min; 95°–55°C, 1 min 30 s; 55°C, 30 s; 55°–60°C, 30 s; 60°C, 2 min 30 s.

Detection of Amplified HIV-1 DNA Using AE-labeled Probes. The hybridization protection assay using AE-labeled oligonucleotide probes and reagents are commercially available (AccuSearch kit, GenProbe). Two overlapping AE-labeled oligonucleotide probes, *gag* 1 and *gag* 2, were used to reduce the potential loss in signal resulting from mismatches between the probe and SK38/SK39-amplified HIV-1 DNA (30). Amplified DNA (25 μl) and an equal volume of a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.45% NP-40, 0.45% polysorbate (Tween) 20, and 0.1 mg/ml gelatin were added to a polypropylene test tube (Stockwell Scientific, Alnut, CA). The mixture was heated at 95°C for 5 min in a water bath to allow dissociation of double-strand DNA and was immediately placed in ice water to maintain the separated DNA as single-strand conformation. Probe mixture (50 μl) containing 0.02 pmol of each probe, 0.1 M lithium succinate buffer, pH 4.7, 2% (wt/vol) lithium lauryl sulfate, 1.2 M $LiCl_2$, 20 mM EDTA, and 20 mM EGTA was

added. Hybridization of the probe and amplified DNA was performed at 60°C for 30 min, and the reaction was quickly terminated by cooling in ice water for 2 min. After the addition of hydrolysis buffer (300 μl) containing 0.6 M sodium borate, pH 8.5, 1% Triton X-100 was added, and the mixture was vortexed briefly and placed at 60°C for another 10 min to hydrolyze AE groups on unhybridized probes. Samples cooled on ice (2 min) and warmed to room temperature (2 min) were analyzed by a luminometer (Leader-50, GenProbe) equipped with automatic injection of detection reagent I (containing 0.1% hydrogen peroxide and 1 mM nitric acid) and detection reagent II (containing 1 N sodium hydroxide and a surfactant component). Quantities of emitted photons were measured by the luminometer, and the results were expressed as relative light units. Using dilutions of 8E5 T cells, the AE-labeled probes were able to detect 1–1,000 copies of HIV *gag* gene per PCR (30).

Superoxide Assay. Neutrophils or PBMCs obtained from healthy, HIV-seronegative donors or U1, U937, or HL60 promyelocytic cells in 0.1 ml of complete medium or HBSS (pH 7.2) were added to triplicate wells of a 96-well flat bottom microtiter plate. Assays for superoxide generation were performed (20, 31). Cells incubated (37°C, 5 min) with cytochrome C (0.025 ml, 0.001 mol/liter) were either cocultured with U1 cells (2 × 10⁵ to 2 × 10³) or treated with medium or with medium containing PMA (5 μM), CAT (58 μg/ml, 7 U per μg of protein), and/or SOD (300 U/ml); superoxide was monitored at the designated times. For all conditions, simultaneous controls containing 0.025 ml of 0.1 mg/ml SOD were set up in parallel. Absorbance of the cell suspensions was measured at 540 and 550 nm using an IBM-compatible 386 computer-assisted automated microtiter plate reader (model EL340; Bio-Tek Instruments Inc., Winooski, VT). The OD at 550 and 540 nm was compared in duplicate samples with and without SOD, and superoxide production was calculated as the SOD-inhibitable reduction of cytochrome C, using the extinction coefficient of 2.11/mmol/liter/cm (20).

Culture and Differentiation of HL60 Promyelocytic Cells. HL60 cells (CCL240; ATCC) were cultured in complete medium. Cells were differentiated by 1.3% DMSO over 6–9 d. Differentiation by DMSO is associated with morphologic maturation from promyelocytes to bands and neutrophils with the capacity to phagocytose, secrete primary granular enzymes, and rapidly generate ROIs in response to PMA and zymosan (31).

Detection of CMV. Healthy laboratorians were screened by EIA (Diamedix Corp.) for the presence of CMV-specific IgM and IgG antibodies at the clinical laboratory of The New York Hospital. PBMCs and PMNs from CMV serology-tested donors were obtained as previously described and used for cocultures or for detection of CMV DNA. PBMCs, PMNs, or HL60 cells were dissolved in DNA lysis buffer at 4 × 10⁷ cells per ml and were treated for 16 h with proteinase K as previously described. Human embryonic fibroblasts (HEFs; Baxter Bartels, Issaquah, WA) were infected with CMV (1993 proficiency testing isolate [sample 603], Laboratory for Virology, Wadsworth Center for Laboratory Research, New York State Department of Health, Albany, NY) and cultured for 6 d, at which time maximal numbers of cells showed cytopathic effects characteristic of intracellular CMV propagation. Supernatant and cells from five culture tubes concentrated by centrifugation at 200,000 g for 2 h were dissolved in DNA lysis buffer and treated with proteinase K as previously described. Purified DNA was obtained by extraction with phenol–chloroform and ethanol precipitation and suspended in 10 mM Tris, 1 mM EDTA, pH 7.4 buffer. Approximately 1.5–5.5 μg of DNA from CMV-infected HEFs or healthy donors was used to perform PCR to detect the

major immediate early antigen (IEA) using 50 pmol of each primer complementary to the IEA (nucleotide 2038–2300; upstream primer: 5'-GGT GCT CAC GCA CAT TGA TC-3'; downstream primer: 5'-AGA CCT TCA TGC AGA TCT CC-3') (32–34). The mixture was subjected to 50 cycles of amplification. Each cycle was performed at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. 20 μl of each amplified sample was electrophoretically separated on 1.5% agarose gel, stained with ethidium bromide, photographed, and transferred onto nylon by diffusion. The membrane was probed with 5' or 3' ^{32}P end-labeled oligonucleotide complementary to the internal sequence of the IEA (probe: 5'-CTA GTG TGA TGC TGG CCA AGC GGC CTC TGA-3'). PCR performed on DNA from CMV-infected HEFs (1.5–5.5 μg) diluted with 1.5 μg of DNA from PMNs of a CMV-seronegative donor was positive for CMV and was similar to samples not mixed with DNA from CMV-seronegative PMNs. CMV-positive samples contained a 262-bp fragment and were positive by Southern analysis.

Cytokine Assays. Commercial EIAs were used for detection of TNF-α and IL-6 (Genzyme Corp.). Interference was not detected by EIA in RPMI 1640 with 10% FBS. The following cytokines did not cross-react with the TNF-α assay kit: murine TNF-α, IL-3, IL-1-α and IL-1-β, IL-4, platelet-derived growth factor (PDGF), IL-2, IL-6, GM-CSF, epidermal growth factor (EGF), basic fibroblast growth factor (FGF), IFN-γ, and TNF-β. The following cytokines did not cross-react with the IL-6 assay kit: TNF-α, EGF, basic FGF, GM-CSF, IFN-γ, IL-1α and IL-1-β, IL-2, IL-4 G-CSF, PDGF, and insulin-like growth factor 1. The assay was performed as described by the manufacturer, absorbance was read at 450 nm (Bio-Tek Instruments Inc.), and a standard curve was constructed to quantitate IL-6 concentrations in controls and undiluted or serially diluted samples of culture supernatants.

Statistical Analysis. Data were analyzed using analysis of variance (ANOVA) and Kruskal-Wallis statistics, the respective parametric and nonparametric tests for repeat measures of multiple treatments (SAS software, version 6.04; SAS Institute Inc., Cary, NC). In addition, comparisons between two groups with single measure were analyzed by Student's *t* test of paired samples and Wilcoxon signed rank, the respective parametric and nonparametric tests. The alpha level was set at 0.05. Values are expressed as mean ± SEM, unless otherwise indicated. *n* defines the number of individual experiments, each using a different single donor.

Results

PMNs from HIV-seronegative Donors Induce HIV Replication by Mononuclear Cells from HIV-seropositive Patients. We investigated the interaction among PMNs and HIV-infected mononuclear cells because PMNs are the predominant cells recruited during acute STD. Coculture with mitogen-stimulated PBMCs from HIV-seronegative donors and IL-2 is the standard method to induce HIV replication from HIV-seropositive patients' mononuclear cells (35). Using this coculture of "mixed lymphocytes," the reported rates of positive viral cultures varied from 70 to 100% (35). As expected, we did not detect HIV p24 antigen when mononuclear cells from HIV-seropositive patients were cultured alone in medium or in medium containing 10% IL-2 (*n* = 3 and 13; Fig. 1). In contrast, PMNs from HIV-seronegative donors in the absence of exogenous IL-2 triggered HIV p24 production by mononuclear cells obtained from 17 patients (Fig. 1). The PMN-induced production of HIV p24 antigen by HIV-infected
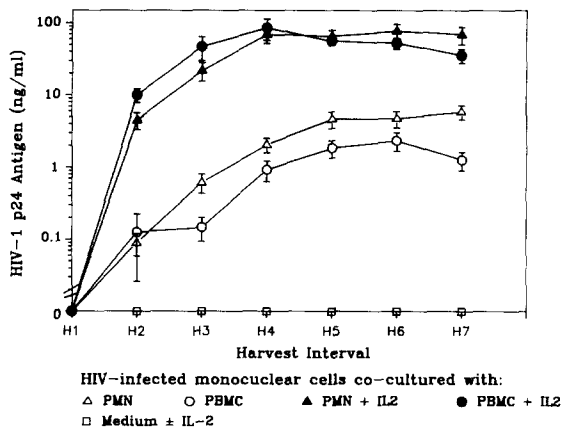

Figure 1. PMNs from HIV-seronegative donors induced HIV replication by mononuclear cells from HIV-infected patients. Illustrated are the results (mean ± SEM) of 17 separate HIV-infected patients' mononuclear cells (2 × 10⁶) cocultured with heterologous PMNs or PBMCs (each 2 × 10⁶) from low risk, HIV-seronegative volunteers in medium containing or lacking 10% IL-2. A significant increase over time in the amount of HIV p24 antigen was observed for PMNs and PBMCs cocultured with patients' mononuclear cells in medium containing or lacking IL-2 (*p* <0.05, Kruskal-Wallis statistics). In the absence of IL-2, the production of p24 antigen in cocultures with PMNs was significantly higher than the production with PBMCs (*p* < 0.05, Kruskal-Wallis statistics). PMNs (2 × 10⁶) and PHA-stimulated PBMCs (2 × 10⁶) obtained from HIV-seronegative, low risk volunteers were cultured with PBMCs of HIV-infected patients (2 × 10⁶ PBMC) for 21 d with one-half volume medium change every 2–4 d for harvest (*H*) intervals 1–7. In data not shown, lower dilutions of HIV-infected patients' PBMCs (2 × 10⁵ to 2 × 10³) cocultured with IL-2 and PMNs or PBMCs from HIV-seronegative donors produced similar amounts of HIV p24 antigen (*n* = 6).

mononuclear cells increased significantly over time (*p* <0.05, Kruskal-Wallis statistics, *n* = 17).

Continuous p24 antigen production by HIV-infected patients' mononuclear cells required weekly addition of PMNs. Without further addition of PMNs, a burst of p24 antigen reached by harvest 3 rapidly fell to undetectable levels (*n* = 3). The requirement for the weekly addition of fresh PMNs is due to loss of PMNs from cell death. When PMNs were cultured alone, 96 ± 1% (SD) of the PMNs were viable at the initiation of culture. By day 3, only 0.3% of the PMNs were viable in medium containing or lacking IL-2, and >80% of the cells' nuclei had disintegrated when compared with the first day of culture. By day 7 of culture, almost no nuclei were observed.

Even though HIV-seronegative donors' PMNs or PBMCs induced similar kinetics of p24 antigen production, PMNs in medium lacking IL-2 induced significantly greater amounts of p24 antigen than PBMCs from the same donors (Fig. 1, *p* <0.05, Kruskal-Wallis statistics). In the presence of 10% IL-2, similar levels of p24 antigen were induced by PMNs and PBMCs. The lack of constitutive production of p24 antigen by HIV-infected patients' mononuclear cells, the requirement for the weekly addition of PMNs, and the induction of p24 production by PMNs in medium without IL-2 provide evidence that PMNs triggered HIV replication from HIV-infected mononuclear cells.

Cell-free Supernatants from Cocultures of PMNs or PBMCs

with Mononuclear Cells of HIV-infected Patients Contain Replication-competent HIV. Cell-free viral supernatants were tested for replication-competent HIV because myeloperoxidase of neutrophils had been reported to deactivate HIV (36). Fig. 2 illustrates the propagation of HIV in a second round of viral cultures using cell-free supernatants of each coculture condition shown in Fig. 1. A time-dependent increase in new p24 antigen was detected in secondary cultures using the cell-free supernatants from cocultures of patients' mononuclear cells with either PMNs or PBMCs from HIV-seronegative donors (n = 4 separate patients). The kinetics of p24 antigen production in the second round of cocultures were comparable to those in the primary culture even though only 200 μl of the 1,500-μl total volume of the cell-free supernatant from the primary culture was used.

To confirm further that the HIV in the primary culture supernatant was infectious, the presence of HIV proviral DNA resulting from the reverse transcription of viral RNA in the cells of the secondary cultures was evaluated. HIV provirus was detected by gag gene PCR and probes in the secondary cultured cells' DNA (Table 1). The finding of increasing p24 production and HIV provirus in the secondary cultures indicates that the supernatants from primary cultures of HIV-infected patients with either PMNs or PBMCs from HIV-seronegative donors contained replication-competent HIV.

Effect of PMNs and C. trachomatis on HIV Production by U1 Cells. Since PMNs from HIV-seronegative donors can independently trigger HIV replication by mononuclear cells from HIV-infected patients, we questioned whether C. trachomatis could additionally increase HIV replication. Chroni-
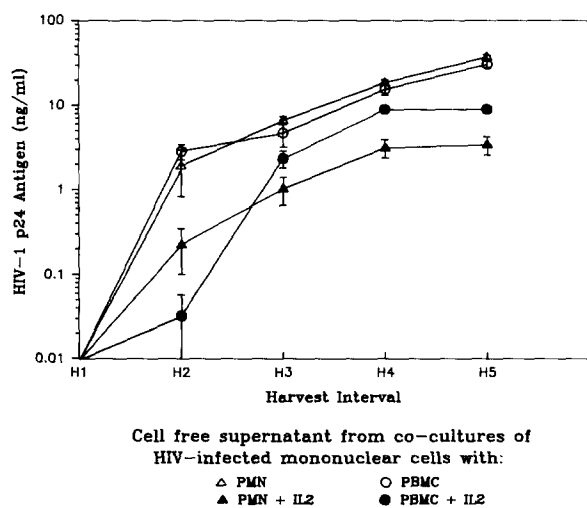

Cell free supernatant from co-cultures of HIV-infected mononuclear cells with:

△ PMN   ○ PBMC
▲ PMN + IL2   ● PBMC + IL2

Figure 2. Propagation of HIV from coculture supernatants of PMNs or PBMCs from HIV-seronegative donors with HIV-infected patients' mononuclear cells. Cell-free supernatants (200 μl) from cocultures of HIV-infected patients' mononuclear cells with HIV-seronegative donors' PMNs or PBMCs from Fig. 1 were used for a second round of viral culture using PHA-stimulated HIV-seronegative donor PBMCs. A time-dependent increase in p24 production was seen in the second round of viral cultures (p = 0.05, ANOVA and Kruskal-Wallis statistics). The results are the mean ± SE of four different patients' primary cultures, each with two donors, representing 32 separate secondary cultures. One-half volume medium was changed every 2–4 d for harvest (H) intervals 1–5.

Table 1. Cell-Free Supernatants from Cocultures of HIV-Infected Patients' Mononuclear Cells with PMNs or PBMCs from HIV-Seronegative Donors Contain Replication-Competent Virus

Propagation of HIV from cocultures of HIV-infected patients' mononuclear cells with	Copies of HIV per 10⁶ cells from secondary culture
PMNs	70 ± 18
PBMCs	25 ± 10
PMNs + IL-2	20 ± 10
PBMCs + IL-2	100 ± 35

Second-round viral cultures were performed with PHA-stimulated HIV-seronegative donor PBMCs and cell-free primary culture supernatants from HIV-seropositive patients' cells cocultured with PMNs or PBMCs from HIV-seronegative donors and without or with IL-2. After 21 d of culture, DNA was extracted from donor PBMCs of secondary culture and amplified by PCR for HIV gag gene. The copies of HIV provirus were determined by comparison with dilutions of 8E5 T cells. The results are the mean ± SE of four separate patients' primary cultures, representing 32 secondary cultures.

cally HIV-infected cell lines were used because they permit standardization of viral load, since the cell lines used contain either a single or a double copy of the HIV genome.

The interactions between chronically HIV-infected monocytic cell line, PMNs, and C. trachomatis are illustrated in Fig. 3. HIV p24 antigen production induced by PMNs increased in a time-dependent manner (n = 19, p <0.05, ANOVA and Kruskal-Wallis statistics). PMNs significantly enhanced the overall production of p24 antigen by U1 cells by 100-fold or more. The enhancement of HIV replication by PMNs was at least 10-fold greater than that by PBMCs (p <0.05, ANOVA and Kruskal-Wallis statistics). The higher production of HIV p24 antigen by U1 cells cocultured with PMNs, compared with PBMCs in medium without IL-2, was similar to the induction of HIV observed for HIV-infected patients' mononuclear cells in medium lacking IL-2. Furthermore, C. trachomatis in the presence of PMNs additionally increased up to 10-fold HIV replication in U1 cells and was significantly greater than that in U1 plus PMNs or U1 plus PBMCs (p <0.05, ANOVA and Kruskal-Wallis statistics). In contrast, C. trachomatis in the absence of PMNs did not enhance p24 antigen production by U1 cells (n = 3; data not shown).

The enhancement of HIV replication by PMNs was also observed with lower (2 × 10³) or higher (2 × 10⁵) U1 cell concentrations. However, the differences were less at higher U1 cell concentrations because of constitutive production of p24 antigen (n = 3; data not shown). Using HIV-infected patients' PBMCs, C. trachomatis in the presence of PMNs induced amounts of p24 antigen that were greater than those in cocultures containing PMNs and HIV-infected mononuclear cells from the same patients (n = 3; data not shown). Therefore, chlamydia induction of HIV required PMNs, because p24 antigen production by U1 cells or HIV-infected patients'
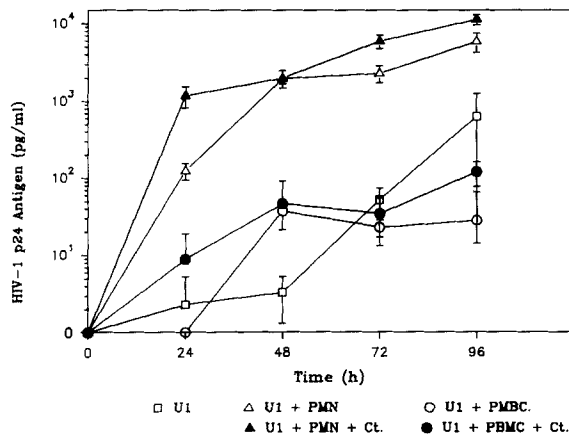
Figure 3. PMNs and *C. trachomatis* enhanced HIV replication in HIV-infected U1 monocytic cells. U1 cells (2×10^4) in complete medium lacking IL-2 were cocultured with PMNs (2×10^6) or PBMCs (2×10^6) from HIV-seronegative donors and with or without five ID_{50}s of *C. trachomatis* (*Ct.*). Significantly higher p24 antigen was produced by U1 cells cultured with PMNs than U1 cells alone or U1 cells plus PBMCs ($n = 17$, $p < 0.05$, ANOVA and Kruskal-Wallis statistics). The addition of *C. trachomatis* to U1 cells in the presence of PMNs further increased p24 antigen production above that of U1 cells with PMNs; *C. trachomatis* had no effect on p24 antigen production by U1 cells or U1 cells plus PBMCs ($n = 5$, $p < 0.05$, ANOVA and Kruskal-Wallis statistics). Statistically significant increase over time in the amount of p24 antigen was observed for U1 cells, U1 cells plus PMNs, and U1 cells plus PMNs and *C. trachomatis* ($n = 19$, $p < 0.05$, ANOVA and Kruskal-Wallis statistics), but not for U1 cells plus PBMCs or U1 cells plus PBMCs and *C. trachomatis*.
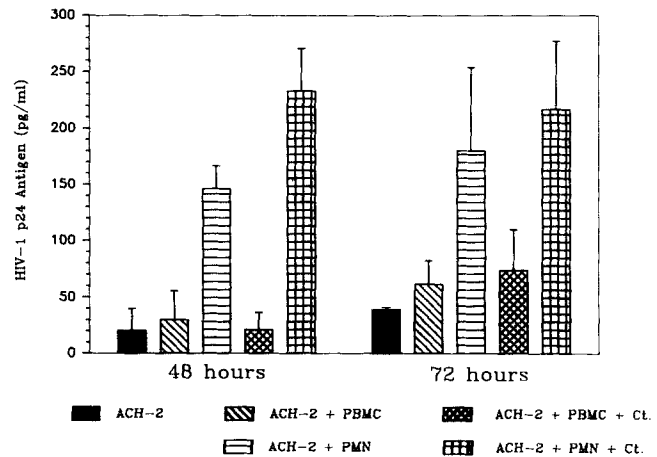

Figure 4. The effect of PMNs or PBMCs from HIV-seronegative donors and *C. trachomatis* on HIV replication by T cell lines. Illustrated is the time-dependent production of p24 antigen by ACH-2, chronically HIV-infected T cells. ACH-2 (2×10^4) and B-HIV1 (2×10^4) cells in medium lacking IL-2 were cultured with PMNs (2×10^6) or PBMCs (2×10^6) and with or without five ID_{50}s of *C. trachomatis* (*Ct.*). Compared with ACH-2 cells alone, significantly greater amounts of p24 antigen were demonstrated in ACH-2 cells cocultured with PMNs but not with PBMCs. Chlamydia additionally increased p24 antigen production by ACH-2 cells cocultured with PMNs and not with PBMCs ($p < 0.05$, Student's *t* test, $n = 4$). For B-HIV1 cells, experiments were conducted in medium containing 1% FBS and similar results were observed ($n = 2$; data not shown).

cells cultured with chlamydia alone was similar to that by U1 or HIV-infected patients' cells, which was, respectively, low or absent.

Effect of PMNs, PBMCs, and C. trachomatis on Chronically HIV-infected T and B Cell Lines. The interactions between PMNs or PBMCs and chronically HIV-infected T and B cell lines were investigated. PMNs enhanced HIV replication by ACH-2 T cells. Basal HIV p24 antigen production by 2×10^5 ACH-2 cells at 24, 48, and 72 h was 125 ± 40, 382 ± 85, and 850 ± 95 pg/ml, respectively ($n = 3$). Coculture of 2×10^5 ACH-2 cells with 2×10^6 PMNs increased HIV p24 antigen production by 5-, 14-, and 12-fold, respectively ($p < 0.05$ compared with basal). In Fig. 4, lower numbers of ACH-2 cells at 2×10^4 were used to study the interaction of ACH-2 cells with PMNs or PBMCs and *C. trachomatis* and to approximate the ratio of HIV-infected cells to PMNs present in semen. Basal production of p24 antigen by 2×10^4 ACH-2 cells was very low even at 48 and 72 h of culture. HIV p24 antigen production by ACH-2 cells cocultured with PMNs or with PMNs and chlamydia was increased by 8- and 12-fold, respectively ($p < 0.05$ compared with basal). In contrast, PBMCs or PBMCs with chlamydia had less effect on HIV replication by ACH-2 cells. The ability of PMNs or PMNs with chlamydia to increase HIV replication by B-HIV1 cells, a chronically HIV infected B cell line, was similarly observed ($n = 2$; data not shown).

Effect of CMV-negative PMNs on HIV replication. CMV infects both PBMCs and PMNs and has been reported to

enhance HIV replication (33, 34, 37). Although both PMNs and PBMCs harbor CMV during acute CMV infection, PBMCs are the major reservoir after the acute phase, and PMNs have been shown to be free of CMV by PCR even in CMV IgG-seropositive persons (34, 38). To exclude the involvement of CMV carried by PMNs in the enhancement of HIV replication, PMNs, obtained from donors who tested negative for CMV by serology, and HL60 promyelocytes were further tested for CMV by PCR using oligonucleotide primers and probe for the IEA gene of CMV. HEFs infected with a strain of CMV and one of six (17%) PBMC samples from CMV-seropositive laboratorians were positive for CMV DNA by PCR and Southern analysis, whereas PMNs from four CMV-seronegative donors, five CMV-seronegative laboratory workers, and HL60 cells were negative. CMV-negative PMNs similarly enhanced HIV p24 antigen production by U1 cells and ACH-2 cells ($n = 3$ CMV-seronegative and IEA gene–negative donors). Basal p24 antigen production by 2×10^4 U1 cells was 50 ± 32 pg/ml at 48 h of coculture. In the presence of PMNs from CMV-seronegative donors, HIV p24 antigen increased 25-fold ($1,250 \pm 150$ pg/ml).

ROIs Generated by PMNs or U1 Cells Trigger Viral Replication. PMNs are recruited into the genital tract by chlamydia infection. Since PMNs and mononuclear phagocytes generate ROIs during phagocytosis of microbes, we evaluated whether ROIs are also generated when PMNs from HIV-seronegative donors interact with HIV-infected U1 cells (Fig. 5). Unstimulated U1 cells or PMNs produced small amounts of superoxide that correlated with cell numbers (Fig. 5 *A*). PMA, a stimulus known to induce the oxidative burst, triggered rapid su-
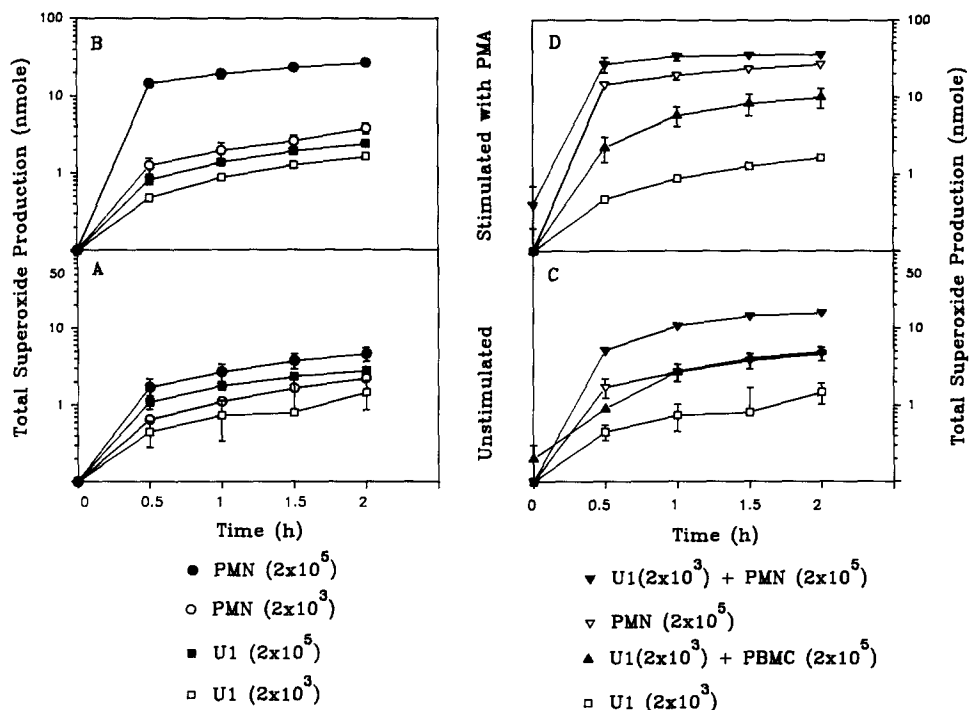
Figure 5. Induction of oxidative burst activity by U1 cells. PMNs or PBMCs (cell number per well as indicated) from HIV-seronegative donors in medium or cocultured with U1 cells (2 × 10^3 per well), or U1 cells (cell number per well as indicated) were stimulated or not with PMA (5 μM) for the designated time. All conditions were assayed in triplicate, and superoxide production by U1 cells or in response to U1 coculture is illustrated ($n = 3$–6).

peroxide production by PMNs, whereas U1 cells required more than 2 h (Fig. 5 B). When measured after 24 h of PMA stimulation, U1 cells generated 24.6 ± 2.1 nmol of superoxide per 2 × 10^5 cells, an amount 1.5-fold greater than basal production ($n = 3$). SOD abrogated both PMA-stimulated and basal production of superoxide by U1 cells. Cocultures of 2 × 10^5 PMNs with 2 × 10^3 U1 cells triggered amounts of superoxide that were greater than those for either cell alone (Fig. 5, C and D). Superoxide production by PMNs and U1 cells was significantly higher than that by U1 cells cocultured with PBMCs at the same cell concentration (p <0.05, Student's t test, $n = 3$; Fig. 5, C and D). The higher production of superoxide by PMNs was further increased by PMA. The larger amount of ROIs produced by PMNs may explain the greater production of p24 antigen induced by HIV-seronegative PMNs than by PBMCs in cocultures with HIV-infected patients' mononuclear cells (Fig. 1). The induction of superoxide production from PMNs and monocytic cells was not dependent on HIV infection, since PMNs cocultured with HIV-uninfected parental cells, U937 monocytic cells, or A3.01 T cells generated amounts of superoxide similar to those generated by PMNs cocultured with U1 cells ($n = 2$; data not shown).

We next examined whether ROIs produced endogenously or delivered by PMNs induced HIV replication, because exogenously added hydrogen peroxide is reported to trigger HIV replication in an HIV-infected T cell line (39–41). To provide evidence that ROIs mediate increasing HIV p24 production, SOD and CAT were used, because SOD catalyzes the transformation of superoxide to hydrogen peroxide and CAT breaks down hydrogen peroxide to molecular oxygen and water. Fig. 6 illustrates the effect of exogenous SOD

and CAT on HIV p24 antigen production. In the presence of SOD and CAT, the production of HIV by U1 cells triggered by PMA was reduced by >92% at 72 h. For cocultures of PMNs with U1 cells, SOD and CAT blocked viral production by 49–69% ($p < 0.05$, Student's t test, $n = 9$–12; Fig. 6, C and D). Individually, SOD or CAT were slightly less effective. Removal of ROIs by SOD and CAT resulted in diminished HIV replication because in the presence of heat-inactivated CAT and SOD, U1 cells stimulated with PMA or cultured with PMNs produced HIV at levels comparable to control levels ($n = 4$ and 6, respectively).

To evaluate the role of ROIs in the enhancement of HIV replication, the HL60 promyelocytic cell line was used. HL60 cells have been shown to produce undetectable amounts of superoxide, which was also confirmed in our laboratory (31). Culture of HL60 cells with 1.3% DMSO for 6–9 d resulted in cell differentiation associated with basal and PMA stimulated superoxide production. Basal and PMA (1 μM)-induced superoxide production by immature HL60 versus DMSO-differentiated HL60 cells was 0.6 ± 0.2 and 0.4 ± 0.2 versus 0.6 ± 0.3 and 38.2 ± 3.2 nmol per 2 × 10^5 cells at 120 min ($n = 2$, mean ± SD). Therefore, DMSO-differentiated HL60 cells required PMA to trigger the production of superoxide, as previously reported (31).

To evaluate the contribution of ROIs in the induction of HIV replication, U1 cells (2 × 10^4) were cultured with immature HL60 or DMSO-differentiated HL60 cells (2 × 10^6). At 48 h, basal p24 antigen production by U1 cells cocultured with immature HL60 cells was 106 ± 73 ($n = 2$, mean ± SD). To induce maximal production of superoxide, DMSO-differentiated HL60 U1 cells were stimulated with PMA (1 μM) for 5 min and washed twice to remove PMA.

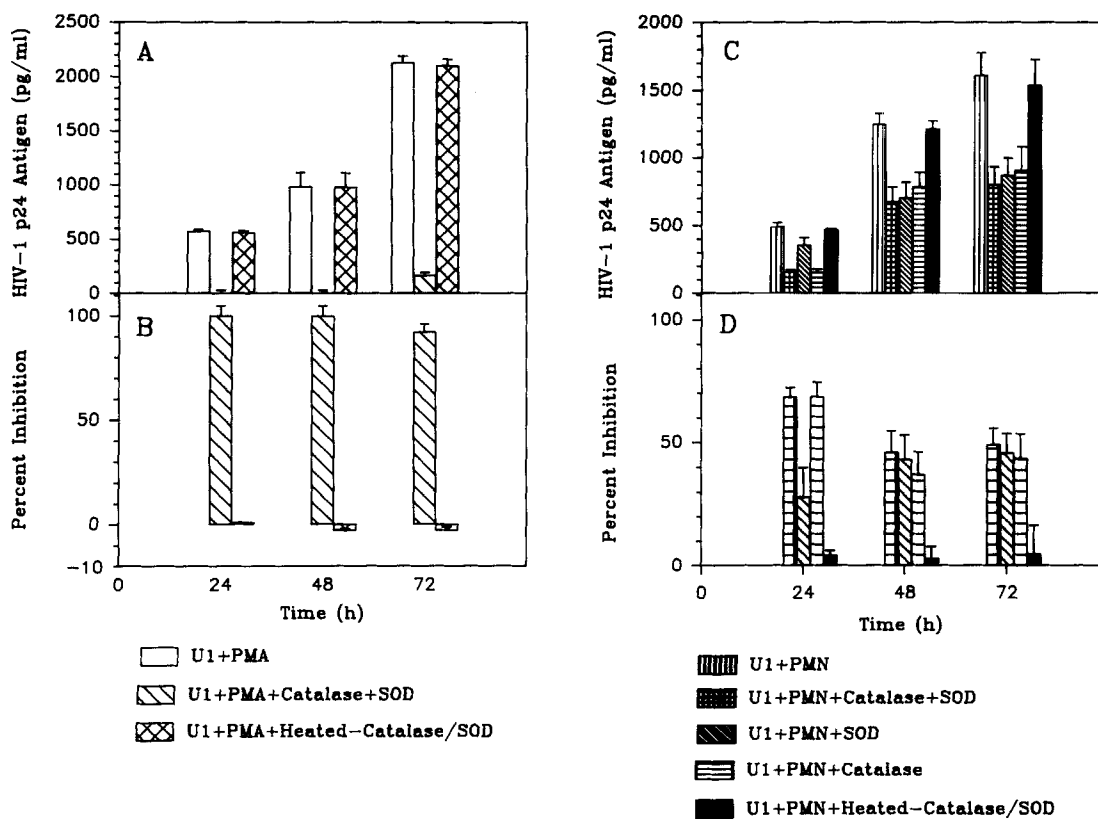**Figure 6.** The effect of CAT and SOD on HIV replication. U1 cells (2 × 10⁴) were stimulated with PMA (50 ng/ml) or PMNs (2 × 10⁶) from HIV-seronegative donors in complete medium or medium containing SOD (300 U/ml), CAT (58 μg/ml), or heat-inactivated SOD (300 U/ml) and CAT (58 μg/ml). One-half volume supernatant was exchanged daily with either medium or medium containing SOD, CAT, or heated SOD + CAT. Illustrated is the production of p24 antigen by U1 cells stimulated by PMA or by PMNs (*A* and *C*, respectively) and the percent inhibition by either SOD, CAT, or SOD + CAT (*B* and *D*, respectively). SOD + CAT significantly inhibited p24 antigen production by U1 cells stimulated by PMA or PMNs (p <0.05, Student's paired t test, n = 9 and 12, respectively). Production of p24 antigen in cocultures containing heated SOD + CAT was comparable to that by cells stimulated with PMNs or PMA (n = 6 and 4, respectively).

HIV p24 antigen production by U1 cells cocultured with DMSO-matured and PMA-stimulated HL60 cells was enhanced by 25-fold (5,853 ± 390 pg/ml). The addition of 300 U/ml SOD and 58 μg/ml CAT to DMSO-matured and PMA-stimulated HL60 cells abrogated the enhancement and resulted in p24 antigen production similar to that of chronically HIV-infected cells cocultured with immature HL60 cells or DMSO-matured HL60 cells: respectively, 487 ± 245 versus 106 ± 73 or 152 ± 107 pg/ml (n = 2, mean ± SD).

Contact between PMNs and U1 Cells Is Required for Induction of HIV Replication. To characterize further the mechanism for the induction of HIV replication, exogenous hydrogen peroxide was added to U1 cells. However, exogenously added hydrogen peroxide from 10 μM to 3 mM did not stimulate the production of p24 antigen. To explain this finding, we examined whether cell–cell contact and local delivery of ROIs triggered HIV replication (Table 2). PMNs separated from U1 cells by a 0.2-μm membrane insert (Millipore Corp.) and PMNs disrupted by freeze–thawing had no effect on HIV replication in U1 cells. Therefore, the induction of HIV replication by PMNs requires direct contact between U1 cells and viable PMNs to deliver ROIs.

Production of Soluble Factor by PMNs Cocultured with U937 Promonocytic Cells. Since exogenous SOD and CAT did not completely inhibit p24 antigen production induced by PMNs, experiments were performed to evaluate whether contact between PMNs and monocytic cells triggered the release of soluble factors. For these experiments we used U937 cells, the monocytic cell line from which U1 cells were derived after infection with a molecular clone of HIV. Cell-free supernatants from cocultures of PMNs and U937 cells, when added to U1 cells, induced a time-dependent increase in HIV p24 antigen (p <0.05, ANOVA; Table 2; Fig. 7). Supernatants from cocultures of freeze–thawed PMNs and U937 cells had no effect on HIV replication by U1 cells. Furthermore, p24 antigen production by U1 cells treated with supernatants from cocultures of PMNs and U937 cells containing SOD and CAT was reduced by 57% (p <0.05, ANOVA and Kruskal-Wallis statistic, n = 4). The maximum residual SOD and CAT may be 6.25% at the end of culture, since these enzymes were not additionally added to U1 cells. To evaluate whether soluble factors were present in the supernatant of PMN and U937 cocultures, fractionation of the supernatant was performed using molecular sieve centrifugation (Amicon

Table 2. *Induction of HIV Replication of U1 Cells Requires Contact with PMNs from HIV-Seronegative Donors*

Conditions	HIV p24 antigen production (pg per ml) by U1 cells	
	24 h	48 h
PMNs	128 ± 64	1,883 ± 312
Membrane-separated PMNs	0	0
Freeze–thawed PMNs	0	0
Supernatant of PMNs + U937 cells	327 ± 199	1,655 ± 454

PMNs (2 × 10⁶), freeze–thawed PMNs (10 ×, −70°C), and PMNs separated from U1 cells by a 0.2-μm membrane insert (Millipore Corp.) were cultured with U1 cells (2 × 10⁴). Cell-free supernatant (0.75 ml) from cocultures of PMNs (2 × 10⁶) and U937 cells (2 × 10⁶) at 48 h were added to U1 cells (2 × 10⁴, total volume of 1.5 ml), and one-half volume exchanges with complete medium were performed. The amounts of p24 antigen in the supernatants were assayed. Constitutive production of p24 antigen by U1 cells was subtracted from each condition (*n* = 2–6).

centrifugation system). Over 90% of the activity for induction of p24 antigen production by U1 cells resided in the <30,000 molecular size fraction of the coculture supernatants from PMNs and U937 cells as compared with the higher molecular size fraction (*n* = 2).

Induction of TNF-α and IL-6. The finding of a soluble factor(s) in the supernatant of PMN and U937 cell cocultures that enhanced p24 production by U1 cells led to the measurement of TNF-α and IL-6. These cytokines have been reported to enhance HIV production by U1 cells (42–44). Assays for TNF-α and IL-6 were performed on stored supernatants from HIV-infected patients' mononuclear cells cocultured with HIV-uninfected PMNs or PBMCs (Fig. 1), from PMN and U1 cell cocultures (Table 2), and from PMN and U937 cell cocultures (Fig. 7). A time-dependent increase in TNF-α and IL-6 was observed in the supernatants of PMNs or PBMCs from HIV-seronegative donors cocultured with HIV-infected patients' mononuclear cells (*p* <0.05 ANOVA, *n* = 4 separate patients; Fig. 8 *A*). The kinetics of TNF-α and IL-6 generation in Fig. 8 *A* paralleled those of p24 antigen production illustrated in Fig. 3. TNF-α and IL-6 were assayed in the supernatants of the U1 and PMN cocultures from experiments presented in Table 2. A fivefold increase was observed in TNF-α, and IL-6 was observed between 24 h and 72 h in U1 cells cocultured with PMNs but not with disrupted (freeze–thawed) PMNs (*n* = 3; Fig. 8 *B*). The amounts of cytokines detected paralleled p24 antigen production shown in Table 2.

Supernatants of the U937 cells and PMNs from experiments illustrated in Fig. 7 were assayed for TNF-α and IL-6. High levels of TNF-α and IL-6 were found in the supernatants from cocultures of PMNs and U937 cells but not in supernatants of cocultures of disrupted PMNs and U937 cells

(*n* = 8; Fig. 8 *C*). Furthermore, SOD and CAT, but not heated SOD/CAT abrogated the production of TNF-α and IL-6 by PMNs and U937 cells (*p* <0.05 compared with no SOD or CAT, Student's *t* test, *n* = 3 separate experiments). In contrast, the amount of p24 antigen produced by U1 cells triggered by supernatants from U937 cells and PMNs in medium containing SOD/CAT was decreased by 53% (Fig. 7). These results suggested that additional factors are involved in the induction of HIV replication.

Discussion

In this in vitro model, PMNs from HIV-seronegative donors induced HIV replication by mononuclear cells of HIV-infected patients even in the absence of IL-2. PMNs triggered HIV replication, because mononuclear cells from HIV-infected patients cultured alone did not produce measurable p24 antigen and because the addition of fresh PMNs was required to sustain the production of p24 antigen. PMNs also enhanced HIV replication by three different lineages of chronically HIV-infected cell lines. *C. trachomatis* in the presence of PMNs further enhanced HIV p24 production by U1 and other chronically HIV-infected cell lines; in contrast, *C. trachomatis* without PMNs had no independent effect on HIV replication.

Although these experiments strongly implicate PMNs as a mediator of viral replication, the possibility that dendritic cells are involved is not excluded. The finding that the PBMC
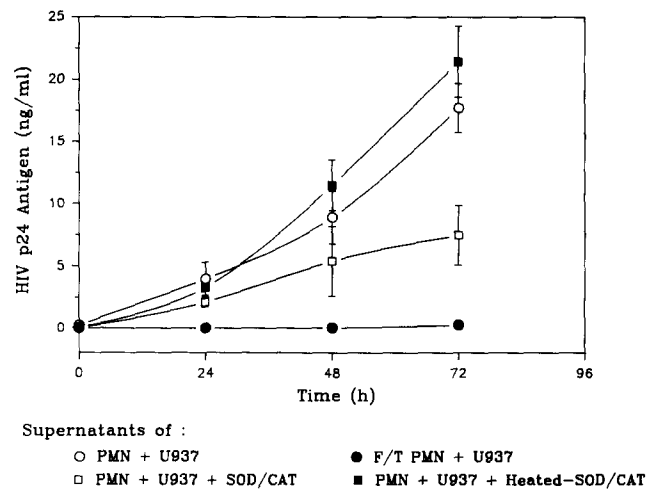

Supernatants of :

O PMN + U937   ● F/T PMN + U937

□ PMN + U937 + SOD/CAT   ■ PMN + U937 + Heated–SOD/CAT

Figure 7. The effect of SOD and CAT on the production of a soluble factor(s) from U937 cells cocultured with PMNs from HIV-seronegative donors. The promonocytic U937 cells not containing HIV (2 × 10⁴) were cocultured with PMNs or freeze–thawed (F/T) PMNs (2 × 10⁶) in medium or in medium containing 300 U/ml SOD and 58 μg/ml CAT (SOD/CAT) or heat-inactivated SOD/CAT. At 48 h of culture, cell-free supernatants (750 ml) from each of these conditions were added to HIV-infected U1 cells. Increasing amounts of p24 antigen were detected in cocultures of U1 cells with supernatants from PMNs + U937 cell, or PMNs + U937 cells + heated-SOD/CAT, but were not detected in coculture of U1 cells with supernatants from freeze–thawed PMNs + U937 cells (*p* <0.05, ANOVA, *n* = 4). The presence of SOD/CAT in the PMN and U937 cocultures reduced p24 antigen production by U1 cells (compared with no SOD/CAT; *p* <0.05, ANOVA, *n* = 4).
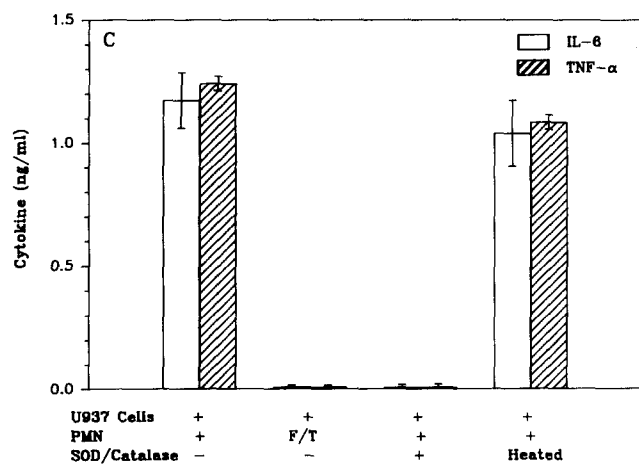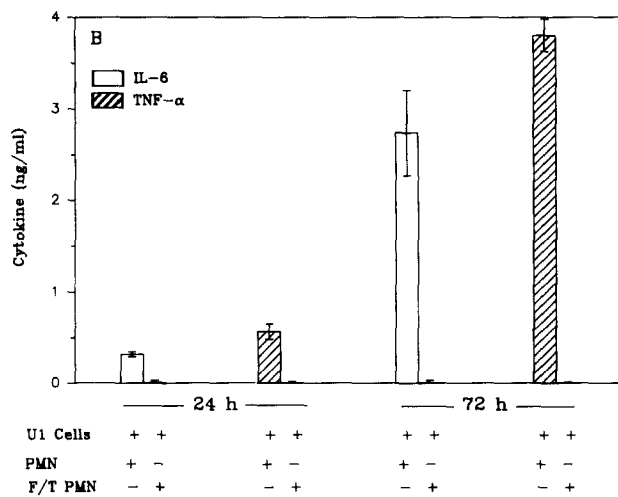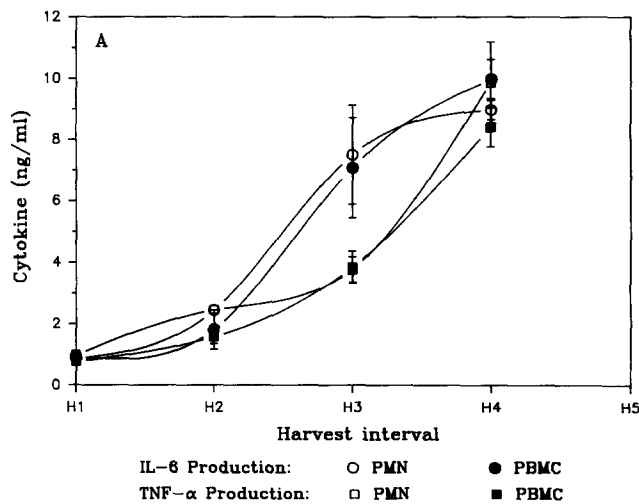
A

IL–6 Production: O PMN   ● PBMC
TNF–α Production: □ PMN   ■ PBMC

Harvest interval

B

IL–6
TNF–α

	24 h		72 h	
U1 Cells	+ +	+ +	+ +	+ +
PMN	+ –	+ –	+ –	+ –
F/T PMN	– +	– +	– +	– +

C

IL–6
TNF–α

U937 Cells	+	+	+	+
PMN	+	F/T	+	+
SOD/Catalase	–	–	+	Heated

Figure 8. The production of TNF-α and IL-6. Illustrated is the amount of TNF-α and IL-6 measured in coculture supernatants of PMNs or PBMCs from HIV-seronegative donors with mononuclear cells of HIV-infected patients (Fig. 1), PMNs with U1 cells (Table 2), and PMNs with U937 cells (Fig. 7). A time-dependent production of TNF-α and IL-6 was detected in supernatants of HIV-infected patients' mononuclear cells cocultured with PMNs or PBMCs (A) and in supernatants of U1 cells cocultured with PMNs but not with freeze–thawed (F/T) PMNs (B) ($p < 0.05$, Student's t test for four separate patients each with two donors and three separate U1 experiments). Similar levels of TNF-α and IL-6 were detected in supernatants from PMNs and U937 cells cocultured in medium or in medium containing heat-inactivated 300 U/ml SOD and 58 μg/ml CAT. (C) Barely detectable amounts of TNF-α and IL-6 were found, respectively, in supernatants of U937 cells cocultured with PMNs in the presence of SOD/CAT and with freeze–thawed PMNs ($n = 3$). One-half volume medium was changed every 2–4 d for harvest (H) intervals 1–5.

fraction, which contains most of the dendritic cells in blood, was less effective than PMNs in inducing HIV replication in patients' cells and cell lines suggests that the PMN is an inducer of HIV replication. In addition, it may not be necessary to exclude a role of dendritic cells in these in vitro experiments because the inflammatory exudate of STDs in the genital tract is likely to have dendritic cells present as a result of recruitment or breakdown of the mucosa.

Cell-free myeloperoxidase of PMNs has been reported to inactivate HIV in vitro (36). Our data indicate that HIV in the cell-free supernatant from cocultures of PMNs and patient PBMCs was replication competent. Evidence for replication competence included the propagation of virus in a second round of viral culture and the detection of HIV provirus in the nuclear extracts from these cells, indicating the reverse transcription of viral RNA to DNA. HIV may have remained infectious despite the presence of PMNs because of one or more of the following reasons: (a) most of the virus is in the fluid phase whereas myeloperoxidase is usually found within phagolysosomes; (b) extracellular myeloperoxidase may be less active in the presence of 10% FBS compared with serum-free conditions previously reported (36); and (c) the

amounts of virus produced may have overwhelmed the available myeloperoxidase.

Epidemiologic studies have defined that concurrent infection with many STDs, and in particular C. trachomatis infection, is associated with subsequent HIV seroconversion. However, a causal relationship between coinfection with other STDs and enhanced HIV transmission has not been demonstrated. Our findings suggest that PMNs trigger HIV replication by HIV-infected mononuclear cells and that C. trachomatis in the presence of PMNs additionally enhances HIV replication. Based on these in vitro observations, it is likely that women with a chlamydia infection have an increased risk for HIV infection because PMNs recruited by C. trachomatis induce the production of infectious viruses from the HIV-infected sex partner's mononuclear cells ejaculated in semen. Chlamydia in this environment may further augment HIV replication. Since PMNs from HIV-seronegative persons enhance HIV replication, it is possible that recruitment of PMNs into the female genital tract by any etiology may increase the risk for HIV transmission. Thus, the presence of PMNs may contribute to the increased risk for HIV seroconversion in women with trichomoniasis or gonorrhea (1, 7, 45, 46). Not exam-

ined in these experiments is whether autologous PMNs in an HIV-infected individual enhance HIV replication, making the genital secretions from persons coinfected with HIV and another STD more infectious.

One mechanism by which PMNs induced HIV replication is the generation of ROIs, because exogenous SOD and CAT partially or completely abrogated p24 antigen production induced by PMNs, PMA, or differentiated HL60 cells stimulated with PMA. The finding that local delivery of ROIs by PMNs triggered HIV replication extends the previous observations that exogenously added hydrogen peroxide induced HIV replication (39, 40). These investigators demonstrated that HIV replication induced by hydrogen peroxide is mediated by NF-κB transcriptional regulatory proteins (40, 41). In contrast to these reports, in our system the exogenous hydrogen peroxide did not induce HIV p24 antigen production. It is possible that the presence of 10% serum in the culture medium may have reacted with ROIs and therefore reduced the amount of ROIs reaching HIV-infected cells. It is possible that HIV-infected monocytic cells used in our system behave differently from T cell lines (39, 40). The failure of hydrogen peroxide to replace PMNs when SOD and CAT partially blocked the action of PMNs suggests that ROIs are necessary but not sufficient. Cell–cell contact may provide the necessary stimulus because p24 antigen production was abrogated in cocultures of U1 cells separated from PMNs by a porous membrane (Table 2). In this setting C. trachomatis triggers greater p24 antigen production because of greater amounts of ROIs generated by PMNs during phagocytosis of the microbe.

In addition to ROIs, cell–cell contact may provide another signal for the induction of HIV replication. This is supported by the lack of viral replication when U1 cells were separated by a membrane from PMNs or cocultured with disrupted PMNs. Furthermore, in cocultures of U1 cells with PMNs,

SOD and CAT inhibited only 69% of HIV p24 antigen production (Table 2; Fig. 6 B). In contrast, SOD and CAT inhibited >92% of the p24 production triggered by PMA (Fig. 6 A).

Cell–cell interaction also triggers the production of cytokines, such as TNF-α and IL-6, that feed back to enhance HIV production (42–44). Large amounts of TNF-α and IL-6 were detected in supernatants of cocultures containing PMNs with either patient mononuclear, U1, or U937 cells (Fig. 8, A–C). Under these coculture conditions, the time course and the amounts of HIV p24 antigen generated paralleled the production of these cytokines (Figs. 1 and 7; Table 2). The production of IL-6 and TNF-α was also inhibited by SOD and CAT. In addition to TNF-α and IL-6, other soluble factors are present because the supernatants from coculture of U937 cells and PMNs in medium containing SOD and CAT had barely detectable amounts of these cytokines while remaining able to induce p24 antigen at a level that was ~50% of the levels in PMN and U937 cell coculture without SOD and CAT.

These observations provide support for programs that target the prevention and prompt treatment of all STDs (7, 45, 46). Condoms, spermicides, or the combination have been advocated to reduce STD and HIV transmission (47–55). Clinical trials of nonoxynol-9, the major active ingredient of spermicides, have not conclusively demonstrated its ability to prevent HIV transmission (49, 56) probably because of the narrow therapeutic and toxicity range, since the mode of action of nonoxynol-9 is the disruption of cell membranes. Studies should be conducted to identify new agents with the ability to prevent STD and HIV transmission while preserving mucosal integrity and to define ways of maintaining healthy female genital epithelium. Our results suggest that local use of antioxidants should also be evaluated for their potential to decrease HIV replication and reduce HIV transmission.

References

1. Mann, J., D.J.M. Tarantola, and T.W. Netter, editors. 1992. A Global Report AIDS in the World. Harvard University Press, Cambridge, MA. pp. 11–132.

2. Wiley, J.A., S.J. Herschkorn, and N.S. Pandian. 1989. Heterogeneity in the probability of HIV transmission per sexual contact: the case of male-to-female transmission in penile-vaginal intercourse. *Statist. Med.* 8:93–102.

3. Hearst, N., and S.B. Hulley. 1988. Preventing the heterosexual spread of AIDS. Are we giving our patients the best advice? *J. Am. Med. Assoc.* 259:2428–2432.

4. Peterman, T.A., R.L. Stonebrunner, J.R. Allen, H.W. Jaffe, and J.W. Curran. 1988. The risk of human immunodeficiency virus transmission for heterosexual adults with transfusion-associated infections. *J. Am. Med. Assoc.* 259:55–58.

5. Pepin, J., F.A. Plummer, R.C. Brunham, P. Piot, D.W. Cameron, and A.R. Ronald. 1989. The interaction of HIV infection and other sexually transmitted diseases: an opportunity for intervention. *AIDS.* 3:3–9.

6. Keenlyside, R., A.M. Johnson, and D.C.W. Mabey. 1993. The epidemiology of HIV-1 infection and AIDS in women. *AIDS.* 7(Suppl. 1):S83–S90.

7. Wasserheit, J.N. 1991. Epidemiological synergy: interrelationships between HIV infection and other STDs. *In* AIDS and Women's Reproductive Health. L.C. Chen, editor. Plenum Publishing Corp., New York. 47–72.

8. Plummer, F.A., J.N. Simonsen, D.W. Cameron, J.O. Ndinya-Achola, J.K. Kreiss, M.N. Gakinya, P. Waiyaki, M. Cheang, P. Piot, A.R. Ronald, and E.N. Ngugi. 1991. Cofactors in male-to-female transmission of human immunodeficiency virus type-1. *J. Infect. Dis.* 163:233–239.

9. Hayes, R., and K.F. Schultz. 1992. What proportion of HIV infections are attributable to genital ulcers in sub-Sahara Africa. *8th International Conference on AIDS, Amsterdam.* Abstr. WD 4001.

10. Laga, M., A. Manoka, M. Kivuvu, B. Malele, M. Tuliza, N. Nzila, J. Goeman, F. Behets, V. Batter, M. Alary, et al. 1993. Non-ulcer sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. *AIDS.* 7:95–102.

11. Bowie, W.R., and K.K. Holmes. 1990. Chlamydia trachomatis. *In* Principles and Practice of Infectious Diseases. G.L. Mandell, R.G. Douglas, and J.E. Bennett, editors. 3rd ed. Churchill Livingston, New York. 1426–1440.

12. Freund, K.M., C.A. Buttlar, C. Giampaolo, R.S. Phillips, M.D. Aronson, and M.A. Moskowitz. 1992. The use of cervical cytology to identify women at risk for chlamydial infection. *Am. J. Prev. Med.* 8:29–36.

13. Krieger, J.N., R.W. Coombs, A.C. Collier, S.O. Ross, K. Chaloupka, D.A. Cummings, V.L. Murphy, and L. Corey. 1991. Recovery of human immunodeficiency virus type 1 from semen: minimal impact of stage of infection and current antiviral chemotherapy. *J. Infect. Dis.* 163:386–388.

14. Ho, D.D., R.T. Schooley, T.R. Rota, J.C. Kaplan, and T. Flynn. 1984. HTLV-III in the semen and blood of a healthy homosexual man. *Science (Wash. DC).* 226:451–453.

15. Zagury, D., J. Bernard, J. Liebowitch, J.E. Groopman, M. Feldman, and R.C. Gallo. 1984. HTLV-III in cells cultured from semen of two patients with AIDS. *Science (Wash. DC).* 226:449–451.

16. Levy, J.A. 1993. The transmission of HIV and factors influencing progression to AIDS. *Am. J. Med.* 95:86–100.

17. Ashida, E.R., and V.L. Scofield. 1987. Lymphocyte major histocompatibility complex-encoded class II structures may act as sperm receptors. *Proc. Natl. Acad. Sci. USA.* 84:3394–3395.

18. Bagasra, O., M. Freund, J. Weidman, and G. Harley. 1988. Interaction of human immunodeficiency virus with human sperm in vitro. *J. Acquired Immune Defic. Syndr.* 1:431–435.

19. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood; isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest.* 21(Suppl.):77–87.

20. Hatzigeorgiou, D.H., S.H. He, J. Sobel, A. Hafner, K. Grabstein, and J.L. Ho. 1993. Interleukin-6 down-modulates cytokine-enhanced antileishmanial killing. *J. Immunol.* 151: 3682–3692.

21. Metcalf, J.A., J.I. Gallin, W.M. Nauseef, and R.K. Root. 1986. Laboratory Manual of Neutrophil Function. Raven Press, New York. 2–3.

22. Ho, D.D., T. Moudgil, and M. Alam. 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N. Engl. J. Med.* 321:1621–1625.

23. Rothermel, C.D., J. Schachter, P. Lavrich, E.C. Lipsitz, and T. Francus. 1989. Chlamydia trachomatis-induced production of IL-1 by human monocytes. *Infect. Immun.* 57:2705–2711.

24. Caldwell, H.D., and P.J. Hitchcock. 1981. Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. *Infect. Immun.* 31:1161–1176.

25. Clouse, K.A., D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barstad, J. Kovacs, A. Fauci, and T.M. Folks. 1989. Monokine regulation of human immunodeficiency virus-1 expression in chronically infected human T cell clone. *J. Immunol.* 142:431–438.

26. Folks, T.M., J. Justement, A. Kinter, S. Schnittman, J. Orenstein, G. Poli, and A.S. Fauci. 1988. Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J. Immunol.* 140: 1117–1122.

27. Laurence, J., B. Grimison, C. Rodriquez-Alfageme, and S.M. Astrin. 1993. A model system for regulation of chronic HIV-1 infection in peripheral B lymphocytes. *Virology.* 196:433–441.

28. Rogers, M.F., C.-Y. Ou, M. Rayfield, P.A. Thomas, E.E. Schoenbaum, E. Abrams, K. Krasinki, P.A. Selwyn, J. Moore, A. Kaul, et al. 1989. Use of the polymerase chain reaction for early detection of the proviral sequence of human immunodeficiency virus in infants born to seropositive mothers. *N. Engl. J. Med.* 320:1649–1654.

29. Folks, T.M., D. Powell, M. Lightfoote, S. Koenig, A.S. Fauci, S. Benn, A. Rabson, D. Daugherty, H.E. Gendelman, M.D. Hoggan, et al. 1986. Biological and biochemical characterization of a cloned Leu-3⁻ cell surviving infection with the acquired immune deficiency syndrome retrovirus. *J. Exp. Med.* 164:280–290.

30. Ou, C.Y., S.H. McDonough, D. Cabanas, T.B. Ryder, M. Harper, J. Moore, and G. Schochetman. 1990. Rapid and quantitative detection of enzymatically amplified HIV-1 DNA using chemiluminescent oligonucleotide probes. *AIDS Res. Hum. Retroviruses.* 6:1323–1329.

31. Newburger, P.E., M.E. Chovaniec, J.S. Greenberger, and H.J. Cohen. 1979. Functional changes in human leukemic cell line HL-60: a model for myeloid differentiation. *J. Cell Biol.* 82:315–322.

32. Akrigg, A., G.W.G. Wilkinson, and J.D. Oram. 1985. The structure of the major immediate early gene of human cytomegalovirus strain AD169. *Virus Res.* 2:107–121.

33. Gerna, G., M. Parea, E. Percivalle, D. Zipeto, E. Silini, G. Barbarini, and G. Milanesi. 1990. Human cytomegalovirus viremia in HIV-1-seropositive patients at various clinical stages of infection. *AIDS (Phila.).* 4:1027–1031.

34. Gerna, G., D. Zipeto, E. Percivalle, M. Parea, M.G. Revello, R. Maccario, G. Peri, and G. Milanesi. 1992. Human cytomegalovirus infection of the major leukocyte subpopulations and evidence for initial viral replication in polymorphonuclear leukocytes from viremic patients. *J. Infect. Dis.* 166:1236–1244.

35. Bylund, D.J., U.H.M. Ziegner, and D.G. Hooper. 1992. Review of testing for human immunodeficiency virus. Laboratory Immunology II. *Clin. Lab. Med.* 12:305–333.

36. Klebinoff, S., and R.W. Coombs. 1992. Viricidal effects of polymorphonuclear leukocytes on human immunodeficiency virus-1. Role of the myeloperoxidase system. *J. Clin. Invest.* 89:2014–2017.

37. Laurence, J. 1990. Molecular interactions among herpes viruses and human immunodeficiency viruses. *J. Infect. Dis.* 162:338–346.

38. Taylor-Wiedeman, J., G.P. Hayhurst, J.G.P. Sissons, and J.H. Sinclair. 1993. Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals. *J. Gen. Virol.* 74:265–268.

39. Legrand-Poels, S., D. Vaira, J. Pincemail, A. van de Vorst, and J. Piette. 1990. Activation of human immunodeficiency virus type 1 by oxidative stress. *AIDS. Res. Hum. Retroviruses.* 6:1389–1397.

40. Schreck, R., P. Rieber, and P.A. Baeuerle. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO (Eur. Mol. Biol. Org.) J.* 10:2247–2258.

41. Schreck, R., R. Grassmann, B. Fleckenstein, and P.A. Baeuerle. 1992. Antioxidants selectively suppress activation of NF-κB by human T cell leukemia virus type-1 tax protein. *J. Virol.* 66:6288–6293.

42. Griffin, G.E., K. Leung, T.M. Folks, S. Kunkel, and G.J. Nabel. 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. *Nature (Lond.).* 339:70–73.

43. Folks, T.M., J. Justement, A. Kinter, C.A. Dinarello, and A.S. Fauci. 1987. Cytokine induced expression of HIV-1 in a chronically infected promonocytic cell line. *Science (Wash. DC).* 238:800–802.

44. Poli, G., P. Bressler, A. Kinter, E. Duh, W.C. Timmer, A. Rabson, J.S. Justement, S. Stanley, and A.S. Fauci. 1990. Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor α by transcription and post-transcriptional mechanisms. *J. Exp. Med.* 172:151–158.

45. Laga, M., A. Manoka, M. Kivuvu, B. Malele, M. Tuliza, N. Nzila, J. Goeman, F. Behets, V. Batter, M. Alary, et al. 1993. Non-ulcer sexually transmitted disease as risk factors for HIV-1 transmission in women: results from a cohort study. *AIDS.* 7:95–102.

46. Mehendale, S., J. Rodriques, T. Quinn, N. Mawar, A. Divekar, R. Bookmeyer, R. Gangakhedkar, J. Zenilman, A. Rompalo, S. Deshpande, et al. 1993. Risk factors for HIV in high risk participants in the preparation for AIDS/HIV vaccine efficacy study in India. Conference on Advances in AIDS vaccine development. *6th Annual Meeting of the National Cooperative Vaccine Development Groups for AIDS, Berlin.* Abstr. 132.

47. Cates, W., F.H. Stewart, and J. Trussel. 1992. Commentary: the quest for women's prophylactic methods–hope vs science. *Am. J. Public Health.* 82:1479–1482.

48. Cates, W., and K.M. Stone. 1992. Family planning, sexually transmitted diseases, and contraceptive choice: a literature update. *Fam. Plann. Perspect.* 24:75–84; 122–128.

49. Feldblum, P., S. Hira, S. Godwin, J. Kamanga, and G. Mukelabai. Efficacy of spermicide and condom use by HIV-discordant couples in Zambia. Abstract WEC 1085. Presented at the 8th International Conference on AIDS. July 22, 1992. Amsterdam, The Netherlands.

50. Niruthisard, S., R.E. Roddy, and S. Chutivongse. 1992. Use of nonoxynol-9 and reduction in rate of gonococcal and chlamydia cervical infection. *Lancet.* 339:1371–1375.

51. Chvapil, M., W. Droegemueller, J.A. Owen, C.D. Eskelson, and K. Betts. 1980. Studies of nonoxynol-9. I: the effect on the vagina of rabbits and rats. *Fertil. Steril.* 33:445–450.

52. Niruthisard, S., R.E. Roddy, and S. Chutivongse. 1991. The effects of frequent nonoxynol-9 use on the vaginal and cervical mucosa. *Sex. Transm. Dis.* 18:176–179.

53. Rosenberg, M.J., and E.L. Gollub. 1992. Commentary: methods women can use that may prevent sexually transmitted disease, including HIV. *Am. J. Public Health.* 82:1473–1478.

54. Bird, K.D. 1991. The use of spermicide containing nonoxynol-9 in the prevention of HIV infection. *AIDS.* 5:791–796.

55. Miller, C.J., A.G. Hendrickx, N.J. Alexander, P.A. Marx, and A. Gettie. 1992. The effect of virus dose and nonoxynol-9 on the genital transmission of SIV in Rhesus macaques. *Fertil. Steril.* 57:1126–1128.

56. Kreiss, J., E. Ngugi, K. Holmes, J. Ndinya-Achola, P. Waiyaki, P.L. Roberts, I. Ruminjo, R. Sajabi, J. Kimata, T.R. Fleming, et al. 1992. Efficacy of nonoxynol-9 contraceptive sponge use in preventing heterosexual acquisition of HIV in Nairobi prostitutes. *J. Am. Med. Assoc.* 268:477–482.