Activated B Lymphocytes from Human Immunodeficiency Virus–infected Individuals Induce Virus Expression in Infected T Cells and a Promonocytic Cell Line, U1

By Peter Rieckmann, Guido Poli, John H. Kehrl, and Anthony S. Fauci

From the Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary

Freshly isolated B lymphocytes from patients infected with human immunodeficiency virus (HIV), in contrast to B cells from normal controls, were shown to induce viral expression in two cell lines: ACH-2, a T cell line, and U1, a promonocytic cell line, which are chronically infected with HIV, as well as in autologous T cells. In 10 out of 10 HIV-infected individuals with hypergammaglobulinemia, spontaneous HIV-inductive capacity was found with highly purified peripheral blood B cells, whereas peripheral blood or tonsillar B cells from six healthy, HIV-negative donors did not induce HIV expression unless the cells were stimulated in vitro. The induction of HIV expression was observed in direct coculture experiments of B lymphocytes and HIV-infected cells, and could also be mediated by supernatants from cultures of B cells. Significantly higher amounts of interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) were detected in the B cell culture supernatants from HIV-infected patients with hypergammaglobulinemia (IL-6: $\bar{x}$ = 536 pg/ml; TNF-α: $\bar{x}$ = 493 pg/ml), as compared with normal uninfected controls (IL-6: $\bar{x}$ = 18 pg/ml; TNF-α: $\bar{x}$ = 23 pg/ml). Antibodies against these cytokines abolished the HIV-inductive capacity of B cells. We conclude that in vivo activated B cells in HIV-infected individuals can upregulate the expression of virus in infected cells by secreting cytokines such as TNF-α and IL-6, and, therefore, may play a role in the progression of HIV infection.

After infection with the HIV, the causative agent of AIDS, variable periods of clinical latency measured in years are observed in most infected individuals, suggesting that a state of low-level, persistent infection is established (reviewed in reference 1). To delineate the mechanisms regulating virus expression, two chronically HIV-infected cell lines, U1 (promonocytes) and ACH-2 (T cells), which constitutively express low to undetectable levels of virus, were established in our laboratory (2, 3). It has been demonstrated that cytokines such as TNF-α (4) and IL-6 (5) upregulate the expression of HIV in U1 (both cytokines) and ACH-2 (TNF-α only) cells, as well as in other cells or cell lines of T lymphocyte (6) and monocyte (5) origin.

An early and consistent feature of HIV infection is polyclonal B cell activation, characterized by hypergammaglobulinemia (7), and increased numbers of spontaneous Ig-secreting cells in the circulation (7, 8). Despite this state of hyperactivity, certain B cell functions such as in vitro response to *Staphylococcus aureus* Cowan strain 1 (SAC) or PWM and antibody production after antigenic challenge (7) are impaired in HIV-infected individuals.

Several recent reports have demonstrated that in vitro activated normal B cells produce TNF-α (9) and IL-6 (10). In vivo production of IL-6 by germinal center B cells of hyperplastic lymph nodes from patients with Castleman's disease (11) suggested that activated B lymphocytes may be an important source of cytokine production in vivo. Therefore, we investigated the capacity of B lymphocytes from HIV-infected individuals to secrete TNF-α and IL-6, and to induce HIV expression in infected cells.

Materials and Methods

Purification and Activation of B Cells. B cell–enriched populations from tonsils and lymph nodes were obtained as previously described (12). PBL were obtained from 10 HIV-seropositive individuals with hypergammaglobulinemia and six healthy seronegative donors by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation. T lymphocytes were removed as described (12), and the remaining cells were sorted for the B cell–specific antigen CD20 (Leu-16) with an Epics C-flow cytometer (Coulter Electronics Inc., Hialeah, FL). These preparations were routinely >98% Leu-16 (B cell) positive, and <1% Leu-M3

(monocyte), <1% Leu-11b (NK cells), and <1% Leu-5b (CD2, SRBC receptor on T cells) positive as determined by FACS analysis. All mAbs were purchased from Becton Dickinson & Co., Mountain View, CA).

B cells were cultured in the absence or presence of 0.01% formalin-inactivated SAC (Bethesda Research Laboratories, Gaithersburg, MD) and 100 U/ml of rIL-2 (Cetus Corp., Emeryville, CA) at 10^6 cells/ml. Overnight culture supernatants were collected, filtered through an 0.45-μm low protein binding filter (Millipore Continental Water Systems, Bedford, MA), and stored at −70°C until use. Supernatants from unstimulated and activated B cell cultures were tested for the presence of TNF-α and IL-6 by commercially available ELISA kits.

Coculture System. 100 μl of chronically HIV-infected cells (5×10^5/ml) or 100 μl of autologous T cells (10^6/ml) and 100 μl of various concentrations of B lymphocytes (10, 5, 2.5, 1.25, and 0.5×10^5/ml) resuspended in RPMI 1640 + 10% FCS were combined in 96-well plates. Supernatants were collected every 2 d and stored at −70°C. U1 and ACH-2 cells were also incubated at the same concentration in the presence or absence of either PMA (10^{-7} M; Sigma Chemical Co., St. Louis, MO), 100 U/ml of rTNF-α (Genzyme, Boston, MA), 100 U/ml of rIL-6 (Amgen Biologicals, Thousand Oaks, CA), or 25% (vol/vol) B cell supernatants. In the neutralization experiments, goat polyclonal and murine monoclonal anti-IL-6 antibodies (R&D Systems, Minneapolis, MN) and a monoclonal anti-TNF-α antibody (Olympus, Corporation of America, New Hyde Park, NY) were added alone or in combination to B cell supernatant–enriched media and incubated at room temperature for 30 min before addition to the HIV-infected cells. In coculture experiments, the antibodies were directly added at the beginning of the cocultivation with similar results.

Virus Detection. The presence of HIV reverse transcriptase (RT) activity in the culture supernatants was measured by the method of Willey et al. (13). p24 antigen in the culture supernatant was detected with a clone ELISA-based kit (Coulter Immunology, Hialeah, FL).

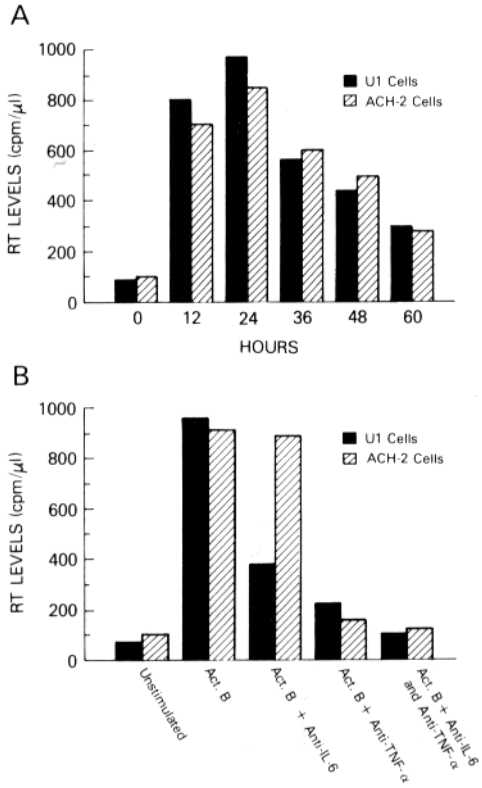

Figure 1. Induction of HIV expression by supernatants from activated normal B cells. (*A*) Effect of supernatants collected at different times from SAC/IL-2-activated tonsillar B cells on the chronically HIV-infected promonocyte cell (U1) and T cell (ACH-2) line. Cells were incubated in media containing 50% filtered B cell supernatant. Reverse transcriptase (RT) activity was measured at its peak induction (4 d post-stimulation). (*B*) Neutralization of supernatants from in vitro activated B cells (Act. B cells) by anticytokine antibodies. B cell supernatant–enriched media were preincubated for 30 min with the antibodies before adding to the infected U1 or ACH-2 cells. RT activity was measured after 4 d (peak activity).

Results

Activated B Cell Supernatants Induce HIV Expression in Infected Cell Lines. In initial studies with fresh tonsillar B cells, obtained from HIV-seronegative individuals, TNF-α and IL-6 secretion could be induced upon stimulation with SAC/IL-2 (data not shown). Supernatants from SAC/IL-2-stimulated B cells were then tested for their HIV-inductive capacity using U1 or ACH-2 cells, and the highest activity was present after 24 h (Fig. 1 *A*). Because TNF-α and IL-6 are known to induce HIV expression in a variety of infected cells, including U1 (both cytokines [4, 5]) and ACH-2 (TNF-α only [4]), these observations suggested that the secreted cytokines represented the major viral-inductive components of B cell supernatants. To further confirm this hypothesis, supernatants from B cells stimulated with SAC/IL-2 were preincubated with anti-TNF-α and/or anti-IL-6 antibodies before their addition to U1 or ACH-2 cells. As shown in Fig. 1 *B*, the anti-TNF-α antibody suppressed HIV expression in both ACH-2 and U1 cells incubated with supernatants from normal activated B cells. The anti-IL-6 antibody reduced B cell–dependent HIV expression in U1 cells, whereas it had no effect on ACH-2 cells, as previously reported (6). A combination of both antibodies completely blocked the virus production in U1 cells stimulated with B cell supernatants (Fig. 1 *B*).

In Vitro Activated Normal B Cells Induce HIV Expression in a Coculture System. Having established that B cell–derived cytokines can induce HIV expression in chronically infected cells, we investigated whether a similar effect could be seen in a direct cell-to-cell coculture system. When U1 or ACH-2 cells were cocultivated with SAC/IL-2 activated B cells, the peak of RT production was usually seen on the fourth day of culture (data not shown). In comparative experiments, the direct B cell coculture was found to be more sensitive than incubation with B cell supernatants in terms of HIV induction in U1 and ACH-2 cells. Therefore, this approach was used in all subsequent experiments.

In Vivo Activated B cells From HIV-infected Individuals Induce HIV Expression in a Coculture System. Having demonstrated that in vitro activation of B lymphocytes can induce HIV expression, we addressed the question whether in vivo activated B cells from HIV-infected individuals with hypergammaglobulinemia can exert similar effects. In all 10 patients

Table 1. *Laboratory Findings of HIV-infected Individuals and Seronegative Healthy Donors*

	HIV-infected individuals (n = 10)*	Healthy seronegative donors (n = 6)
B lymphocytes (number per μl)	128 ± 34	140 ± 46
CD4/CD8	0.6 ± 0.3	1.7 ± 0.4
IgG (mg/dl)	2,010 ± 450	1,089 ± 278
IgA (mg/dl)	436 ± 148	219 ± 89
IgM (mg/dl)	348 ± 104	200 ± 102
TNF-α (pg/ml)‡	493 ± 207	23 ± 4.7
IL-6 (pg/ml)‡	536 ± 226	18 ± 5.4

* Three of the HIV-infected individuals were asymptomatic, two had AIDS-related complex, and five had AIDS.
‡ TNF-α and IL-6 were measured in unstimulated overnight cultures from highly purified peripheral blood B lymphocytes using commercially available ELISA kits.

with IgG levels > 1,600 mg/dl investigated to date (Table 1), highly purified peripheral blood B lymphocytes (>99% $CD20^+$) secreted significant amounts of TNF-α and IL-6 (Table 1). When these cells were cocultivated with U1 or ACH-2 cells, they induced HIV expression without any in vitro stimulation (Fig. 2). In contrast, peripheral blood B cells from six healthy seronegative donors secreted low to undetectable levels of TNF-α and IL-6 (Table 1) and failed to induce HIV expression without in vitro stimulation (Fig. 2). The HIV-inductive effect of patients' B cells in these coculture systems was also blocked by anti-TNF-α and/or anti-IL-6 antibodies as observed with normal, in vitro activated B cells (data not shown). In two patients who underwent axillary lymphadenectomy, B cells obtained from the lymph node were also found to be in vivo activated since they secreted high levels of cytokines and induced HIV expression. This suggests that B cell activation is not confined to circulating cells, but can occur also in lymphoid tissue where B cells can be

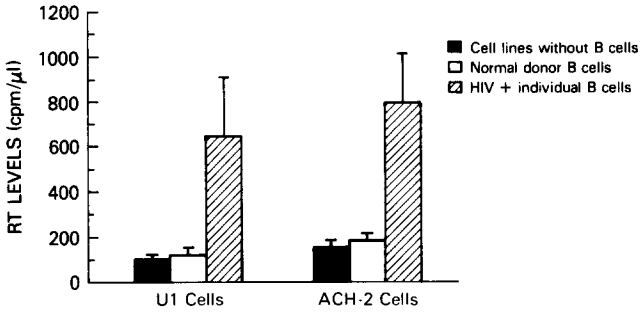

Figure 2. B lymphocytes from HIV-infected individuals but not normal donors induce HIV expression. Peripheral blood B lymphocytes from HIV-seropositive individuals with hypergammaglobulinemia (n = 10) and normal donors (n = 6) were cocultivated with HIV-infected cell lines (U1 or ACH-2) at a 1:1 ratio for 4 d without any stimulants.

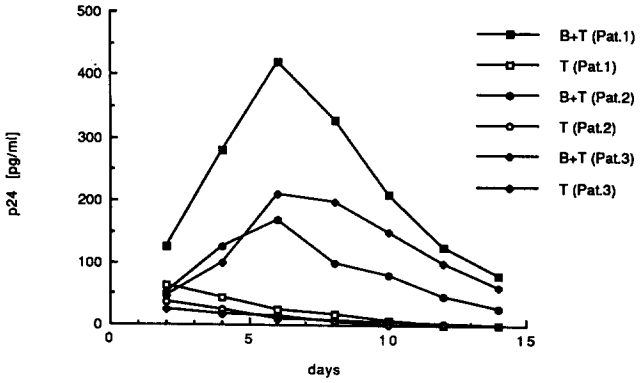

Figure 3. Induction of HIV expression in an unstimulated autologous coculture system. Separated B and T cells from the lymph node (patient 1) or peripheral blood (patients 2 and 3) were cocultured at a ratio of 1:1. Supernatant was collected every 2 d and assayed for p24 antigen (*closed symbols*, B + T coculture; *open symbols*; T cells alone).

in close cell-to-cell contact with HIV-infected cells. Finally, in an unstimulated coculture system of autologous B and T cells (optimal ratio, 1:1) from the same patient, increasing amounts of p24 antigen, which peaked around day 6, were detected in the culture supernatants (Fig. 3). This induction was clearly B cell dependent, as limiting dilution experiments revealed (data not shown). No increase of p24 release was observed if T cells were cultured in the absence of autologous B cells.

Discussion

The present study has demonstrated that activated B lymphocytes, which are present in the circulation of HIV-infected individuals with hypergammaglobulinemia, secrete TNF-α and IL-6 and induce HIV expression in chronically infected cells, as well as in autologous infected T cells. Given the consistent finding of polyclonally activated B cells in HIV-infected individuals (7), this study strongly suggests a potentially important role of cytokine production by these activated B cells in the induction of HIV expression in infected cells. IL-6 and TNF-α production in HIV infection has been attributed mainly to monocytes/macrophages (14, 15), and although the reported levels of cytokines produced by unstimulated overnight cultures of monocytes/macrophages from HIV-infected individuals were four- to eightfold higher than the amounts of IL-6 and TNF-α secreted by B cells, it must be taken into consideration that the adherence of monocytes to tissue culture flasks may increase cytokine production in vitro, as well as the fact that there is a much greater density of B cells compared with macrophages in lymph nodes of patients with HIV-related follicular hyperplasia (16). Our finding that virus can be induced in an unstimulated, autologous B/T cell coculture system is a close correlate to the possible interaction of these two cell types in vivo in lymphatic tissue.

Previous reports have described that HIV (17), HIV-derived proteins, or synthetic peptides from the gag-env region (18)

can exert a direct effect on B lymphocyte proliferation and differentiation. In some HIV-infected individuals, but none of the seronegative controls, we observed an enhancing effect of recombinant gp120 on the ability of B cells to secrete virus-inducing cytokines (P. Rieckmann et al., unpublished observation). It is therefore conceivable that HIV antigen–presenting follicular dendritic cells, present in the lymph nodes of HIV-infected individuals, can activate B cells in vivo to secrete cytokines that then upregulate virus expression in infected cells of the follicle and paracortical regions in a paracrine manner. In addition to its potential role in the regulation of HIV expression, the constitutive production of TNF-α and IL-6 by B cells may also explain in part the elevated Ig levels observed in these patients. In fact, both cytokines are involved in the proliferation and differentiation of B lymphocytes (10, 19), and an autocrine mechanism of B cell differentiation has been recently proposed for IL-6 (10). In addition, we have observed spontaneous TNF-α and IL-6 production in B cells from patients with SLE who were hypergammaglobulinemic. On the other hand, B cells from three HIV-infected individuals without hypergammaglobulinemia failed to spontaneously secrete HIV-inductive cytokines (P. Rieckmann et al., unpublished observation). Furthermore, direct effects of B lymphocytes on the induction of T cell abnormalities can also occur, and a recent report (20) has demonstrated that T lymphocyte dysfunction in a murine retrovirus-induced immunodeficiency syndrome is dependent on the presence of mature B cells.

In conclusion, our findings that in vivo activated B cells from HIV-infected individuals are able to upregulate HIV expression by constitutively secreting TNF-α and IL-6 focus on the importance of B cell activation during the progression of HIV infection. Chronic antigenic stimulation of B cells may account not only for the high Ig levels seen in HIV-infected individuals, but may also directly contribute to the expression of virus. These findings have important implications not only in the delineation of the scope of pathogenic mechanisms of HIV infection, but also may serve as a basis for the design of therapeutic regimens targeted to this process.

We thank H. C. Lane, G. Pantaleo, C. Fox, and S. Koenig for providing the clinical specimens used in this study and for their helpful discussions, as well as M. Baseler for purification of the B cells and M. A. Rust for her editorial assistance.

P. Rieckmann was supported by a fellowship from the Deutsche Forschungsgemeinschaft (DFG).

Address correspondence to Peter Rieckmann, Laboratory of Immunoregulation, Building 10, Room 11B13, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

Received for publication 10 July 1990 and in revised form 24 August 1990.

References

1. Rosenberg, Z.F., and A.S. Fauci. 1990. Immunopathogenic mechanisms of HIV infection: cytokine induction of HIV expression. *Immunol. Today.* 11:176.
2. 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 promonocyte cell line. *Science (Wash. DC).* 238:800.
3. Clouse, K.A., D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barrstad, J. Kovacs, A.S. Fauci, and T.M. Folks. 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *J. Immunol.* 142:431.
4. Poli, G., A. Kinter, J.S. Justement, J.H. Kehrl, P. Bressler, S. Stanley, and A.S. Fauci. 1990. Tumor necrosis factor-alpha functions in an autocrine manner in the induction of human immunodeficiency virus expression. *Proc. Natl. Acad. Sci. USA.* 87:782.
5. 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 transcriptional and post-transcriptional mechanisms. *J. Exp. Med.* 172:151.
6. Matsuyama, T., Y. Hamamoto, G.I. Soma, D. Mizuno, N. Yamamoto, and N. Kobayashi. 1989. Cytocidal effect of tumor necrosis factor on cells chronically infected with human immunodeficiency virus (HIV): Enhancement of HIV replication. *J. Virol.* 63:2504.
7. Lane, H.C., H. Masur, L.C. Edgar, G. Whalen, A.H. Rook, and A.S. Fauci. 1983. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 309:453.
8. Amadori, A., R. Zamarchi, V. Ciminale, A. Del Mistro, S. Siervo, A. Alberti, M. Colombatti, and L. Chieco-Bianchi. 1989. HIV-1-specific B cell activation: a major constituent of spontaneous B cell activation during HIV-1 infection. *J. Immunol.* 143:2146.
9. Sung, S.S.J., L.K.L. Jung, J.A. Walters, W. Chen, C.Y. Wang, and S.M. Fu. 1988. Production of tumor necrosis factor/cachectin by human B cell lines and tonsillar B cells. *J. Exp. Med.* 168:1539.
10. Muraguchi, A., T. Hirano, B. Tang, T. Matsuda, Y. Horii, K. Nakajima, and T. Kishimoto. 1988. The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. *J. Exp. Med.* 167:332.
11. Yoshizaki, K., T. Matsuda, N. Nishimoto, T. Kuritani, L.

Taeho, K. Aozasa, T. Nakahata, H. Kawai, H. Tagoh, T. Komori, S. Kishimoto, T. Hirano, and T. Kishimoto. 1989. Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease. *Blood.* 74:1360.

12. Falkoff, R.J.M., A. Muraguchi, J.X. Hong, J.L. Butler, C.A. Dinarello, and A.S. Fauci. 1983. The effects of interleukin 1 on human B cell activation and proliferation. *J. Immunol.* 131:801.
13. Willey, R.L., D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D. Capon, and M.A. Martin. 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J. Virol.* 62:139.
14. Clouse, K.A., P.B. Robbins, B. Fernie, J.M. Ostrove, and A.S. Fauci. 1989. Viral antigen stimulation of the production of human monokines capable of regulating HIV 1 expression. *J. Immunol.* 143:470.
15. Breen, E.C., A.R. Rezai, K. Nakajima, G.N. Beall, R.T. Mitsuyasu, T. Hirano, T. Kishimoto, and O. Martinez-Maza. 1990. Infection with HIV is associated with elevated IL-6 levels and production. *J. Immunol.* 144:480.
16. Biberfeld, P., Å. Öst, A. Porwit, B. Sandstedt, G. Pallesen, B. Böttiger, L. Morfelt-Mansson, and G. Biberfeld. 1987. Histopathology and immunohistology of HTLV-III/LAV related lymphadenopathy and AIDS. *Acta Pathol. Microbiol. Immunol. Scand. Sect. A Pathol.* 95:47.
17. Yarchoan, R., R.R. Redfield, and S. Broder. 1986. Mechanisms of B cell activation in patients with acquired immunodeficiency syndrome and related disorders. *J. Clin. Invest.* 78:439.
18. Nair, M.P.N., R. Pottathil, E.P. Heimer, and S.A. Schwartz. 1988. Immunoregulatory activities of human immunodeficiency virus (HIV) proteins: effects of HIV recombinant and synthetic peptides on immunoglobulin synthesis and proliferative responses by normal lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:6498.
19. Kehrl, J.H., A. Miller, and A.S. Fauci. 1987. Effect of tumor necrosis factor α on mitogen-activated human B cells. *J. Exp. Med.* 166:786.
20. Cerny, A., A.W. Hügin, R.R. Hardy, K. Hayakawa, R.M. Zinkernagel, M. Makino, and H.C. Morse III. 1990. B cells are required for induction of T cell abnormalities in a murine retrovirus-induced immunodeficiency syndrome. *J. Exp. Med.* 171:315.