Prognosis in HIV-1 Infection Predicted by the Quantity of Virus in Plasma

John W. Mellors,* Charles R. Rinaldo Jr., Phalguni Gupta, Roseanne M. White, John A. Todd, Lawrence A. Kingsley

The relation between viremia and clinical outcome in individuals infected with human immunodeficiency virus–type 1 (HIV-1) has important implications for therapeutic research and clinical care. HIV-1 RNA in plasma was quantified with a branched-DNA signal amplification assay as a measure of viral load in a cohort of 180 seropositive men studied for more than 10 years. The risk of acquired immunodeficiency syndrome (AIDS) and death in study subjects, including those with normal numbers of $CD4^+$ T cells, was directly related to plasma viral load at study entry. Plasma viral load was a better predictor of progression to AIDS and death than was the number of $CD4^+$ T cells.

The prognosis of individuals infected with HIV-1 is variable. In adults, the average time between infection and development of AIDS is 10 to 11 years (*1*), but a significant proportion of individuals (~20%) progresses rapidly to AIDS within 5 years of infection (*2*). At the other extreme, it is estimated that 12% of infected individuals will remain free of AIDS for 20 years (*2*).

Many clinical and laboratory markers have been used to estimate prognosis in HIV-1 infection (*3*). Although the single best predictor of AIDS onset characterized to date is the percentage or absolute number of circulating $CD4^+$ T cells (*4*), a marker that could be used to assess risk before substantial immune destruction has occurred would be preferable. Recent interest has focused on measurement of HIV-1 RNA in cells or plasma for prediction of outcome (*5*, *6*). Previous studies have suggested that the amount of HIV-1 RNA in plasma soon after HIV-1 infection (seroconversion) is a good $CD4^+$ T cell–independent predictor of AIDS risk (*7*). In clinical settings, however, the date and duration of HIV-1 infection are usually not known. We have now investigated the prognostic value of plasma HIV-1 RNA measurements in a large cohort of HIV-1–infected men for whom the duration of infection at study entry was not known.

The study population consisted of 209 HIV-1–infected gay or bisexual men enrolled in the Pittsburgh portion of the Multicenter AIDS Cohort Study (MACS) between April 1984 and March 1985 (*8*, *9*). These 209 men constitute all of the HIV-1–seropositive men enrolled at the Pittsburgh site; thus, there was no selection bias in choosing the study population. Clinical status, $CD4^+$ T cell counts (*10*, *11*), and specimens for laboratory studies were obtained at study entry (baseline) and at follow-up visits every 6 months. Heparinized plasma samples for HIV-1 RNA quantification were processed within 2 to 20 hours of collection and stored at −70°C until testing (*12*). Samples from 180 (86%) of the 209 men were available for testing. HIV-1 RNA was measured with an ultrasensitive branched-DNA signal amplification assay, which has a quantification limit of 500 molecules/ml and a linear dynamic range of up to 1.6×10^6 molecules/ml (*13*, *14*).

Subjects were followed for progression to AIDS (1987 Centers for Disease Control definition) and death. Median follow-up was 5.6 years (range, 0.02 to 10.6 years) for those who developed AIDS, and 10.6 years (range, 3.2 to 11.2 years) for those who remained free of AIDS. None of the subjects had received antiretroviral therapy by study entry or by the 6-month follow-up visit, and only 74 (41%) received antiretroviral therapy at any time during follow-up.

HIV-1 RNA concentrations in plasma samples obtained at study entry (baseline) were normally distributed over a range of <500 to 294,200 molecules/ml (Fig. 1A). In only 11 (6.1%) of 180 samples were baseline HIV-1 RNA concentrations below the quantification limit of the assay. Baseline HIV-1 RNA concentrations were significantly correlated ($P < 0.001$) with the corresponding absolute $CD4^+$ T cell counts, although the association was weak (Spearman's $r = -0.27$) (Fig. 1B). For example, among individuals with 400 to 800 $CD4^+$ T cells/μl, there was an ~400-fold range in HIV-1 RNA concentrations (≤500 to 192,200 molecules/ml). Thus, the $CD4^+$ T cell count in a subject within any $CD4^+$ T cell range was a grossly inaccurate indicator of the level of viremia.

The relations between baseline viral load or baseline $CD4^+$ T cell count and progression to AIDS or death were examined with Kaplan-Meier survival curves (*15*). Kaplan-Meier estimates of the proportion of subjects who progressed to either AIDS or death, stratified by quartiles according to baseline HIV-1 RNA concentrations or $CD4^+$ T cell counts, revealed that baseline viral load provided excellent discrimination of both time to AIDS and time to death (Mantel-Haenszel test, $P < 0.001$) (Fig. 2). For quartiles ranging from the lowest through the highest viral load, the proportion of subjects who progressed to AIDS by 5 years after study entry were 8, 26, 49, and 62%, respectively (Fig. 2A). The median times to development of AIDS for subjects in these viral load quartiles were >10, 7.7, 5.3, and 3.5 years, respectively.

Because death from AIDS was observed a median of 15 months after AIDS diagnosis, the Kaplan-Meier estimates of time to death were greater than those for the time to AIDS. For quartiles ranging from the lowest through the highest viral load, the proportions of subjects who died within 5 years were 5, 10, 25, and 49%, respectively (Fig. 2B). The median estimated survival times in these viral load quartiles were >10, 9.5, 7.4, and 5.1 years, respectively. In contrast to the close relation between baseline viral load and outcome, baseline $CD4^+$ T cell counts failed to show a strong gradient among quartiles for risk of AIDS or death. Among the three quartiles with the highest $CD4^+$ T cell counts, no differences were observed for either time to development of AIDS (Fig. 2C) or time to death (Fig. 2D). Only the quartile of subjects with the lowest $CD4^+$ T cell counts (≤321 cells/μl) was associated with a shorter time to development of AIDS or death ($P = 0.002$).

Additional evidence of the independence of viral load from $CD4^+$ T cell counts in influencing prognosis is shown in Fig. 3. Among subjects with baseline $CD4^+$ T cell counts of ≥500 cells/μl, there was a significant difference ($P < 0.001$) in time to death dependent on whether the baseline HIV-1 RNA concentration was greater than or less than the median value of 10,190 molecules/ml (Fig. 3A). The median time to death of subjects with a baseline HIV-1 RNA concentration of >10,190 molecules/ml was 6.8 years. In contrast, the median time to death of subjects with a baseline HIV-1 RNA concentration of ≤10,190 molecules/ml could not be accurately estimated because only

J. W. Mellors, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA; Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA; and Veterans Affairs Medical Center, Pittsburgh, PA 15240, USA.
C. R. Rinaldo Jr., Department of Pathology, School of Medicine, and Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA.
P. Gupta, Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA.
R. M. White and J. A. Todd, Chiron Corporation, Emeryville, CA 94608, USA.
L. A. Kingsley, Department of Infectious Diseases and Microbiology and Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA.

*To whom correspondence should be addressed at University of Pittsburgh Graduate School of Public Health, 403 Parran Hall, Pittsburgh, PA 15261, USA.

30% had died within 10 years. This major difference in outcome was evident despite the fact that the median $CD4^+$ T cell count for both groups was ~780 cells/μl. Similarly, among subjects with baseline $CD4^+$ T cell counts of <500 cells/μl, a significantly ($P < 0.001$) shorter survival time was evident among subjects with a baseline HIV-1 RNA concentration greater than the median value (17,320 molecules/ml), again despite similar baseline $CD4^+$ T cell counts (Fig. 3B).

To assess whether two consecutive measurements of viral load or $CD4^+$ T cell counts provided more prognostic information than did single determinations, we compared the mean results of the first two study samples (obtained at study entry and the 6-month follow-up visit) and ranked them by quartile (Fig. 4). Outcome discrimination was not improved by stratifying survival curves according to mean $CD4^+$ T cell counts (Fig. 4B), as compared to single baseline $CD4^+$ T cell counts (Fig. 2D). In contrast, a clearer separation was obtained when survival curves were stratified according to mean HIV-1 RNA concentrations (Fig. 4A), as compared with single baseline HIV-1 RNA values (Fig. 2B). Among subjects in the highest viral load quartile, a shorter time to death was evident with mean HIV-1 RNA measurements (median survival, 3.9 years) than with single baseline HIV-1 RNA measurements (median survival, 5.1 years). These results indicate a poorer prognosis for subjects with persistently high viral load.

The prognosis for subjects with persistently high viral load was explored further by excluding from the analysis those subjects in whom HIV-1 RNA concentrations decreased by >80% from baseline values by the 18- or 24-month follow-up visit (Fig. 4, C and D). These subjects ($n = 29$) were considered to be in the recovery phase of initial HIV-1 infection at study entry because viral load had not yet fallen to a post-serconversion nadir. Among subjects in the highest viral load quartile, this analysis reduced the estimated proportion with 10-year survival from 20 to ≤5% and reduced the median survival time from 5.1 to 2.5 years (Figs. 2B and 4C). In contrast, exclusion of these 29 subjects had no effect on survival when stratified by baseline $CD4^+$ T cell counts (Fig. 4D). These results provide additional evidence that persistently high viral load is almost always associated with more rapid disease progression.

The independent effects of $CD4^+$ T cell count and viral load on survival were formally examined with Cox proportional hazard models (Table 1) (*16*). The adjusted relative hazard of death was 1.55 ($P < 0.001$) for each threefold increase in baseline HIV-1 RNA concentration, and 1.03 ($P > 0.05$) for each decrease of 100 cells/μl in $CD4^+$ T cell count. The unadjusted relative hazard (1.06) associated with a 100-cell decrease in $CD4^+$ T cell count was statistically significant ($P < 0.05$), but it failed to remain so after controlling for HIV-1 RNA concentration. Hence, in comparing two individuals whose T cell counts differ by 100 cells/μl at baseline, these results indicate that the individual with a threefold higher HIV-1 RNA concentration faces a 1.5-fold greater risk of death within 10 years. The risk of death was assessed as a function of changes over time in both viral load and $CD4^+$ T cell count with time-dependent Cox proportional hazards analysis (Table 1). Consistent with the time-fixed analysis, the time-dependent analysis showed the adjusted relative hazard of death was 1.57 ($P < 0.001$) for each threefold increase in HIV-1 RNA concentration from the baseline value. In this instance, however, the relative hazard of death associated with a 100-cell decrease in $CD4^+$ T cell count from baseline remained statistically significant even after controlling for HIV-1 RNA values, albeit at a lower hazard (1.33).

Because the follow-up of subjects in our study was longer than that for any previous study of viral load, an analysis of long-term outcome of HIV-1 infection was possible. Such analysis showed a marked gradient of risk of disease progression and death that was directly related to the initial quantity of virus in plasma. Our observation that this risk gradient was still evident 10 years after the baseline determination emphasizes the critical influence of viral load on the course of HIV-1 infection. The Kaplan-Meier survival curves stratified by HIV-1 RNA quartile (Figs. 2 and 4) show prognostic discrimination similar to that of surgical staging procedures for cancer, including traditional staging systems for Hodgkin's disease (*17*) and the Duke classification for colorectal carcinoma (*18*), both of which are based on anatomic and pathological assessments of the extent of cancer invasion and spread. For HIV-1 infection, a similar degree of prognostic discrimination can be determined with a simple test on plasma.

Initial cross-sectional analyses of HIV-1–infected individuals showed a direct relation between the extent of immunodeficiency and viral load, measured as infec-

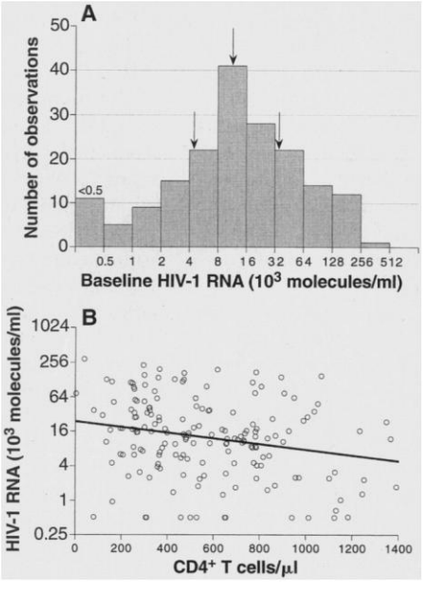
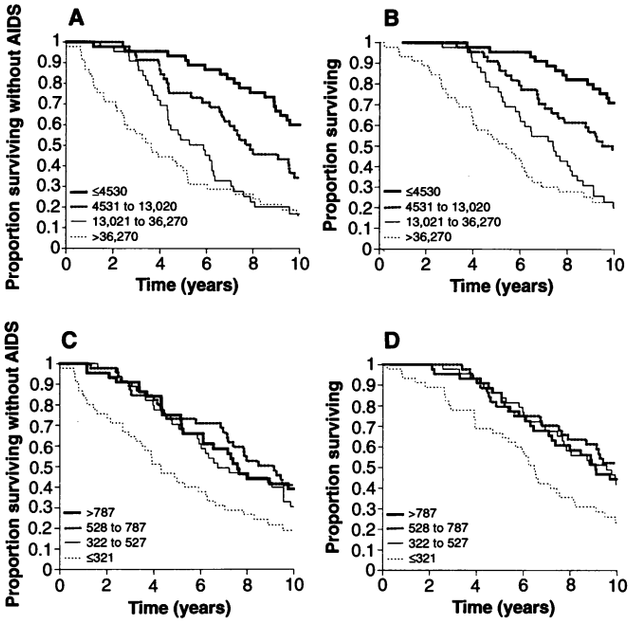


Fig. 1. (left) (**A**) Frequency distribution of HIV-1 RNA concentrations (log_2 scale) in baseline plasma samples. Arrows indicate location of 25th, 50th, and 75th percentiles. (**B**) Relation between baseline $CD4^+$ T cell counts and plasma HIV-1 RNA concentration (log_2 scale). The linear regression line is shown (Spearman's $r = -0.27$; $P < 0.001$). **Fig. 2. (right)** Relations between baseline markers and prognosis. Kaplan-Meier curves for AIDS-free survival (**A**) and survival (**B**) stratified by baseline HIV-1 RNA quartiles (molecules per milliliter), and for AIDS-free survival (**C**) and survival (**D**) stratified by baseline $CD4^+$ T cell quartiles (cells per microliter).



tious virus or viral nucleic acid (*19*). Studies of HIV-1 seroconverters established that the pattern of viremia after initial infection was predictive of clinical outcome (*7*). Individuals with persistently high viremia after seroconversion were at markedly increased risk of AIDS development (*7*). Saksela *et al.* (*5*) showed that the amount of intracellular HIV-1 mRNA in peripheral blood mononuclear cells of seroprevalent HIV-1–infected men predicted the likelihood of progression to AIDS even in individuals with >600 $CD4^+$ T cells/μl. Our study confirms and extends these observations by examining the risk of AIDS and death associated with the level of plasma viremia over a 10-year period.

In clinical practice, a $CD4^+$ T cell count of <500 cells/μl is commonly used as a trigger to initiate antiretroviral therapy. This practice requires reevaluation in light of our observations that subjects with $CD4^+$ T cell counts of ≥500 cells/μl can progress as rapidly to AIDS and death as those with much lower counts, depending on the extent of viremia. Specifically, 50% of individuals in our study with ≥500 $CD4^+$ T cells/μl (median, 780 cells/μl) and >10,900 HIV-1 RNA molecules/ml died within 6 years of study entry, compared with only 5% with similar $CD4^+$ T cell counts and HIV-1 RNA concentrations of ≤10,900 molecules/ml. Indeed, the rate of disease progression in subjects with ≥500 $CD4^+$ T cells/μl may exceed that in subjects with <500 cells/μl depending on the plasma viral load. Thus, treatment strategies should not be based solely on $CD4^+$ T cell numbers.

Studies by Wei *et al.* (*20*) and Ho *et al.* (*21*) have shown that viremia in HIV-1 infection is sustained by rapid, high-level viral replication, requiring continuous reinfection and destruction of $CD4^+$ T cells. Further kinetic analyses (*22*) indicate that the rate constant of viral clearance does not vary according to disease stage; thus, the extent of viremia depends on the rate of virus production. The factors that influence virus production in an individual are ill defined, but our study shows that a higher level of viremia—and, by inference, greater virus production—is associated with a poorer prognosis. Higher virus production may result in more rapid exhaustion of the capacity of the immune system to replenish $CD4^+$ T cells.

Several recent studies have examined whether reductions in viral load, measured as HIV-1 RNA, in response to antiretroviral therapy correlate with delayed disease progression to AIDS and death. O'Brien *et al.* (*23*) have shown that reductions in plasma HIV-1 RNA concentration in response to zidovudine treatment account for a substantial portion (59%) of the benefit of this drug in delaying the onset of AIDS. A decrease in HIV-1 RNA was a better predictor of outcome than was an increase in the $CD4^+$ T cell count. Two other studies of combination drug therapy have also demonstrated that 0.5 to 1.0 $\log_{10}$ reductions in HIV-1 RNA in response to treatment cor-

Table 1. Time-fixed and time-dependent Cox proportional hazards models of survival. Each analysis shows the relative risk of death associated with a 1-unit change in the covariates (a threefold increase in HIV-1 RNA concentration and a decrease of 100 cells/μl in $CD4^+$ T cell count). The time-fixed covariate model uses only the baseline marker measurements (n = 180). The time-dependent covariate model uses all available marker measurements (total number of measurements, 984; average number of measurements per subject, 5.5).

Variable	Relative hazard of death (95% confidence intervals)			
	Time-fixed covariates		Time-dependent covariates	
	Unadjusted	Adjusted	Unadjusted	Adjusted
HIV-1 RNA (threefold increase)	1.50* (1.36–1.82)	1.55* (1.34–1.80)	1.95* (1.72–2.21)	1.57* (1.34–1.82)
$CD4^+$ T cell count (100 cell/μl decrease)	1.06† (1.00–1.12)	1.03 (0.96–1.09)	1.62* (1.45–1.80)	1.33* (1.18–1.50)

*P < 0.001. †P < 0.05.

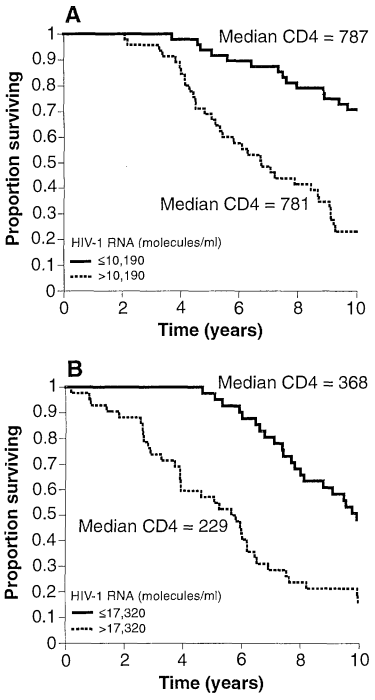


Fig. 3. Independence of the relation between prognosis and baseline HIV-1 RNA of the baseline $CD4^+$ T cell count. Kaplan-Meier survival curves stratified by median HIV-1 RNA concentration for subjects with baseline $CD4^+$ T cell counts of ≥500 cells/μl (**A**) or <500 cells/μl (**B**). The median baseline $CD4^+$ T cell count for each group is shown adjacent to the corresponding survival curve.

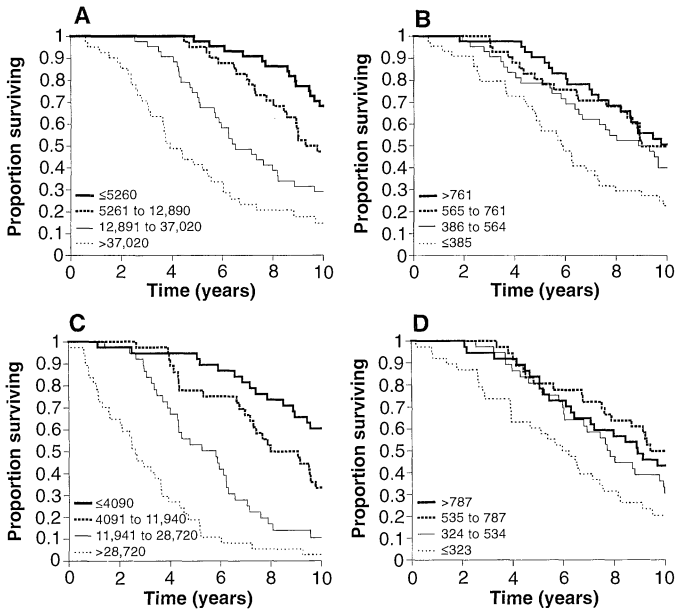


Fig. 4. (**A** and **B**) Kaplan-Meier survival curves stratified by quartiles of the mean of the first two HIV-1 RNA measurements (molecules per milliliter) (A) or $CD4^+$ T cell counts (cells per microliter) (B). Survival time for each of the 172 subjects for whom two consecutive samples were available was calculated from the number of years from the midpoint between the two viral measurements and the time of death. (**C** and **D**) Survival curves excluding subjects with a >80% decrease in HIV-1 RNA from baseline (n = 29) stratified by quartiles of single baseline HIV-1 RNA measurement (C) or single baseline $CD4^+$ T cell count (D).

relate with delayed disease progression and death (*24*).

Collectively, these data indicate that the extent of viremia, measured as HIV-1 RNA, is the best available surrogate marker of HIV-1 disease progression. Several of the rational criteria for demonstrating the adequacy of a surrogate marker as put forward by DeGruttola *et al.* (*25*) appear to have been met: (i) Base-line HIV-1 RNA concentrations are highly predictive of prognosis. (ii) There is a strong time-dependent prognostic relation between HIV-1 RNA and outcome. And (iii) reduced concentrations of HIV-1 RNA, in response to antiretroviral therapy, are predictive of improved prognosis (*24*). Use of HIV-1 RNA as a surrogate marker should help guide future therapeutic research and individual patient management.

REFERENCES AND NOTES

1. A. Muñoz *et al.*, *Am. J. Epidemiol.* **130**, 530 (1989).
2. A. Muñoz *et al.*, *J. AIDS Hum. Retrovir.* **8**, 496 (1995).
3. C. M. Tsoukas and N. F. Bernard, *Clin. Microbiol. Rev.* **7**, 14 (1994).
4. J. L. Fahey *et al.*, *N. Engl. J. Med.* **322**, 166 (1990).
5. K. Saksela, C. Stevens, P. Rubinstein, P. E. Taylor, D. Baltimore, *Ann. Intern. Med.* **123**, 641 (1995).
6. E. Hogervorst *et al.*, *J. Infect. Dis.* **171**, 811 (1995).
7. J. W. Mellors *et al.*, *Ann. Intern. Med.* **122**, 573 (1995); S. Jurrians *et al.*, *Virology* **204**, 223 (1994); D. R. Henrard *et al.*, *JAMA* **274**, 554 (1995).
8. Details of the recruitment and characteristics of the MACS cohort have been described elsewhere (*9*). The MACS protocol was approved by the Biomedical Institutional Review Board of the University of Pittsburgh. All subjects gave written informed consent after the potential consequences of study participation were explained.
9. R. A. Kaslow *et al.*, *Am. J. Epidemiol.* **126**, 310 (1987).
10. T lymphocyte subsets were measured in whole blood by staining with fluorescent dye–conjugated monoclonal antibodies specific for CD3, CD4, and CD8 (Becton Dickinson, Mountain View, CA) as described (*11*). The total number of $CD4^+$ T cells was determined by multiplying the percentage of lymphocytes that were $CD4^+$ T cells by the total lymphocyte count.
11. J. Giorgi *et al.*, *Clin. Immunol. Immunopathol.* **55**, 173 (1990).
12. For subjects developing AIDS, the samples tested were from study entry, the first follow-up visit (6 months), the most recent visit before AIDS diagnosis, and equally spaced visits in between. For AIDS-free subjects, the samples tested were from study entry, the 6-month follow-up visit, the last available visit, and equally spaced visits in between.
13. Developed by Chiron, the assay measures HIV-1 RNA associated with viral particles that are separated from 1.0-ml plasma samples by centrifugation at 23,500*g* for 1 hour at 4°C. Performance characteristics of the assay have been described elsewhere (*14*). For our study, the interassay coefficient of variation for the positive control samples tested with each batch of experimental samples was 18%. All samples were coded and the assay operators were blinded to clinical outcomes.
14. J. Todd *et al.*, *J. AIDS Human Retrovir.* **10**, S35 (1995).
15. E. L. Kaplan and P. Meier, *J. Am. Stat. Assoc.* **53**, 457 (1958). Kaplan-Meier analyses were performed with Statistica version 4.5 (StatSoft, Tulsa, OK).
16. D. R. Cox and D. Oakes, *Analysis of Survival Data* (Chapman & Hall, London, 1984). Cox proportional hazards analyses were performed with Splus version 3.3 (StatSci Division of Mathsoft, Seattle, WA).
17. B. J. Kennedy *et al.*, *Cancer* **56**, 2547 (1985).
18. K. W. Falterman *et al.*, *ibid.* **34**, 951 (1974).
19. D. D. Ho, T. Moudgil, M. Alam, *N. Engl. J. Med.* **321**, 1621 (1989); R. W. Coombs *et al.*, *ibid.*, p. 1626; P. Gupta *et al.*, *Virology* **196**, 586 (1993).
20. X. Wei *et al.*, *Nature* **373**, 117 (1995).
21. D. D. Ho *et al.*, *ibid.*, p. 123.
22. A. S. Perelson, A. U. Neumann, M. Markowitz, J. M. Leonard, D. D. Ho, *Science* **271**, 1582 (1996).
23. W. A. O'Brien *et al.*, *N. Engl. J. Med.* **334**, 426 (1996).
24. W. Freimuth *et al.*, paper presented at the Third Conference on Retroviruses and Opportunistic Infections (Infectious Disease Society of America), Washington, DC, 28 February to 1 March 1996; S. Hammer *et al.*, *ibid.*
25. V. DeGruttola, R. Gelman, S. Lagakos, in *AIDS Clinical Trials*, D. Finkelstein and D. A. Shoenfeld, Eds. (Wiley, New York, 1995), pp. 129–142.

26. We thank the participants and staff of the Pitt Men's Study for their dedication and support. Supported by Public Health Service contract N01-AI-72632 and cooperative agreement U01-AI-35041 and by grant support from the National Institutes of Health (R01 AI34301-01 and AI34294-01A2), the Medical Research Service of the Department of Veterans Affairs, and the Pathology Education and Research Foundation of the University of Pittsburgh.

20 March 1996; accepted 17 April 1996

Regulation of T Cell Receptor Signaling by Tyrosine Phosphatase SYP Association with CTLA-4

Luc E. M. Marengère, Paul Waterhouse, Gordon S. Duncan, Hans-Willi Mittrücker, Gen-Sheng Feng, Tak W. Mak*

The absence of CTLA-4 results in uncontrolled T cell proliferation. The T cell receptor–specific kinases FYN, LCK, and ZAP-70 as well as the RAS pathway were found to be activated in T cells of *Ctla-4*$^{-/-}$ mutant mice. In addition, CTLA-4 specifically associated with the tyrosine phosphatase SYP, an interaction mediated by the SRC homology 2 (SH2) domains of SYP and the phosphotyrosine sequence Tyr-Val-Lys-Met within the CTLA-4 cytoplasmic tail. The CTLA-4–associated SYP had phosphatase activity toward the RAS regulator $p52^{SHC}$. Thus, the RAS pathway and T cell activation through the T cell receptor are regulated by CTLA-4–associated SYP.

Activation through the T cell receptor (TCR) results in increased surface expression of CTLA-4 (*1*). Although CTLA-4 is homologous to the T cell costimulatory molecule CD28 (*2*), CTLA-4–deficient mice have constitutively activated T cells (*3*). Thus, in contrast to CD28, CTLA-4 appears to be a negative regulator of T cell activation (*3*). Initial events in TCR signaling involve the protein tyrosine kinases FYN, LCK, and ZAP-70 (*4*). We now show that FYN, LCK, and ZAP-70, as well as the RAS pathway, are constitutively activated in the T cells of *Ctla-4*$^{-/-}$ mutant mice. This identifies CTLA-4 as a negative regulator of proximal events after activation through the TCR.

We investigated the most proximal effectors in TCR signaling events, the tyrosine kinases FYN, LCK, and ZAP-70, comparing tyrosine kinase activities in primary T cells isolated from lymph nodes of *Ctla-4*$^{-/-}$ mice. The activities of all three tyrosine kinases were increased in homozygous mutant T cells, as shown by elevated autophosphorylation and increased phosphorylation of coimmunoprecipitating proteins (Fig. 1A). ZAP-70 is tyrosine phosphorylated after TCR stimulation (*5*); we also observed constitutive phosphorylation of ZAP-70 in mutant T cells (Fig. 1B). Increased protein phosphorylation in mutant T cells did not reflect elevated expression of tyrosine kinases, as protein immunoblot analyses of immunoprecipitated FYN, LCK, and ZAP-70 showed similar quantities in wild-type and mutant cells (Fig. 1C). Thus, the tyrosine kinases associated with the proximal events in TCR signaling are hyperactive in T cells in the absence of CTLA-4.

In mutant T cells analyzed ex vivo, we observed a drastic increase in tyrosine phosphorylation of 16- and 50-kD proteins and a moderate increase in phosphorylation of 23-, 36-, and 140-kD proteins (Fig. 2A). The 23-, 36-, and 140-kD proteins are targets of tyrosine phosphorylation after TCR activation (*6*). When the antigen receptor is stimulated, the 16-kD TCR subunit CD3ζ and the 50-kD adapter SRC homology and collagen (SHC) are hyperphosphorylated at tyrosine residues (*6*). As previously reported, CD3ζ has a low amount of constitutive phosphorylation (*7*). Together these data raised the possibility that the 16- and 50-kD hyperphosphorylated proteins in mutant cells might be CD3ζ and $p52^{SHC}$,

L. E. M. Marengère, P. Waterhouse, G. S. Duncan, H.-W. Mittrücker, T. W. Mak, AMGEN Institute, Ontario Cancer Institute, Departments of Immunology and Medical Biophysics, University of Toronto, Toronto, Ontario, M5G 2C1, Canada.
G.-S. Feng, Department of Biochemistry and Molecular Biology and Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202–5121, USA.
*To whom correspondence should be addressed.