A Mathematical Model of Combined Drug Therapy of HIV Infection

DENISE E. KIRSCHNER[a] and G. F. WEBB[b,*]

[a]*Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109;*
[b]*Department of Mathematics, Vanderbilt University, Nashville, TN 37240 USA*

A mathematical model of the HIV infected human immune system is presented. The model consists of a system of ordinary differential equations for the populations of uninfected $CD4^+$ T cells, infected $CD4^+$ T cells and free virus in the blood. The model is constructed, and parameters chosen, so that turnover rates and life spans for these populations agree with clinical data. The model is used to simulate chemotherapy treatment of HIV infection. The simulations are based upon preliminary clinical reports of treatment with combinations of antiviral drugs involving standard reverse transcriptase inhibitors and newly developed protease inhibitors. The models incorporate the appearance of drug-resistant viral strains, which is the key limiting factor in the effectiveness of HIV chemotherapy. The simulations focus upon the timing of treatment initiation.

Keywords: HIV, immune system, AIDS, $CD4^+$ T cells, combined therapy, drug resistance, mathematical model

INTRODUCTION

The dynamics of the HIV-infected immune system involve the interaction of $CD4^+$ T cells and virus populations. Recent clinical studies (Ho *et al.*, 1995; Wei *et al.*, 1995) have produced new insight into the dynamics of these populations during HIV infection. These studies identified the turnover rates and life spans of both $CD4^+$ T cells and virus by measuring their rate changes in patients subjected to strong antiviral agents. These studies have led to a new conceptual view of the HIV infected immune system as a hyperdynamic process. In this conceptual model, large numbers of $CD4^+$ T cells and virus are gained or lost each day as the total $CD4^+$ T-cell population slowly depletes over the course of years.

The recent clinical determination of this dynamical information promotes the development of mathematical modeling for the pathology and therapy of HIV infection. The goal of such mathematical models is to assist qualitative understanding of the complex interactions of $CD4^+$ T cells and virus at the population level. The model we present here derives

from our earlier studies (Kirschner and Webb, 1996; 1997a; 1997b) in that it assumes certain forms for the terms in the differential equations representing population interactions. These equations are constructed so that their solutions produce CD4$^+$ T-cell counts and viral titers, as well as their turnover rates and life spans, consistent with present clinical knowledge of their values before and after treatment.

Our primary goal here is to model recently developed treatment regimens for combined drug therapy of HIV. Recent reports of ongoing clinical trials show improved benefit for combined drug therapy with newly developed protease inhibitors (ritonavir and indinavir) together with standard reverse transcriptase inhibitors (zidovidine), didanosine and delavirdine) (Travis, 1996; Stephenson, 1996). Although final clinical reports are not yet available, preliminary reports indicate that combined therapy yields (1) viral titers falling to $\leq$ 1% of pre-treatment values after a few weeks of treatment and (2) CD4$^+$ T-cell counts rising by $\geq$ 100/mm^3 (normal CD4$^+$ T-cell counts lie between 800/mm^3 and 1200/mm^3 in blood plasma). Furthermore, during combined drug therapy, these improved levels are sustained for months.

These results compare very favorably to standard monotherapy treatment with reverse transcriptase inhibitors alone, which typically yield CD4$^+$ T-cell counts increasing by only about 30/mm^3, peaking at 3 months and returning to baseline within 1 year (Fischl et al., 1990). With combined therapy, the appearance of resistance virus, which is a key limiting factor in HIV therapy, is apparently forestalled much longer than with monotherapy. It is generally recognized that monotherapy with reverse transcriptase inhibitors alone extends long-term survival by about 1 year (Enger et al., 1996). The extension of long-term survival with combined therapy is not yet known, but is hoped that it will be significantly longer than with monotherapy.

In the simulations we present here, we focus on two key issues in treatment strategy: (1) the time at which treatment is initiated and (2) the time at which drug-resistant virus appears. With standard reverse transcriptase inhibitors, it is generally

recognized that early initiation of treatment (when CD4$^+$ T-cell counts are > 500/mm^3) and late initiation of treatment (when CD4$^+$ T-cell counts are < 200/mm^3) is less beneficial than initiation of treatment when CD4$^+$ T-cell counts are between 200/mm^3 and 500/mm^3. It is also recognized that drug-resistant virus replaces drug-sensitive virus within a few weeks after initiation of treatment (Wei et al., 1995). Our simulations for combined treatment show the same conclusion for the timing of combined drug-treatment initiation, i.e. advantageous starting times occur when CD4$^+$ T-cell counts are between 200/mm^3 and 500/mm^3.

Because the time at which resistance appears during combined therapy is not yet clinically established (and is in any case variable), we allow a range of values for the appearance of resistant virus. In all cases, there is an advantage to beginning treatment with middle-level CD4$^+$ T cell counts. Because treatment is limited by the replacement of sensitive virus with resistant virus, the timing of treatment is of major importance in HIV therapy. In future work, the authors will investigate other treatment-strategy issues in combined drug therapy, such as interrupting treatment before the development of resistance and sequencing different drugs rather than combining them.

THE MODEL

In the equations below, $T(t)$ represents the uninfected CD4$^+$ T-cell population at time t, $T_s(t)$ represents the drug-sensitive infected CD4$^+$ T-cell population at time t, $T_r(t)$ represents the drug-resistant infected CD4$^+$ T-cell population at time , $V_s(t)$ represents the drug-sensitive virus population at time t, $V_r(t)$ represents the drug-resistant virus population at time t and $V(t) = V_s(t) + V_r(t)$ represents the total virus population at time t. All these populations are measured in the blood plasma, which constitutes only 2% of their total, the rest residing in the lymphoreticular tissue. The equations for the model without treatment are as follows:

$$T'(t) = S(t) - \mu_T T(t) + \frac{pT(t)V_s(t)}{(C + V_s(t))}$$
$$- k_s V_s(t)T(t) \qquad (1)$$

$$T'_s(t) = k_s V_s(t) T(t) - \mu_{T_i} T_s(t)$$

$$- \frac{p_i T_s(t) V_s(t)}{(C_i + V_s(t))} \qquad (2)$$

$$V'_s(t) = N p_i T_s(t) \frac{V_s(t)}{(C_i + V_s(t))}$$

$$- k_v T(t) V_s(t) + \frac{G_s V_s(t)}{(B + V_s(t))}. \qquad (3)$$

In equation (1) $S(t)$ represents the source of uninfected CD4$^+$ T cells from the thymus or other compartments. It is assumed that there is a degeneration of this source during the course of HIV infection from a normal input of 10 /mm^3 per day to 3/mm^3 per day. Accordingly, we take

$$S(t) = 10.0 - 7.0 \frac{V(t)}{(B_s + V(t))},$$

where B_s is a saturation constant. In equation (1) $\mu_T T(t)$ is the mortality rate of uninfected CD4$^+$ T cells (which we assume live about 100 days), p is a proliferation rate mediating the gain of CD4$^+$ T cells due to the immune response term $[pT(t)V_s(t)]/[(C + V_s(t))]$ (C is a saturation constant and k_s is the infection rate of CD4$^+$ T cells by virus; it is assumed that the rate of infection is governed by the mass action term $k_s V_s(t) T(t)$).

In equation (2) there is a gain term $k_s V_s(t) T(t)$ of CD4$^+$ T cells infected by drug-sensitive virus, a loss term $\mu_{T_i} T_s(t)$ due to the mortality of these cells (we assume that infected CD4$^+$ T cells live about 2 days) and a loss term $[p_i T_s(t) V_s(t)]/[C_i + V_s(t)]$ due to their bursting caused by invading virus (p_i is a proliferation constant and C_i is a saturation constant).

In equation (3) the virus population is increased by the bursting term $[N p_i T_s(t) V_s(t)]/[C_i + V_s(t)]$ (where we take $N = 10$ as the number of viable virions produced in each bursting). In equation (3) the virus population is decreased by the immune response term $k_v T(t) V_s(t)$ (we assume that CD4$^+$ T cells are in 1:2 proportion with CD8$^+$ T cells, which are responsible for actual elimination of the virus). In equation (3) there is an external input of virus from compartments outside the blood (spleen, lymph nodes, etc.), which is represented by the term $[G_s V_s(t)]/[B + V_s(t)]$. This external input exerts

a major influence upon the virus production and the overall course of the disease. This is because 98% of all T cells, and hence viral production, are in these other compartments (Lafeuillade *et al.*, 1996). As the parameter G_s increases, the qualitative behavior of the system changes from the existence of a stable uninfected steady state (*disease-free state*), to the existence of a stable infected steady state (*latency*) and finally to the collapse of the CD4$^+$ T-cell population (*progression to AIDS*). These three types of behavior are illustrated in Figure 1. In each case, it is assumed that the initial population values are $T(0) = 1000.0$, $T_s(0) = 0.0$ and $V_s(0) = 10.0$.

In the treatment simulations presented here we assume that treatment starts at some time t_0 with initial values $T(t_0)$, $T_s(t_0)$ and $V_s(t_0)$ obtained from the equations above, and with no resistant strain, namely $T_r(t_0) = 0.0$ and $V_r(t_0) = 0.0$. The value of G_s is at a level that results in the progression to AIDS. We assume that drug-resistant virus appears at a time $t_r > t_0$. The equations for the model with treatment are as follows:

$$T'(t) = S_0(t) - \mu_T T(t) + \frac{pT(t)V(t)}{(C + V(t))}$$

$$- (\mu k_s V_s(t) + k_r V_r(t)) T(t) \qquad (4)$$

$$T'_s(t) = \mu k_s V_s(t) T(t) - \mu_{T_i} T_s(t)$$

$$- \frac{p_i T_s(t) V(t)}{C_i + V(t)} \qquad (5)$$

$$T'_r(t) = k_r V_r(t) T(t) - \mu_{T_i} T_r(t)$$

$$- \frac{p_i T_r(t) V(t)}{C_i + V(t)} \qquad (6)$$

$$V'_s(t) = \frac{\rho q(t) N p_i T_s(t) V(t)}{C_i + V(t)}$$

$$+ \frac{(1 - q(t)) N p_i T_r(t) V(t)}{C_i + V(t)}$$

$$- k_v T(t) V_s(t) + \eta \frac{G_s V_s(t)}{B + V(t)} \qquad (7)$$

$$V'_r(t) = \frac{q(t) N p_i T_r(t) V(t)}{C_i + V(t)} + \frac{(1 - q(t)) N p_i T_s(t) V(t)}{C_i + V(t)}$$

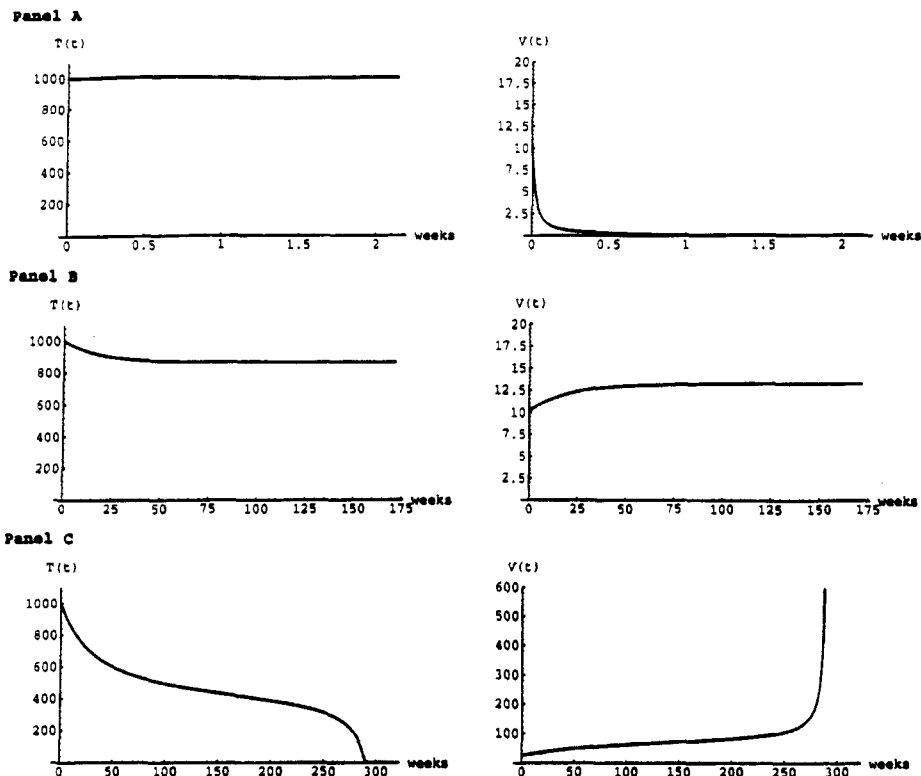$$- k_v T(t) V_r(t) + \frac{G_r V_r(t)}{B + V(t)} \qquad (8)$$

FIGURE 1   CD4$^+$ T-cell counts and viral titers without treatment. Panel 1A $=$ uninfected steady state (G_s $=$ 80.0), Panel 1B $=$ infected steady state (G_s $=$ 180.0), Panel C $=$ progression to AIDS (G_s $=$ 330.0).

In equations (4)–(8), treatment is modeled as a constant reduction in various viral rates during the time of treatment (we do not model drug pharmacokinetics or metabolic variations). During treatment, the loss of uninfected CD4$^+$ T cells in equation (4) and the consequent gain of sensitive virus-infected CD4$^+$ T cells in equation (5) are multiplied by a treatment parameter μ $<$ 1.0. This effect of treatment may be interpreted as the effect of a reverse transcriptase inhibitor, which reduces infection of new cells, but does not block production of virus from cells already infected and past the stage at which reverse transcription occurs (Nowak and Bonhoeffer, 1995).

During treatment, the term corresponding to the production of sensitive virus due to bursting in equation (7) is multiplied by a treatment parameter ρ $<$ 1.0. This effect of treatment may be interpreted as the effect of a protease inhibitor, which acts later in the cell interphase of the virus life cycle to

block the production of new virus particles. During treatment, the external sensitive virus input term in equation (7) is multiplied by a treatment parameter η $<$ 1.0. This effect of treatment further inhibits the production of new sensitive virus, since treatment represses replication of virus that enters the blood from external compartments.

When treatment begins, we assume that the source term of CD4$^+$ T cells in equation (4) has the value $S_0(t)$ $=$ minimum$\{S(t), S(t_0)\}$, where t_0 is the time at which treatment is initiated. This assumption means that the external input of CD4$^+$ T cells does not increase once treatment begins. We thus assume that there is no direct enhancement of CD4$^+$ T-cell production or recruitment due to treatment, but only an indirect effect due to a reduced rate of their infection. This assumption is controversial, since it has been advocated that the increase in CD4$^+$ T cells induced by antiviral treatment is a direct result of CD4$^+$ T-cell production which is stimulated in

the bone marrow (Agur *et al.*, 1995) or by the direct release of CD4$^+$ lymphocytes from trapping in the lymphoidal tissue (Sprent and Tough, 1995).

In our model of treatment, we assume that there is a known time t_r (which can vary) at which resistant virus appears. Before t_r, there is no significant level of resistant virus present. After t_r there is a proportion $q < 1.0$ of sensitive virus produced that remain faithful to the sensitive type and a proportion $1.0 - q$ that mutate to the resistant type. This phenomenon is incorporated into the equation for the sensitive virus (7) (analogous relationships hold for the equation for the resistant virus, equation (8)). In both of these equations, $q(t) = 1.0$ for $t < t_r$ and $q(t) = q$ for $t > t_r$. We assume that the external input constant G_r in equation (8) is slightly less that G_s in equation (7), which means that the wild

type (sensitive virus) has an evolutionary advantage over the mutant type (resistant virus). In Table I, we provide a list of parameter values for the models with and without treatment. In Table II, we provide a comparison of various quantitative values of the models and clinical data (model values are computed using average disease state values for CD4$^+$ T cells, virus and infected T cells as $T(t) = 435/mm^3$, $V(t) = 72/mm^3$ and $T_s(t) = 12/mm^3$, respectively, at a typical time $t = 150$ weeks as in Figure 1, Panel C).

THE SIMULATIONS

We first provide simulations for the model without treatment, equations (1)–(3). In Figure 1, we show

TABLE I Parameter values for models with and without treatment

Parameters and constants	Values
μ_T = mortality rate of uninfected CD4$^+$ T cells	0.01/day
μ_{T_i} = mortality rate of infected CD4$^+$ T cells	0.5/day
k_s = rate CD4$^+$ T cells are infected by sensitive virus	2.5×10^{-4} mm^3 day
k_r = rate CD4$^+$ T cells are infected by resistant virus	2.5×10^{-4} mm^3 day
k_v = rate CD8$^+$ T cells kill virus	0.01/mm^3 day
p = proliferation rate of uninfected CD4$^+$ T cells	0.5/mm^3 day
p_i = proliferation rate of infected CD4$^+$ T cells	0.5/mm^3 day
G_s = rate of input of external sensitive virus to the blood	330.0 mm^3/day
G_r = rate of input of external resistant virus to the blood	$0.9995 \times G_s$
q = probability of mutation	0.999
N = number of viable virus produced in bursting	10.0
C = half-saturation constant of uninfected CD4$^+$ T cells	188.0/mm^3
C_i = half-saturation constant of infected CD4$^+$ T cells	188.0/mm^3
B = half-saturation constant of external virus input	8.0 mm^3
B_s = half-saturation constant of CD4$^+$ T-cell source	55.0 mm^3
μ = treatment parameter	0.9
η = treatment parameter	0.15
ρ = treatment parameter	0.05

TABLE II Comparison of quantitative values and clinical data

Parameter	Model values	Clinical data values
CD4$^+$ T-cell turnover rate	12/mm^3 per day	7/mm^3 per day (Ho *et al.*, 1995) 8/mm^3 per day (Wei *et al.*, 1995)
Plasma virus turnover rate	300/mm^3 per day	136/mm^3 per day (Ho *et al.*, 1995) 2060/mm^3 per day (Wei *et al.*, 1995)
Life span of infected CD4$^+$ T cell	1.6 days	1 to 2 days (Coffin, 1995)
Life span of virus	0.23 days	0.3 day (Perelson *et al.*, 1996)
Percent of CD4$^+$ T-cells infected	3%	5% (Embretson *et al.*, 1993)

the qualitative behavior of solutions for three different values of the external viral input parameter G_s: Figure 1A — locally stable uninfected steady state, Figure 1B — locally stable infected steady state and Figure 1C — progression to AIDS. Figure 1A demonstrates that the immune system can eradicate the disease, but only if the external viral source is extremely low. Figure 1B demonstrates

that a long period of non-progression may ensue, provided that the external input of virus into the blood from other compartments is low. Figure 1C shows a typical gradual decline of the CD4$^+$ T-cell population for a progressor, when the external input of virus from other compartments is high.

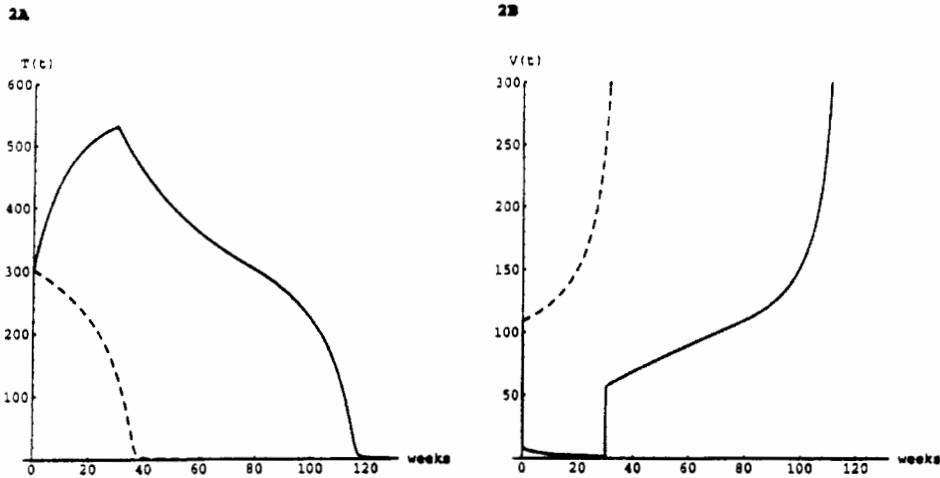In Figure 2, we provide a typical combined treatment simulation for the model equations (4)–(8).

FIGURE 2   CD4$^+$ T-cell count (A) and viral titer (B) when treatment starts with $T(t) = 300/\text{mm}^3$. Dashed curve represents non-treatment progression.

FIGURE 3   CD4$^+$ T-cell count (A) and viral titer (B) when treatment starts with $T(t) = 500/\text{mm}^3$. Dashed curve represents non-treatment progression.
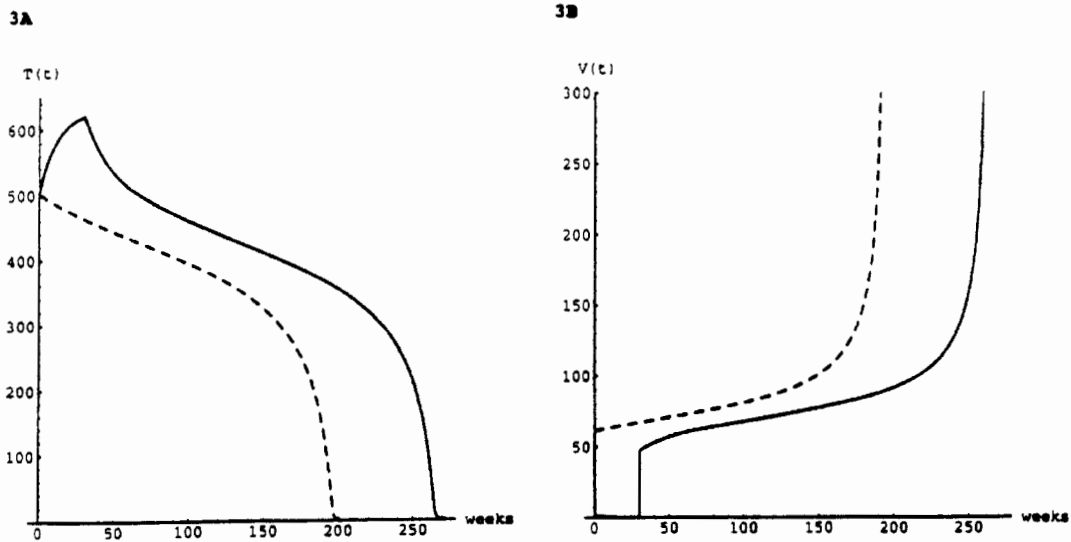
FIGURE 4   CD4$^+$ T-cell count (A) and viral titer (B) when treatment starts with $T(t) = 200/mm^3$. Dashed curve represents non-treatment progression.
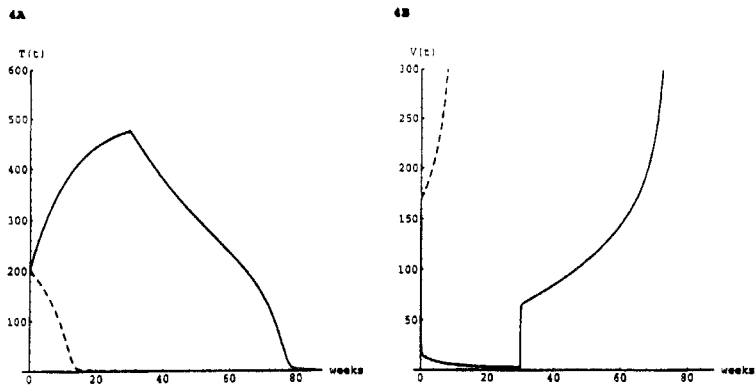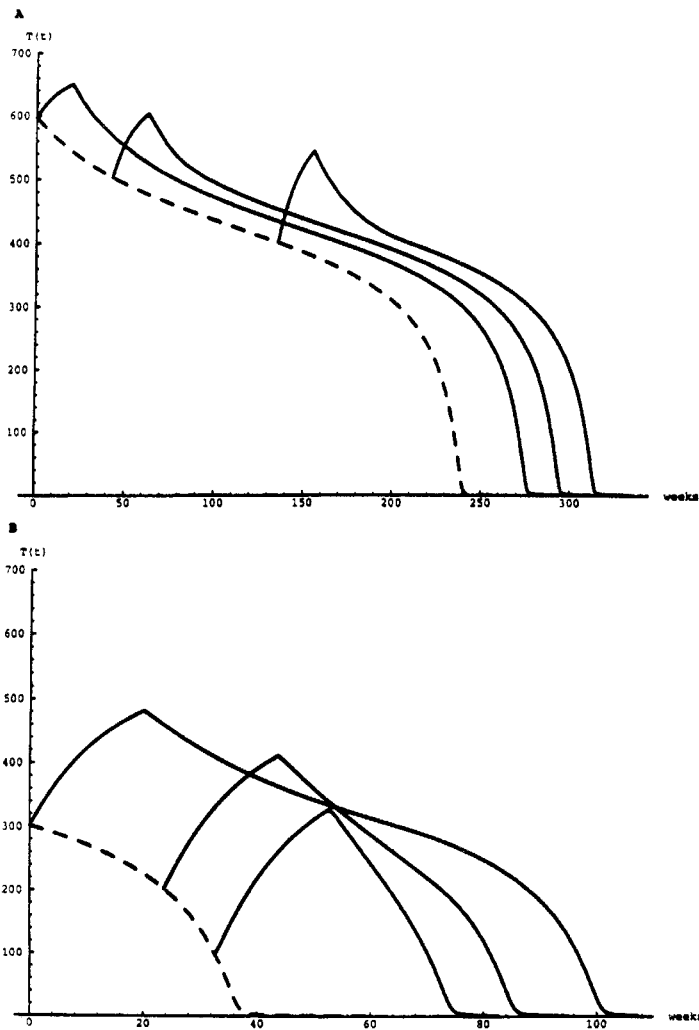

FIGURE 5   Composite CD4$^+$ T-cell counts for treatment starting at (A) 600, 500 and 400/mm^3 and (B) 300, 200 and 100/mm^3 with resistance appearing at $t_r = 20$ weeks after treatment starts. Dashed curve represents non-treatment progression.
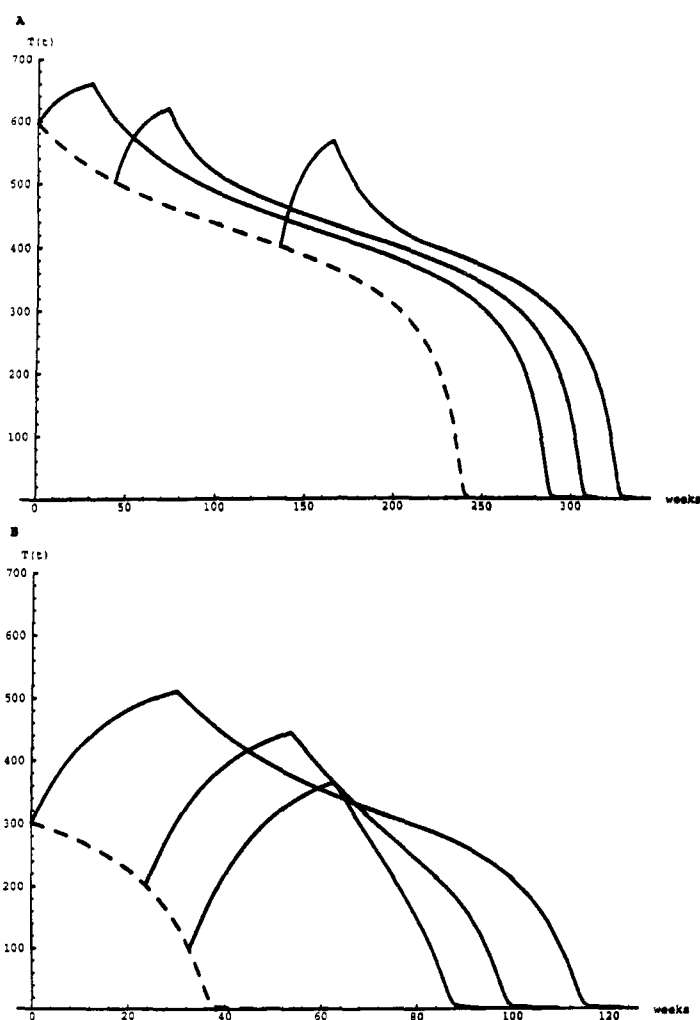
FIGURE 6   Composite CD4$^+$ T-cell counts for treatment starting (A) 600, 500 and 400/mm^3 and (B) 300, 200 and 100/mm^3 with resistance appearing at t_r = 30 weeks after treatment starts. Dashed curve represents non-treatment progression.

Treatment begins when the CD4$^+$ T-cell count is 300/mm^3 and continues until the CD4$^+$ T-cell population collapses. Resistance appears at t_r = 30 weeks after treatment starts. The dashed curve in Figure 2A shows the CD4$^+$ T-cell count without treatment for comparison. The curve in Figure 2B shows the total virus population $V(t) = V_s(t) + V_r(t)$. After the appearance of resistant virus at time t_r the virus is all resistance. The dashed curve in Figure 2B shows the sensitive virus population values without treatment. Figures 3 and 4 show similar simulations for CD4$^+$ T-cell counts at 500/mm^3 and

200/mm^3 at treatment initiation (t_r = 3 weeks in both).

The success of treatment here is based on longer survival times equated to CD4$^+$ T-cell counts, thus we present the following simulations. In Figure 5 we show a composite graph of CD4$^+$ T-cell count simulations for treatment starting when counts are 600, 500 and 400/mm^3 (Figure 5A) and 300, 200 and 100/mm^3 (Figure 5B). The time when resistance appears is t_r = 20 weeks. In Figures 6 and 7 we provide similar composite graphs for resistance appearing at t_r = 30 weeks and t_4 = 40 weeks,
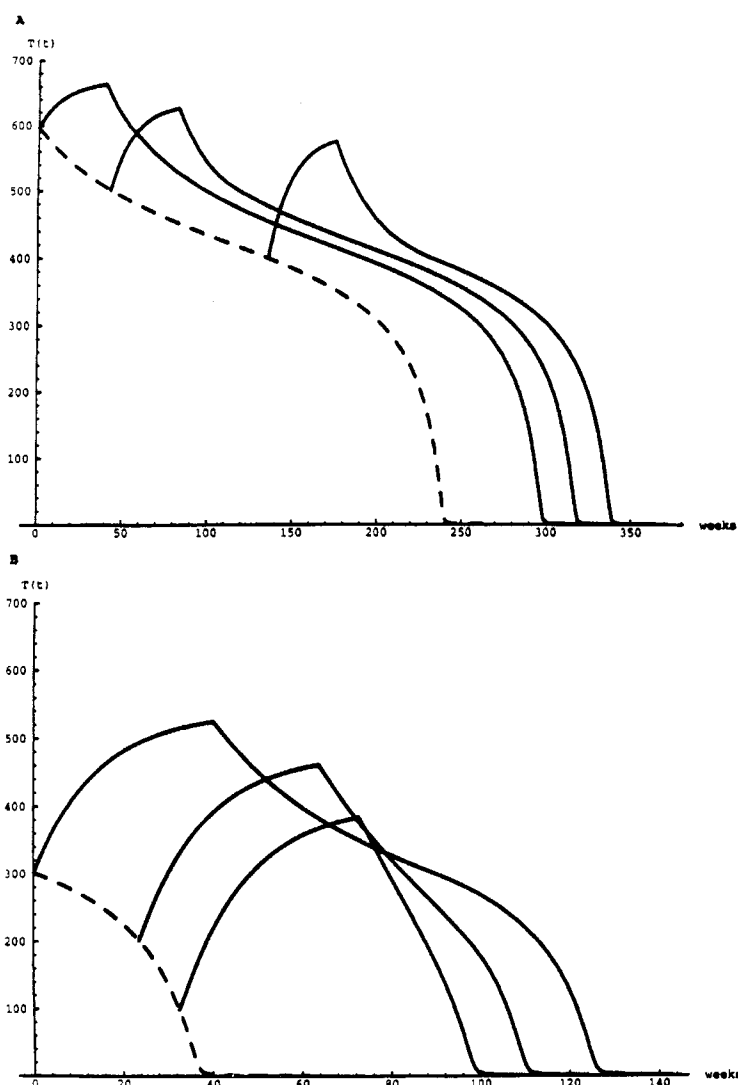
FIGURE 7 Composite CD4$^+$ T-cells counts for treatment starting at (A) 600, 500 and 400/mm^3 and (B) 300, 200 and 100/mm^3 with resistance appearing at $t_r = 40$ weeks after treatment starts. Dashed curve represents non-treatment progression.

respectively. From Figures 5,6 and 7 it appears that the greatest survival benefit occurs if treatment is initiated when CD4$^+$ T-cell counts are between 300/mm^3 and 400/mm^3 and this benefit is independent of the time that resistance appears. It is also apparent that long-term survival benefit is not correlated to treatment-induced increases in CD4$^+$ T-cell counts, which increase as CD4$^+$ T-cell starting values decrease (this correlation is consistent with data given by Ho et al. (1995)).

The explanation for the advantage of beginning treatment when CD4$^+$ T-cell counts are at a middle level in these simulations lies in the assumptions of the model. When CD4$^+$ T-cell counts are high at treatment start, they do not depart as much from baseline values, and consequently return to baseline more quickly when resistance appears. As CD4$^+$ T-cell counts become lower, and then treatment is initiated, the return to baseline takes longer and survival length increases. When CD4$^+$ T-cell counts

are very low at start of treatment, the population is now overcome more rapidly by the high viral load as resistant virus replaces sensitive virus. In a separate study of the authors (Kirschner and Webb, 1997b), we model resistance in a different way, and the results indicate that early, strong treatment yields the most benefit. In these simulations, the deteriorating source of $CD4^+$ T-cells is the critical factor in the disadvantage of late initiation of treatment, since there is a much lower input of these cells during treatment ($S(t_0)$ is close to the limiting value of $3/mm^3$ per day).

Acknowledgement

References

Agur, Z., Tagliabue, G., Schechter, B. and Ubezio, P. (1995). "AZT effect on bone marrow — a new perspective on the Concorde Trials", J. Biol Sys., **3**, 241-251.

Coffin, J. M. (1995). "HIV population Dynamics in vivo: implications fir genetic variation, pathogenesis and therapy", Science **267**, 483-489.

Embretson, J. (1993). "Analysis of HIV infected tissues by amplification and in situ hybridization reveals latent and permissive infections at single-cell resolution, Proc, Nat. Acad. Sci., **90**, 357.

Enger, D., Graham, N., Peng, Y., Chmiel, J. S., Kingsley, L. A., Detels, R. and Munoz, A. (1996). "Survival from early, intermediate, and late stages of HIV infection", J. Amer. Med. Assoc., **275**, 1329-1334.

Fischl, M. A., Richman, D. D., Hansen, N., Collier, A. C., Carey, J. T. and Para, M. F. (1990). "The safety and efficacy of AZT in the treatment of subjects with mildly symptomatic HIV Type 1", Ann. Int. Med., **112**, 727-737.

Haynes, B. F., Pantaleo, G. and Fauci, A. S. (1996). "Toward an understanding of the correlations of protective immunity to HIV infection", Science, **271**, 324-328.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M. and Markowitz, M. (1995), "Rapid turnover of plasma virions and CD4⁺ lymphocytes in HIV-1 infection, Nature, **373**, 123-126.

Kirschner, D. and Perelson, A. (1995). "A model for the immune system response to HIV: AZT treatment studies", In O. Arino, D. Axelrod and M. Kimmel, eds. Mathematical Populations Dynamics. III Theory of Epidemics 1, pp. 295-310 (Wuerz). Winnipeg, Canada.

Kirschner, D. and Webb, G. F. (1996). "A model for treatment strategy in the chemotherapy of AIDS", Bull. Math. Biol., **58**(2), 367-390.

Kirschner, D. and Webb, G. F. (1997a). Understanding drug resistance for monotherapy treatment of HIV infection", Bull. Math. Biol., **59**(4), 763-785.

Kirschner, D. and Webb, G. F. (1997b). "Qualitative differences in HIV chemotherapy between remission and resistance outcomes", Emerging Infections Diseases, (in press).

Lafeuillade, A., Poggi, C., Profizi, N., Tamalet, C. and Costes, O. (1996). "Human immunodeficiency virus type 1 kinetics in lymph nodes compared with plasma", J. Infect. Dis., **174**, 404-407.

McLean, A. and Nowak, N. (1992). "Competition between AZT sensitive and AZT resistant strains of HIV, AID, **6**, 71-79.

Nowak, M. A. and Bonhoeffer, S. (1995). "Scientific correspondence", Science **375**, 193.

Perelson, A. (1989). "Modeling the interaction of the immune system with HIV". In C. Castillo-Chavez, ed. Mathematical and Statistical Approaches to AIDS Epidemiology, Lectures Notes in Biomathematics 83, Springer-Verlag, New York, pp. 350-370.

Perelson, A., Kirschner, D. E. and De Boer, R. J. (1993). "The Dynamics of HIV infection of CD4⁺ T cells", Math. Biosciences, **114**, 81-125.

Perelson, A., Neumann, A. U., Markowitz, M., Leonard, J. M. and Ho, D. D (1996). "HIV-1 dynamics in vivo: virion clearance rate, infected cell lifespan, and viral generation Time", Science, **271**, 1582-1586.

Sprent, J. and Tough, D. (1995). "Scientific correspondence", Nature **375**, 194.

Stephenson, J. (1996). "New anti-HIV drugs and treatment strategies buoy AIDS researchers", J. Amer. Med. Assoc., **275**, 579-580.

Travis, J. (1996). "New drugs, New tests, new optimism mark recent AIDS research", Science News, **149**, 184-186.

Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Saag, M. S. and Shaw, G. M. (1995). "viral dynamics in human immunodeficiency virus type 1 infection", Nature **373**, 117-122.

Wein, L. M., Zenios, S. and Nowak, M. A. "Dynamic multidrug therapies for HIV: a control theoretic approach", J. Theo. Biol., (in press).