

Quantitation of Intracellular Zidovudine Phosphates by Use of Combined Cartridge-Radioimmunoassay Methodology

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This report describes the development of a potentially clinical method to measure the cellular metabolites of zidovudine (ZDV) in patients receiving the drug. This new method combines the use of Sep-Pak cartridges to separate ZDV phosphates with radioimmunoassaying to quantitate ZDV. The detection limit is 0.02 pmol/10⁶ cells, and this assay can measure a wide range of intracellular drug concentrations. The use of the cartridge-radioimmunoassay methodology should prove very useful for in vivo cellular pharmacokinetic studies of ZDV.

Zidovudine (3'-azido-3'-deoxythymidine [ZDV]) is one of five dideoxynucleoside analogs approved for the treatment of human immunodeficiency virus (HIV) infection (1-3, 6, 7, 9-11, 13, 16, 18, 21, 22). ZDV within HIV-infected peripheral blood lymphocytes and monocytes is converted by a series of cellular kinases to the triphosphate (ZDV-TP), the putative inhibitor of HIV reverse transcriptase and HIV replication (5, 12). A simple correlation between ZDV in plasma and clinical response has not been easily determined (15). It is likely that a better understanding of the intracellular pharmacokinetics of ZDV and related anti-HIV agents would lead to improvements in drug therapy for individual patients.

Several methods for the quantitation of intracellular drug

metabolites have recently been described (8, 14, 17, 20). The most widely used method to date is based on a combination of high-performance liquid chromatography (HPLC) and radioimmunoassaying (HPLC-RIA), which determines the cellular concentration of ZDV metabolites after their separation by HPLC and enzymatic dephosphorylation to the parent drug (17). However, this method is quite labor-intensive and poorly suited for most patient studies. The use of cartridges instead of HPLC allows for the quantitative determination of intracellular anabolites and is faster and simpler than the HPLC-RIA method.

Peripheral blood mononuclear cells (PBMC) from healthy human volunteers were isolated by centrifugation on Ficoll-

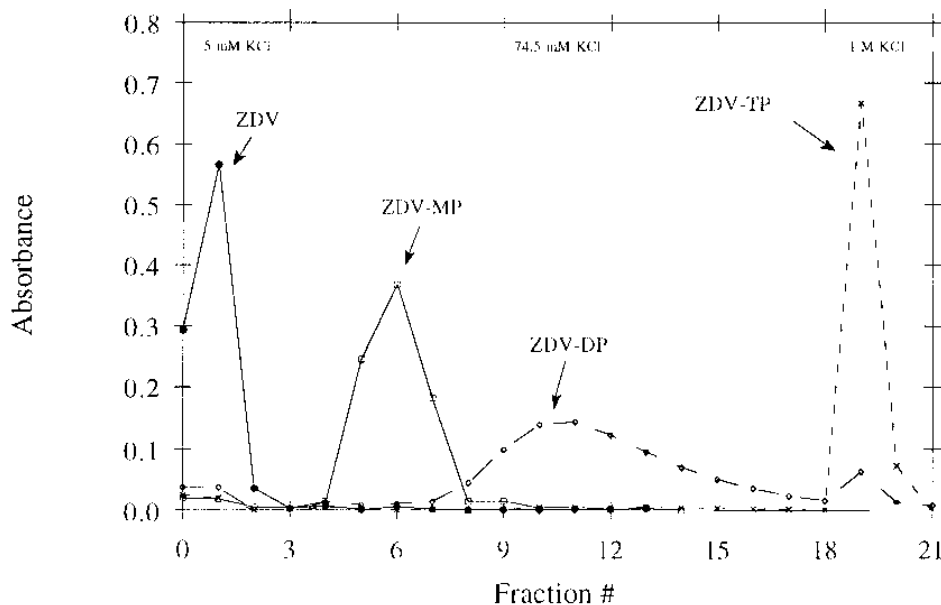


FIG. 1. Separation of ZDV metabolites with anion-exchange cartridges. Standard solutions of ZDV (●), ZDV-MP (□), ZDV-DP (◇), and ZDV-TP (×) were applied to the individual cartridges and eluted with the indicated concentrations of KCl.

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TABLE 1. Recovery of ZDV metabolites from QMA cartridges

Metabolite	% Recovery ^a
ZDV-MP	90.4 91.5 94.0
Mean \pm SD	91.9 \pm 1.8
ZDV-TP	91.3 91.1 90.8
Mean \pm SD	91.1 \pm 0.3

^a Recovery was determined by adding a known amount of radioactive ZDV-MP or ZDV-TP, performing the elution as mentioned previously, and counting the radioactivity in each of the fractions. Recovery values were corrected for the small amount of decay of the radioactive tracer.

Paque. The cells were suspended to a density of 1 million cells per ml in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 25 U of penicillin-streptomycin per ml. The cells were activated by the addition of 5 μ g of phytohemagglutinin (PHA) per ml and 10 U of interleukin 2 per ml for 72 h at 37°C before their use. When quiescent cells were required, the PHA and interleukin 2 were omitted, and the cells were used on the following day.

ZDV metabolites were separated with QMA anion-exchange cartridges (Waters Co., Milford, Mass.). The QMA cartridges were preconditioned with 10 ml of 1 M KCl, which was followed by a 10-ml wash with 5 mM KCl. Samples were loaded onto the cartridges at a rate of 3 ml/min or less, and the void volume that contained ZDV was collected in a conical centrifuge tube. ZDV-MP and ZDV-DP were eluted sequentially with 6 and 11 ml of 74.5 mM KCl, respectively. This was followed by the elution of ZDV-TP with 3.3 ml of 1 M KCl, which was collected in polypropylene test tubes. As shown in Fig. 1, ZDV-MP, -DP, and -TP derivatives can be separated from each other by the QMA cartridge with <8% overlap of DP into the TP peak. The separation is highly reproducible, and recoveries of standard nucleotides from the cartridge were consistently between 91 and 94% (Table 1).

The ZDV nucleotide digestion was done by modification of the method in widespread use. ZDV nucleotide fractions from the QMA cartridges, which were described above, were adjusted to pH 4.0 with 1 M sodium acetate buffer (final concentration, 50 mM). The fractions were treated for about 30 min with only 2 U of the acid phosphatase (Type XA; Sigma Chemical Co., St. Louis, Mo.) per ml rather than 40 U/ml as used previously (17). The samples containing free ZDV were cleaned with Waters C-18 plus cartridges (preconditioned with 10 ml of acetonitrile, 20 ml of HPLC-grade methanol, and 10 ml of Millipore water). Each cartridge was eluted with 10 ml of

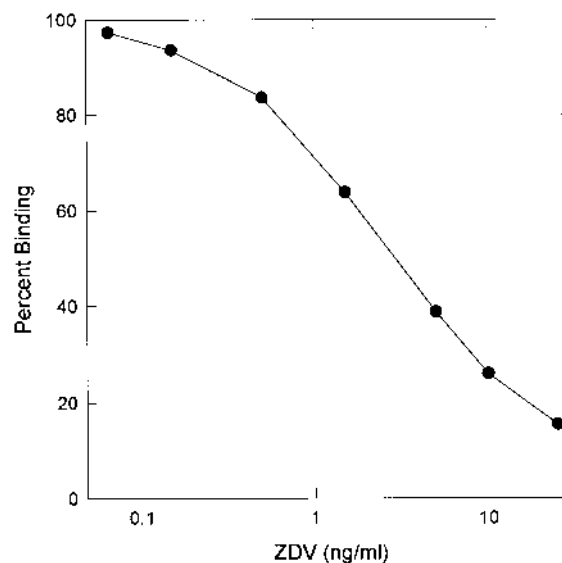


FIG. 2. Standard curve of the ZDV-RIA. The standard curve for measuring ZDV concentrations was constructed with standards corresponding to final concentrations of 0.065, 0.15, 0.5, 1.5, 5.0, 10.0, and 25.0 ng/ml. The data were the means of three determinations for each point.

5% methanol, which removed any residual endogenous thymidine, which was followed by 5.0 ml of acetonitrile. The recovery of ZDV was excellent, with 98.2% \pm 0.06% of the nucleotides being cleaved and recovered as ZDV. These samples were then evaporated to dryness in a Savant Speed Vac, and the level of ZDV was determined by a RIA, as described by Slusher et al. (17). The samples were counted in a Packard Cobra Auto gamma counter. The concentrations of the standard curve were 0.065 to 25 ng of ZDV per ml with daily controls of 0.25, 2.5, and 15 ng/ml. A typical standard curve is shown in Fig. 2. The standard curve was calculated in nanograms of ZDV per milliliter as determined by the RIA. Intracellular data were calculated in picomoles per 10⁶ cells for comparison with the sensitivity of other assays and literature values of intracellular metabolite concentrations. Thus, although this method involves several steps, we obtained ~90% recovery of ZDV nucleotides from the entire procedure.

To test the usefulness of the cartridge-RIA method in measuring intracellular ZDV nucleotides, the levels of these nucleotides in PHA-stimulated PBMC were determined. Stimulated cells from normal donors (1 million cells per ml) were incubated for 4 h with 1, 2, 5, and 10 μ M [³H]ZDV (Moravek Biochemical, Brea, Calif.). After the incubation, cell extracts were prepared and split into two fractions and ZDV metabo-

TABLE 2. Comparison of cartridge-RIA values with HPLC-determined values^a

[ZDV] (μ M)	Level of ZDV-MP (pmol/10 ⁶ cells)			Level of ZDV-TP (pmol/10 ⁶ cells)			Level of ZDV-DP (pmol/10 ⁶ cells)	
	Cartridge-RIA	HPLC	Difference (%)	Cartridge-RIA	HPLC	Difference (%)	Cartridge-RIA	HPLC
1	73.62	70.14	4.96	3.38	2.93	15.36		1.25
2	123.47	135.34	-8.77	3.72	3.71	0.27		1.59
5	289.83	252.28	14.88	3.64	3.74	-2.67		1.64
10	362.18	355.64	1.84	4.52	4.00	13.00		1.91

^a PBMC from normal subjects were stimulated for 72 h with PHA and then incubated for 3 h with the indicated concentrations of ZDV. ZDV-MP and ZDV-TP levels were determined by cartridge-RIA and were compared with levels determined by HPLC. Data represent an experiment that was performed twice with differences of less than 15%.

lite levels were determined in parallel by anion-exchange HPLC (4) and the cartridge-RIA method described above. As shown in Table 2, ZDV-MP was proportionately higher as the dose of ZDV increased. A 10-fold increase from 1 to 10 μM ZDV led to intracellular levels of ZDV-MP of from 74 to 362 pmol/ 10^6 cells and a less-than-2-fold increase in ZDV-DP and ZDV-TP levels (from 1.3 to 1.9 pmol/ 10^6 cells and from 3.5 to 4.5 pmol/ 10^6 cells, respectively). As shown in Table 2, the differences noted between the levels of ZDV-MP and ZDV-TP determined by the cartridge-RIA and HPLC measurements were less than 15%. ZDV-DP concentrations represented only $\sim 2\%$ of the total ZDV nucleotides formed and were not measured routinely by the cartridge-RIA method.

The reproducibility of the cartridge-RIA was assessed in determinations with ZDV-free PBMC samples spiked with the ranges of ZDV-MP and ZDV-TP concentrations indicated in Table 3. From these three experiments, we could determine several useful parameters, including percent difference from expected concentration, sample variation, and standard deviation of interassay determinations. Table 3 shows that the determined amounts of ZDV-MP and ZDV-TP were close to the expected amounts of these two nucleotides. Intraassay variations (standard deviation for ZDV-MP or the range for ZDV-TP) were indicated by \pm values and were generally less than 20% of the measured values. The interassay measurements were reliable, as indicated by the percent coefficients of variation of less than 20%, as shown in Table 3. These results indicated that this is a reliable method for the determination of ZDV-MP and ZDV-TP levels over the indicated ranges.

The data reported here show that a streamlined Sep-Pak cartridge-RIA method permits the quantitation of intracellular ZDV phosphates in cells without the use of radiolabeled drugs. Thus, in this study we describe the application of anion-exchange cartridges, instead of HPLC, to separate all ZDV phosphates, significantly accelerating sample analysis. For example, HPLC requires 60 to 90 min to separate ZDV phosphates, while this separation can be done in <25 min by using a QMA cartridge. Sample processing efficiency can be increased further by using a vacuum manifold, permitting simultaneous handling of multiple samples. In addition, the use of KCl instead of phosphate buffer for elution of ZDV phosphates was important because it eliminated any inhibition of the acid phosphatase reaction seen with phosphate buffers. This reduced how much phosphatase was needed for digestion of ZDV phosphates from 40 U/ml, as used previously, to 2 U/ml. Additionally, the incubation time was reduced by 16-fold from 8 h to 30 min.

The accuracy and reproducibility of this assay were assessed with several known concentrations of ZDV-MP and ZDV-TP in PBMC extracts measured on separate days. The variation from expected values was less than 13% when ZDV-MP was measured. The difference from expected values when ZDV-TP levels were measured increased with decreasing concentrations to a maximum difference of 32% when 0.050 pmol/ 10^6 cells was measured. The differences from the target concentrations were slightly less than predicted values when ZDV-MP levels were measured and were slightly higher than predicted values when ZDV-TP levels were measured. The sample and interassay precision was good, with percent coefficients of variation of less than 20% when either ZDV-MP or ZDV-TP levels were determined. The limit of detection for the cartridge-RIA assay was dependent on the limit of detection of the ZDV-RIA. The limit of detection for ZDV-RIA is 0.065 ng/ml, which corresponded to a limit of detection of 0.02 pmol/ 10^6 cells by the cartridge-RIA. The limit of quantitation is the minimum concentration at which the percent error was 20% or less. The

TABLE 3. Determinations of metabolites in spiked PBMC samples^a

Day and parameter	ZDV-MP level with the following target amts (pmol/ 10^6 cells):						ZDV-TP level with the following target amts (pmol/ 10^6 cells):					
	0.70	1.50	3.00	4.00	12.00	0.050	0.080	0.120	0.150	0.502		
Day 1	0.684 \pm 0.020	1.348 \pm 0.117	3.011 \pm 0.216	3.927 \pm 0.039	11.48 \pm 0.381	0.059 \pm 0.021	0.077 \pm 0.005	0.148 \pm 0.033	0.163 \pm 0.001	0.485 \pm 0.001		
Day 2	0.602 \pm 0.017	1.239 \pm 0.014	2.647 \pm 0.051	3.615 \pm 0.257	10.60 \pm 0.783	0.060 \pm 0.011	0.102 \pm 0.022	0.120 \pm 0.015	0.149 \pm 0.029	0.505 \pm 0.044		
Day 3	0.597 \pm 0.026	1.308 \pm 0.021	2.573 \pm 0.115	3.476 \pm 0.230	10.74 \pm 0.105	0.067 \pm 0.002	0.112 \pm 0.001	0.146 \pm 0.007	0.184 \pm 0.055	0.438 \pm 0.074		
Mean	0.628	1.298	2.744	3.673	10.94	0.062	0.097	0.138	0.165	0.476		
% Difference	-10.16	-13.47	-8.49	-8.31	-8.83	32.46	21.03	15.16	10.10	-5.17		
SD	0.0489	0.0551	0.2345	0.2320	0.473	0.004	0.018	0.016	0.018	0.034		
% Coefficient of variation	7.79	4.24	8.54	6.32	4.32	6.45	18.55	11.59	10.91	7.14		

^a PBMC extracts were spiked with the indicated target amounts of ZDV-MP or ZDV-TP, and concentrations were determined by cartridge-RIA. The \pm values are the intra-assay standard deviations and ranges for ZDV-MP and ZDV-TP, respectively. The indicated mean is the 3-day interassay mean. The percent difference is the difference between the measured level and the expected level $\times 100$. The SD is the interassay standard deviation over 3 days. The percent coefficient of variation is (interassay SD/interassay mean) $\times 100$.

limit of quantitation was 0.08 pmol/10⁶ cells as determined from the accuracy values shown in Table 3. Therefore, under these conditions, the cartridge-RIA method is reliable for the determination of metabolite levels higher than 0.08 pmol/10⁶ cells. Finally, the sensitivity of the assay to changes in intracellular drug concentration was shown at pharmacologically relevant drug levels. Thus, as shown in Table 2, with ZDV concentrations approximating levels in plasma of 1 to 10 μM, different concentrations in intracellular ZDV-MP and ZDV-TP could be measured by the cartridge-RIA as readily as by the radiolabeling method. In addition, small numbers of PBMC were used with the assay (1 × 10⁷ to 2 × 10⁷), which could be obtained from 10 to 20 ml of blood. There are several potential uses for our assay for *in vivo* pharmacokinetic studies of ZDV-MP and ZDV-TP in HIV-infected patients. Recently, it has been suggested (19) that the accumulation of ZDV-MP may be important in the cytotoxicity of ZDV. Moreover, with the increasing use of combination therapy, assays such as that described here will be needed to study the intracellular interactions of different drugs and to correlate these observations with antiviral activity and clinical toxicity. The improvement in sample-processing efficiency renders this method well-suited for such clinical studies.

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REFERENCES

- Boucher, F. D., J. F. Modlin, S. Weller, A. Ruff, M. Mirochnick, S. Pelton, C. Wilfert, R. McKinney, Jr., M. J. Crain, M. M. Elkins, M. R. Blum, and C. G. Prober. 1993. Phase I evaluation of zidovudine administered to infants exposed at birth to the human immunodeficiency virus. *J. Pediatr.* **122**:137-144.
- Fischl, M. A., C. B. Parker, C. Pettinelli, M. Wulfschlag, M. S. Hirsch, A. C. Collier, D. Antoniskis, M. Ho, D. D. Richman, E. Fuchs, T. C. Merigan, R. C. Reichman, J. Gold, N. Steigbigel, G. S. Leoung, S. Rasheed, and A. Tsatis. 1990. A randomized controlled trial of a reduced daily dose of zidovudine in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **323**:1009-1014.
- Fischl, M. A., D. D. Richman, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, R. T. Schooley, G. G. Jansky, D. T. Durack, and D. King. 1987. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N. Engl. J. Med.* **317**:185-190.
- Fridland, A., M. C. Connelly, and R. Ashmun. 1990. Relationship of deoxynucleotide changes to inhibition of DNA synthesis induced by the antiretroviral agent 3'-azido-3'-deoxythymidine and release of its monophosphate by human lymphoid cells (CCRF-CEM). *Mol. Pharmacol.* **37**:665-670.
- Furman, P. A., J. A. Fyfe, M. H. Sinclair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333-8337.
- Ingrand, D., J. Weber, C. A. B. Boucher, C. Loveday, C. Robert, A. Hill, N. Cammack, and the Lamivudine European HIV Working Group. 1995. Phase I/II study of 3TC (lamivudine) in HIV-positive, asymptomatic or mild AIDS-related complex patients: sustained reduction in viral markers. *AIDS* **9**:1323-1329.
- Kline, M. W., L. M. Dunkle, J. A. Church, J. T. Goldsmith, A. T. Harris, M. E. Federici, M. E. Schultze, L. Woods, D. F. Loewen, S. Kaul, A. Cross, V. L. Rutkiewicz, H. M. Rosenblatt, I. C. Hanson, and W. T. Shearer. 1995. A phase I/II evaluation of stavudine (d4T) in children with human immunodeficiency virus infection. *Pediatrics* **96**:247-252.
- Kuster, H., M. Vogt, B. Joos, V. Nadai, and R. Luthy. 1991. A method for the quantification of intracellular zidovudine nucleotides. *J. Infect. Dis.* **164**:773-776.
- Lambert, J. S., M. Seidlin, R. C. Reichman, C. S. Plank, M. Laverly, G. D. Morse, C. Knupp, C. McLaren, C. Pettinelli, F. T. Valentine, and R. Dolin. 1990. 2',3'-Dideoxyinosine (ddI) in patients with the acquired immunodeficiency syndrome or AIDS-related complex. *N. Engl. J. Med.* **322**:1333-1340.
- Larder, B. A., S. D. Kemp, and P. R. Harrigan. 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. *Science* **269**:696-699.
- Meng, T. C., M. A. Fischl, and D. D. Richman. 1990. AIDS clinical trials group: phase I/II study of combination 2', 3'-dideoxycytidine and zidovudine in patients with acquired immunodeficiency syndrome (AIDS) and advanced AIDS-related complex. *Am. J. Med.* **88**(Suppl. 5B):27S-30S.
- Mitsuya, H. K., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder. 1985. 3'-Azido-3'-deoxythymidine (BWA A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**:7096-7100.
- Murphy, R. 1995. Clinical aspects of human immunodeficiency virus disease: clinical rationale for treatment. *J. Infect. Dis.* **171**:S81-S87.
- Robbins, B. L., J. Rodman, C. McDonald, R. V. Srinivas, P. M. Flynn, and A. Fridland. 1994. Enzymatic assay for measurement of zidovudine triphosphate in peripheral blood mononuclear cells. *Antimicrob. Agents Chemother.* **38**:115-121.
- Sale, M., S. M. Sheiner, P. Volberding, and T. F. Blasche. 1993. Zidovudine response relationships in early human immunodeficiency virus infection. *Clin. Pharmacol. Ther.* **54**:556-566.
- Skowron, G., S. A. Bozzette, L. Lim, C. B. Pettinelli, H. H. Schaumburg, J. Arezzo, M. A. Fischl, W. G. Powderly, D. J. Gocke, D. D. Richman, J. C. Pottage, Jr., D. Antoniskis, G. F. McKinley, N. E. Hyslop, Jr., G. Ray, G. Simon, N. Reed, M. L. Lofaro, R. B. Uttamchandani, L. D. Gelb, S. J. Sperber, R. L. Murphy, J. M. Leedom, M. H. Grieco, J. Zachary, M. S. Hirsch, S. A. Spector, J. Bigley, W. Soo, and T. C. Merigan. 1993. Alternating and intermittent regimens of zidovudine and dideoxycytidine in patients with AIDS or AIDS-related complex. *Ann. Intern. Med.* **118**:321-330.
- Slusher, J. T., S. K. Kuwahara, F. M. Hamazeh, L. D. Lewis, D. M. Kornhauser, and P. S. Lietman. 1992. Intracellular zidovudine (ZDV) and ZDV phosphates as measured by a validated combined high-pressure liquid chromatography-radioimmunoassay procedure. *Antimicrob. Agents Chemother.* **36**:2473-2477.
- Sommadossi, J. P. 1995. Comparison of metabolism and *in vitro* antiviral activity of stavudine versus other 2',3'-dideoxynucleoside analogues. *J. Infect. Dis.* **171**:S88-S92.
- Tornevik, Y., B. Ullman, J. Balzarini, B. Wahren, and S. Eriksson. 1995. Cytotoxicity of 3'-azido-3'-deoxythymidine correlates with 3'-azidothymidine-5'-monophosphate (AZTMP) levels, whereas anti-human immunodeficiency virus (HIV) activity correlates with 3'-azidothymidine-5'-triphosphate (AZTTP) levels in cultured CEM T-lymphoblastoid cells. *Biochem. Pharmacol.* **49**:829-837.
- Toyoshima, T., S. Kimura, S. Muramatsu, H. Takahagi, and K. Shimada. 1991. A sensitive non-isotopic method for the determination of intracellular azidothymidine 5'-mono-, 5'-di- and 5'-triphosphate. *Anal. Biochem.* **196**:302-307.
- Van Leeuwen, R., C. Katlama, V. Kitchen, C. A. B. Boucher, R. Tubiana, M. McBride, D. Ingrand, J. Weber, A. Hill, H. McDade, and S. A. Danner. 1995. Evaluation of safety and efficacy of 3TC (Lamivudine) in patients with asymptomatic or mildly symptomatic human immunodeficiency virus infection: a phase I/II study. *J. Infect. Dis.* **171**:1166-1171.
- Yarchoan, R., R. W. Klecker, K. J. Weinhold, P. D. Markham, H. K. Lyerly, D. T. Durack, E. Gelmann, S. N. Lehrman, R. M. Blum, D. W. Barry, G. M. Shearer, M. A. Fischl, H. Mitsuya, R. C. Gallo, J. M. Collins, D. P. Bolognesi, C. E. Myers, and S. Broder. 1986. Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication to patients with AIDS or AIDS-related complex. *Lancet* **i**:575-580.