Antiviral Research, 18 (1992) 303–316

AVR 00562

Development of resistance to zidovudine in HIV strains isolated from CD4+ lymphocytes and plasma during therapy

Claus Nielsen[a], Peter C. Gøtzsche[b], Carsten Møller Nielsen[a], Jan Gerstoft[b] and Bent Faber Vestergaard[a]

[a]*Department of Virology, Statens Seruminstitut (SSI), Copenhagen, Denmark and*
[b]*Department of Infectious Diseases, Rigshospitalet, Copenhagen, Denmark*

(Received 5 December 1991; accepted 3 February 1992)

Summary

An assay based on production of HIV antigen in cultures of CD4+ lymphocytes infected 'in vitro' with cell-free virus was established. Using this assay it was possible to isolate, propagate and reliably determine the zidovudine susceptibility of HIV isolates from all patients despite differences in cellular tropism and syncytium inducing capacity. Using this assay, differences in zidovudine susceptibility of 52 serial isolates obtained from 16 patients before and after initiation of therapy were examined. HIV with a 10- to 100-fold reduced susceptibility to zidovudine were isolated from 13 patients as early as 4 months after initiation of therapy. Number of months of zidovudine treatment was strongly associated with development of viral resistance, and high CD4 cell counts tended to be associated with lower rates of development of resistance. That patients can harbor mixtures of virus strains with different susceptibility to zidovudine was confirmed by the differences in susceptibility between isolates obtained simultaneously from CD4+ lymphocyte and plasma, and by the differences in susceptibility between virus strains isolated from clones of CD4+ lymphocytes.

Zidovudine; Drug susceptibility assay; HIV-1; CD4+ lymphocyte; Heterogeneity; Resistance

Correspondence to: C. Nielsen, Retrovirus laboratory, Department of Virology, Statens Seruminstitut, Artillerivej 5, DK-2300 Copenhagen S, Denmark.

Introduction

The nucleoside analogue 3′-azido-3′deoxythymidine (zidovudine or AZT) inhibits replication of HIV in vitro (Mitsuya et al., 1985). Zidovudine is triphosphorylated by cellular thymidine kinases, whereafter it inhibits HIV reverse transcriptase (RT) competitively. Its incorporation into viral DNA, instead of thymidine, results in premature termination of DNA chain elongation and inhibition of replication (Furman et al., 1986).

Zidovudine has an effect on the survival of AIDS patients (Fischl et al., 1987) and on the level of p24 antigen and CD4 cell count. However, during prolonged administration of zidovudine, the clinical and immunological benefits of the therapy decline (Dournon et al., 1988; Sette et al., 1989). One explanation for progression of disease, despite therapy, could be development of zidovudine-resistant variants of HIV.

Recently, HIV strains with reduced susceptibility to zidovudine have been isolated from patients under treatment (Larder et al., 1989; Rooke et al., 1989; Land et al., 1990; Larder et al., 1990). In these studies, zidovudine susceptibility of the isolates was determined using assays dependent on CD4+ cell lines (MT-2 and HT4-6C) (Land et al., 1990; Larder et al., 1989; 1990), or by direct inclusion of the drug in the isolation culture medium (Rooke et al., 1989; Land et al., 1990). Assays dependent on CD4+ cell lines reduce the number of isolates testable in the assay to those with tropism for cell lines (approximately 30% of all isolates) (Tersmette et al., 1988). Direct inclusion of zidovudine in the culture medium suffers from the drawback that variations in the infectious titer in the isolation cultures might influence the measurement of zidovudine susceptibility.

The aim of this study is to establish a zidovudine susceptibility assay for HIV isolates using standardized titers of infectious virus and CD4+ donor lymphocytes. CD4+ lymphocytes are susceptible to infection with virus isolates from patients in all stages of immunodeficiency, and the use of these cells more closely reflects the replication of HIV in the human host. Using this assay we wanted to examine whether development of zidovudine resistance, as previously reported for selected isolates with tropism for cell lines (Larder et al., 1989; Land et al., 1990), could be confirmed as a general phenomenon. To this end we have examined differences in zidovudine susceptibility for 52 serial isolates from 16 patients before and after initiation of zidovudine therapy. We have also examined whether heterogeneous populations of virus with different zidovudine susceptibility can exist simultaneously in a patient.

Materials and Methods

Patients and samples

Sixteen patients with AIDS or serious HIV infection who participated in a randomized dose–response study of zidovudine (Nordic Medical Research

Council's HIV therapy group, 1991) were recruited. Six were randomized to 400 mg, 4 to 800 mg, and 6 to 1200 mg zidovudine daily. None of the patients had previously been treated with zidovudine.

Blood samples for virus isolation were obtained before start of treatment and with regular intervals throughout the study. The CD4 cell count was measured at the same intervals.

Isolation of HIV

Peripheral mononuclear blood cells (PMC) from samples of 50 ml heparinized blood, diluted 1:2 in RPMI 1640, were separated by lymphoprep density gradient centrifugation. The PMC were harvested and the plasma-RPMI 1640 mixture was removed and stored at −80°C.

Isolation from PMC was done by co-cultivation of purified CD4+ patient lymphocytes with PHA-stimulated CD4+ lymphocytes purified from HIV antibody-negative blood donors as previously described (Nielsen et al., 1989; 1991). CD4+ lymphocytes were selected by lymphocyte panning with OKT4 monoclonal antibodies (hybridoma supernatant from ATCC clone number CRL 8002).

Isolation from plasma was done by cultivation of 3 ml of thawed plasma-RPMI 1640 mixture with 5×10^6 PHA-stimulated CD4+ lymphocytes in a total volume of 6 ml culture medium (RPMI 1640 supplemented with antibiotics, 10% heat-inactivated fetal calf serum (FCS) (Biochrom), 20 IU/ml recombinant Interleukin-2 (Boehringer Mannheim), and 2 μg/ml polybrene (SIGMA)). The cells were washed after 24 h and cultured in 10 ml culture medium. Half the culture medium was changed twice a week, and 2×10^6 PHA-stimulated CD4+ donor lymphocytes were added once a week.

HIV expression in isolation cultures was monitored by antigen detection in culture supernatants.

Preparation of virus stocks

HIV isolates were propagated for one or two passages in PHA-stimulated CD4+ donor lymphocytes. Three ml of cell-free culture supernatant were incubated with 5×10^6 CD4+ donor lymphocytes overnight. The cells were washed and then cultured as described for isolation cultures. Clarified supernatants, harvested at the burst of antigen production, were stored in aliquots at −80°C.

Determination of infectious virus titer ($TCID_{50}$)

Comparative measurements of the amount of infectious virus and HIV antigen in virus stocks were done in isolates from 6 different patients.

A linear relation between the infectious titer ($TCID_{50}$), determined by endpoint titrations in CD4+ lymphocytes, and the concentration of antigen was found ($r = 0.98$). The relation was only linear if the culture supernatants were harvested at the burst of antigen production.

The infectious titer of the virus stocks, used for zidovudine susceptibility

testing, was therefore determined indirectly by measurements of the HIV antigen concentration. A concentration of 1200 AU/ml of HIV antigen, corresponding to 40 $TCID_{50}$/ ml or 20 $TCID_{50}$ per million of cells, was chosen.

Zidovudine susceptibility assay

Five million purified CD4+ donor lymphocytes were incubated for 3 h at 37°C with 2.5 ml of virus dilution containing 20 $TCID_{50}$ per million cells. After washing, the cells were transferred to a 24-well cell culture plate (Nunc, Denmark, cat. no. 143982) (0.5×10^6 per well) and cultured in duplicate, in 1.5 ml culture medium supplemented with zidovudine at different concentrations (0 μM, 0.1 μM, 1 μM and 10 μM). Zidovudine was dissolved in dimethylsulfoxide and stored as a 10-mM solution at −80°C. Half of the culture medium, supplemented with the different concentrations of zidovudine, was changed every 3rd or 4th day.

The concentration of HIV antigen in the supernatants – determined by ELISA – was normally measured after 14 days of culture. However, in supernatants from cultures with extensive syncytium formation, the antigen concentration was determined when syncytium formation could be observed in the cultures without zidovudine.

HIV antigen detection

HIV antigen in cell-free culture supernatants was measured by a biotin/ avidin potentiated double antibody sandwich ELISA (Nielsen et al., 1987).

Optical densities obtained by ELISA were expressed, relative to dilution series of a standard HIV antigen preparation, in arbitrary units (AU).

Calculation of IC_{50} and IC_{90}

The mean HIV antigen production in the cultures supplemented with each concentration of zidovudine was expressed relative to the mean antigen production in cultures without drug, and the percentage inhibition for the different concentrations was calculated.

50% and 90% inhibitory concentrations of zidovudine (IC_{50} and IC_{90}) were determined by interpolation from the plots of percent inhibition versus zidovudine concentration.

Viral isolates with $IC_{50} < 0.1$ μM were considered sensitive, those with $IC_{50} \geqslant 0.1$ and < 1 μM as partially resistant, and those with $IC_{50} \geqslant 1$ μM as highly resistant.

Statistical methods

Multivariate linear models were used to study the association of viral susceptibility (IC_{50} and IC_{90}) with other variables. As outcome variables, IC_{50}, IC_{90}, log IC_{50} and log IC_{90} were used. As predictor variables, number of months on treatment, randomized dose, occurrence of dose reduction or temporary withdrawal of therapy, CD4 cell count and patient identity were used. Analyses were also done with log CD4 cells and log treatment months

(adding 1 to avoid log zero values), and with only baseline CD4 cell counts, omitting the values during follow-up.

Results

Isolation of HIV

Infectious HIV was isolated from CD4+ lymphocytes in all 52 serial blood samples from 16 patients. Isolation from plasma was attempted in 25 of the latest blood samples (after 7–20 months of therapy), and 12 were found positive.

Evaluation of the zidovudine susceptibility assay

The influence of number of infectious doses ($TCID_{50}$) on zidovudine susceptibility measurements was tested using different concentrations of infectious virus in the assay. The susceptibility of the HIV strain HTLV-IIIB using 10^1, 10^2, 10^3 and 10^4 $TCID_{50}$ per million cells showed a 10-fold difference in IC_{50} between 10^1 and 10^4 $TCID_{50}$ (0.04 μM and 0.4 μM respectively) and a 40-fold difference in IC_{90} (0.08 μM and 3 μM) (Table 1). In order to get comparable susceptibility measurements from different samples, we therefore standardized the infectious titer of the virus stocks used as inoculum in the susceptibility assay.

The susceptibility of 4 patient isolates, obtained before treatment, and HTLV-IIIB was determined using 20 $TCID_{50}$ per million cells. In addition to the 4 concentrations of zidovudine, described in Materials and Methods, 0.001 μM and 0.01 μM were included in the assay. IC_{50} values from all 5 isolates were in the range from 0.01 μM to 0.05 μM (mean 0.03 μM). This indicated that the 4 zidovudine concentrations used in the assay were adequate for susceptibility determination.

Zidovudine susceptibility of serial isolates from patients

HIV strains with reduced susceptibility to zidovudine were isolated from 13 of the 16 patients included in this study (Table 2). Isolates from 6 of the patients were partially resistant and isolates from 7 patients highly resistant.

TABLE 1

Influence of number of infectious doses on zidovudine sensitivity measurements

$TCID_{50}$[a]	IC_{50} (μM)	IC_{90} μM)
10^1	0.04	0.08
10^2	0.04	0.1
10^3	0.08	0.7
10^4	0.4	3

[a]$TCID_{50}$ per million cells of HTLV-IIIB.

TABLE 2

Sensitivity to zidovudine of 52 serial HIV isolates from 16 patients, isolated from CD4+ lymphocytes before and 4–20 months after initiation of therapy

Patient no.	Duration of therapy (months)	Zidovudine dose (mg/day)	CD4 count (per mm^3)	Syncytium-inducing capacity	IC_{50} (μM)	IC_{90} (μM)
393	0	400	120	+	0.03	0.08
	4		240	+	0.03	0.08
	7		54	+	0.04	0.2
	13		135	+	0.05	0.5
	20		120	+	1	6
410	0	400	200	–	0.03	0.08
	4		464	–	0.2	0.9
	8		108	–	2	8
	13		78	–	2	7
	20		27	+	2	>10
411	0	400	200	–	0.04	0.1
	4		374	–	0.04	0.3
	8		210	–	0.6	5
	13		85	–	2	8
416	0	400	100	–	0.03	0.09
	4		135	–	0.04	0.3
	7		120	–	0.05	1
	14		66	–	0.2	4
421	0	400	100	+	0.04	0.1
	4		68	+	0.07	0.6
	8		26	+	0.3	4
422	0	400	30	+	0.03	0.08
	7		16	+	1	6
392	0	800	8	+	0.04	0.1
	9		2	+	4	>10
415	0	800	198	+	0.04	0.1
	4		72	+	0.04	0.1
	7		216	+	0.04	0.2
420	0	800	170	+	0.04	0.1
	5		66	+	0.04	0.09
	8		200	+	0.04	0.09
424	0	800	100	+	0.03	0.08
	4		210	+	0.06	0.5
	7		90	+	2	8
372	0	1200	136	–	0.03	0.08
	4		180	–	0.03	0.08
	9		200	–	0.07	3
	13		70	+	0.9	9
391	0	1200	10	+	0.04	0.2
	6		16	+	0.1	4
	12		12	+	0.7	>10

396	0	1200	5	+	0.04	0.2
	13		0	+	0.6	10
	19		5	+	1	>10
414	0	1200	200	+	0.04	0.3
	4		120	+	0.2	0.7
	12		72	+	0.4	2
419	0	1200	100	–	0.04	0.1
	4		48	–	0.03	0.08
	7		268	–	0.05	0.6
432	0	1200	100	–	0.03	0.08
	7		250	–	0.2	2

Pt. 415 received 800 mg zidovudine per day. Pt. 419 received 1200 mg per day but therapy was stopped by month 3 and 4, and the dose was reduced to 400 mg per day from the 5th month. Pt. 420 received 400 mg per day, but treatment was stopped after 3 months.

Zidovudine susceptibility of HIV isolates, isolated simultaneously from CD4+ cells and plasma

Zidovudine susceptibility of isolates from 8 patients, isolated simultaneously from plasma and CD4+ lymphocytes are shown in Table 3. In 7 patients the zidovudine susceptibility of cell and plasma isolates were identical. However, in one patient (no. 411) the zidovudine susceptibility of the plasma isolate was 10-

TABLE 3

Zidovudine sensitivity of HIV strains, isolated simultaneously from CD4+ cells and plasma

Patient no.	Duration of therapy (months)	CD4+ cells		Plasma	
		IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)
372	9	0.07	3	0.09	2
392	9	4	>10	4	>10
396	19	1	>10	3	>10
410	8	2	8	3	8
	13	2	7	3	8
	20	2	>10	1	9
411	8	0.6	5	0.04	0.4
	13	2	8	0.3	4
416	7	0.05	1	0.04	0.9
	14	0.2	4	0.1	3
419	7	0.05	0.6	0.04	0.2
422	7	1	6	1	6

No difference in replicative and syncytium-inducing capacity was found between HIV isolated simultaneously from CD4+ cells and plasma.

TABLE 4

Sensitivity to zidovudine of HIV isolated from clones of CD4+ cells from the same patient

Isolate	IC_{50} (μM)	IC_{90} (μM)
Isolate[a]	0.2	2
4A-2[b]	0.9	6
4A-3[b]	1	10
4B-6[b]	0.03	0.08
4D-3[b]	0.08	0.9

[a]Isolated from 4×10^6 patient CD4+ cells; [b]isolated from 10^4 patient CD4+ cells.

fold higher than the susceptibility of the cell isolate after 8 months of therapy. In samples from this patient after 13 months of therapy, the zidovudine susceptibility of isolates from both cells and plasma had decreased compared to previous isolates, but the difference between the isolates remained, the plasma isolate being 10-fold more susceptible than the cell isolate.

Zidovudine susceptibility of HIV isolated from clones of CD4+ lymphocytes

The heterogeneity of virus strains in CD4+ lymphocytes from a single patient, with regard to their zidovudine susceptibility, was evaluated by comparing the susceptibility of HIV isolated from clones of the patient's cells. Isolation of HIV was attempted from 48 cultures consisting of either 10^4 or 10^3 patient CD4+ lymphocytes (culture was done as described in Material and Methods).

Four positive cultures (isolate: 4A-2, 4A-3, 4B-6 and 4D-3) were obtained from cultures consisting of 10^4 CD4+ patient cells while all cultures consisting of 10^3 patient cells were negative. This indicates that the lowest amount of patient cells giving a positive culture (a clone) was 10^4. In addition infectious virus was obtained from two standard cultures consisting of 4×10^6 patient

TABLE 5

Sensitivity to zidovudine of HIV isolates passed continuously in purified CD4+ donor lymphocytes

	Isolate		
	34c	48c	92c
Passage number	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
0[a]	2	0.09	0.3
1	3	0.06	0.1
2	2	0.05	0.3
3	1	0.05	0.4
4	3	0.1	0.1
5	3	0.05	0.3
6	1	0.1	0.3
7	4	0.06	0.08
8	3	0.05	0.1
9	2	0.05	0.2
10	3	0.06	0.1

[a]Isolate before passage.

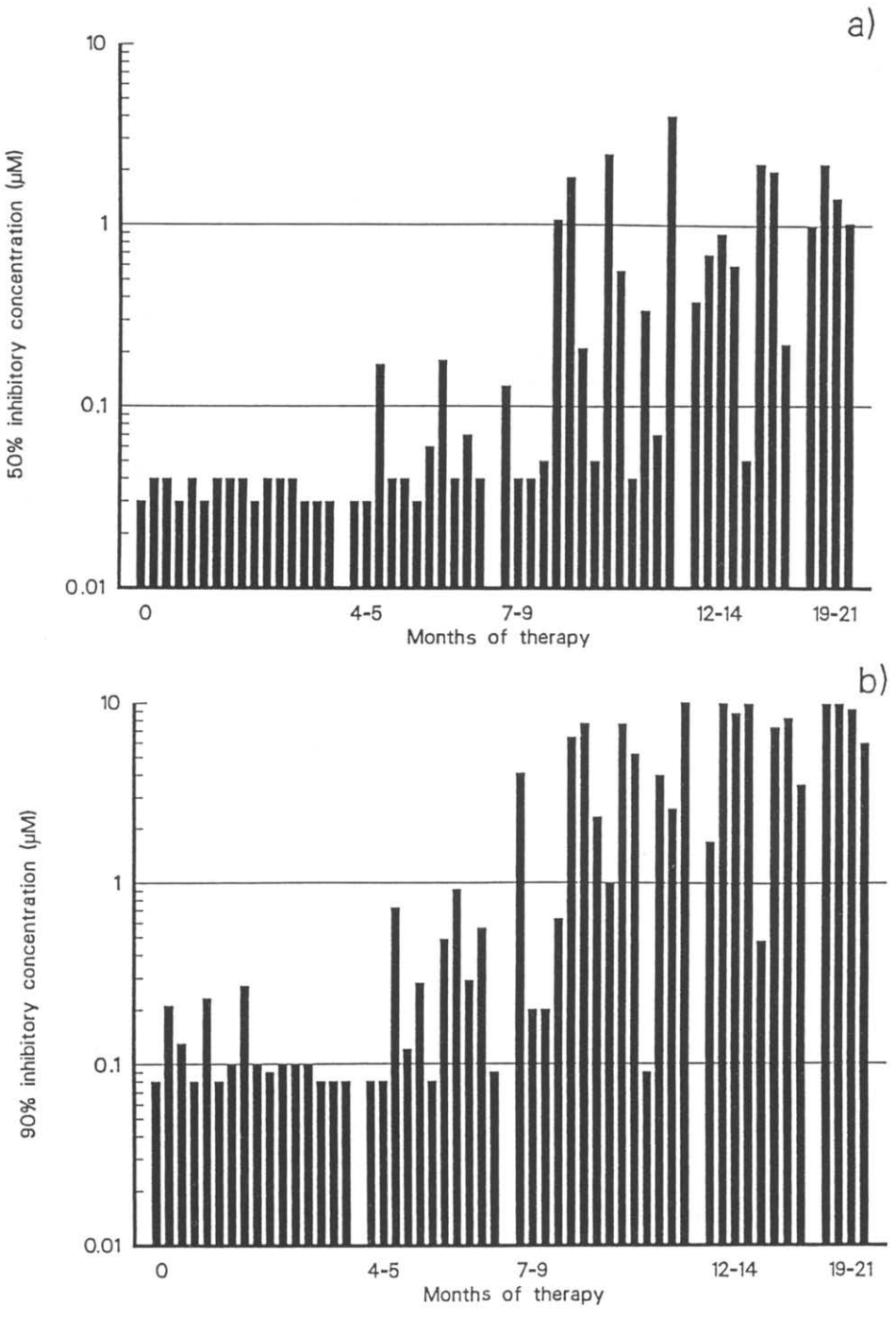

Fig. 1. Association between number of months of zidovudine therapy and development of viral resistance. 50% (a) and 90% (b) inhibitory concentrations versus months of therapy are shown for 52 serial HIV isolates.

CD4+ cells.

Results from the susceptibility measurements (Table 4) showed a substantial difference in the zidovudine susceptibility between the 4 virus strains isolated from cell clones, while the susceptibility of the two standard isolates was equal (IC_{50}: 0.2 μM).

Susceptibility to zidovudine of HIV isolates passed in purified CD4+ donor lymphocytes

The stability of zidovudine-resistant phenotypes of isolates was evaluated by comparing the susceptibility of 3 isolates (undiluted culture supernatant) passed 10 times in CD4+ donor lymphocytes without addition of zidovudine to the culture medium, as described in Material and Methods. The period of time for all 10 passages was approximately 6 months. Before passage, isolate 34c was highly resistant (IC_{50}: 2 μM), isolate 48c was only slightly resistant (IC_{50}: 0.09 μM), and isolate 92c was partially resistant (IC_{50}: 0.3 μM).

The results (Table 5) show that the resistant phenotype of all 3 virus isolates was stable during the passages. (The regression coefficients of the linear plots of IC_{50} versus number of passages were not significantly different from zero (95% confidence limit).)

Association of viral susceptibility with months of treatment, dose, and CD4 cell counts

The best regression models, with highest determination coefficients, were obtained with log IC_{90} as outcome variable, rather than log IC_{50} or the arithmetic values. Further, the normality assumption for the residuals was best met with logarithmic values.

In all statistical models, number of months on zidovudine therapy was a strong predictor of development of viral resistance ($P < 0.00001$ for log IC_{50} and log IC_{90}) (Fig. 1). The occurrence of dose reductions or treatment interruptions did not contribute significantly to the statistical models ($P \geqslant 0.20$) (the coefficients were negative, implying less risk of development of resistance if the dose was interrupted).

Serial CD4 cell counts over time showed a significantly negative correlation with development of viral resistance in 5 out of 8 regression analyses, and there was a tendency towards slower development of resistance with a higher baseline CD4 count (median $P = 0.14$ for the 8 analyses).

Since the dose was automatically dropped from all regression models because of high correlation with other variables, the effect of the randomized dose was studied by using only the last recorded susceptibility value for each of the 16 patients. The dose was not associated with development of resistance ($0.21 < P < 0.46$), whereas the tendency towards slower development of resistance with higher baseline CD4 counts was still apparent ($0.03 < P < 0.13$).

Discussion

An assay based on production of HIV antigen in cultures of CD4+ lymphocytes infected 'in vitro' with cell-free virus was established. Using this assay it was possible to isolate, propagate and reliably determine the zidovudine susceptibility of HIV isolates from all patients despite differences in cellular tropism and syncytium inducing capacity (Table 2).

Our data show that HIV with a 10- to 100-fold reduced susceptibility to zidovudine can be isolated from patients as early as 4 months after initiation of therapy. These results correspond to results reported previously in selected isolates with tropism for cell lines (Larder et al., 1989; Land et al., 1990; Boucher et al., 1990). Thus, development of resistant HIV strains can be considered a general phenomenon and not a phenomenon restricted to isolates with tropism for cell lines, predominantly found in patients in late stage of disease.

We found a strong association between number of months of zidovudine treatment and development of viral resistance. The median time to development of resistant strains was 8 months with a range of 4–20 months, which is in agreement with other studies (Boucher et al., 1990; Richmann et al., 1990).

High CD4 cell counts tended to be associated with lower rates of development of resistance. These results are in accord with findings in North American patients (Richmann et al., 1990), and the explanation may be that mutations causing resistance are more likely to emerge with an increased number of replicative events, which has been shown to be correlated with declining CD4 counts (Schnittman et al. 1990; Venet et al., 1991). A relationship between the zidovudine dose used for therapy and development of resistance was not established. The trend in our study was towards faster development of resistance with lower doses, whereas the opposite trend was noted in the American study (Richmann et al., 1990).

A substantial difference in susceptibility between isolates obtained simultaneously from CD4+ lymphocytes and plasma was demonstrated in 1 of 8 patients. This suggests that 'in vitro' reactivated latent virus (virus from cells) and 'in vivo' activated virus (virus from plasma) can display important differences in zidovudine susceptibility, and that patients can harbor mixtures of virus strains with different susceptibility to zidovudine. These findings were further supported by the difference in susceptibility found in the virus strains isolated from clones of CD4+ lymphocytes.

Studies of the stability of the zidovudine-resistant phenotypes replicating without the selection pressure from zidovudine showed that the susceptibility of the isolates did not change during 10 continuous 'in vitro' passages in CD4+ lymphocytes for a duration of 6 months. If this 'in vitro' phenomenon also can be applied to the 'in vivo' situation, transmission of stable resistant virus strains could have serious implications for future antiviral treatment of HIV-infected individuals.

Our studies of the influence of number of infectious doses on zidovudine measurements showed that a 1000-fold difference in virus titer resulted in a 10-fold difference in measured zidovudine susceptibility. This means that reliable results concerning susceptibility only can be obtained when the virus inoculum used in the test system has been standardized. We used an HIV-1 antigen capture ELISA, predominantly reacting with p24, to standardize the amount of infectious virus. This procedure is much less laborious than endpoint titration in cell cultures. Since the virus stocks produced as described in this study are of relatively low titers, estimation of virus titer by antigen detection is sufficiently accurate for standardization of the virus inoculum.

A difference in syncytium-inducing capacity of isolates could possibly affect the susceptibility determination in our assay. Extensive syncytium formation will result in a reduced production of virus antigen, especially in cultures without zidovudine, resulting in an apparently higher degree of resistance in syncytium-inducing than in non-syncytium-inducing isolates. To reduce the influence of this drawback, concentrations of antigen in cultures of syncytium-inducing isolates were tested when formation of syncytia was be observed in the cultures without zidovudine.

The applicability of the zidovudine susceptibility assay described in this study is supported by two facts: (1) all pre-therapy isolates were found sensitive, and (2) the progressive development of isolates with reduced susceptibility to zidovudine was only correlated to months of treatment and not to the replicative and the syncytium-inducing capacity of the isolates.

In this study we have described a method to measure zidovudine susceptibility of HIV from all patients undergoing therapy with this compound. HIV with reduced susceptibility to zidovudine was demonstrated in almost all patients receiving the drug without interruption, which may explain why the effect of zidovudine appears to be transitory.

Detection of virus drug resistance will be increasingly necessary in the future when new antiretroviral agents become available. PCR amplification of mutations associated with reduced drug susceptibility will probably be the method of choice, but until more is known about these mutations it will be necessary to use reliable assays, applicable to isolates from patients in all stages of immunodeficiency.

Acknowledgements

We thank Anne Lyhning Jensen, Alice Stengade and Merete Simonsen for excellent technical assistance; Lone Jensen for collecting the blood samples; Birte Rothstein for secretarial help; and John-Erik S. Hansen for critical reading of the manuscript and helpful suggestions. This work was supported by the Danish Medical Research Council (Grant 127941), and the Danish Insurance Association (Grant 03.2821:42).

References

Boucher, C.A.B., Tersmette, M., Lange, J.M.A., Kellam, P., De Goede, R.E.Y., Mulder, J.W., Darby, G., Goudsmit, J. and Larder, B.A. (1990) Zidovudine sensitivity of human immunodeficiency viruses from high-risk, symptom-free individuals during therapy. Lancet 336, 585–590.

Dournon, E., Rozenbaum, W., Michon, C., Perronne, C., De Truchis, P., Bouvet, E., Levacher, M., Matheron, S., Gharakhanian, S., Girard, P.M., Salmon, D., Leport, C., Dazza, M.C. and Regnier, B. (1988) Effect of zidovudine in 365 consecutive patients with AIDS or AIDS-related complex. Lancet II, 1297–1299.

Fischl, M.A., Richman, D.D., Grieco, M.H., Gottlieb, M.S., Volberding, P.A., Laskin, O.L., Leedom, J.M., Gropman, J.E., Mildvan, D., Schooley, R.T., Jackson, G.G., Durack, D.T. and King, D. (1987) The AZT collaborative working group: the efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. New. Engl. J. Med. 317, 185–191.

Furman, P.A., Fyfe, J.A., St. Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Nusinoff Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H. and Barry, D.W. (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.

Land, S., Treloar, G., McPhee, D., Birch, C., Doherty, R., Cooper, D. and Gust, I. (1990) Decreased in vitro susceptibility to zidovudine of HIV isolates obtained from patients with AIDS. J. Infect. Dis. 161, 326–329.

Larder, B.A., Darby, G. and Richman, D.D. (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243, 1731–1734.

Larder, B.A., Chesebro, B. and Richman, D.D. (1990) Susceptibilities of zidovudine-susceptible and resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay. Antimicrob. Agents Chemother. 34, 436–441.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St. Clair, M.H., Nusinoff, Lehrman, S., Gallo, R.C., Bolognesi, D., Barry, D.W. and Broder, S. (1985) 3′-Azido-3′deoxythymidine (BW 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.

Nielsen, C., Petersen, J.L., Nielsen, C.M., Pedersen, C., Mathiesen, L.R. and Vestergaard, B.F. (1989) Isolation of HIV using T-Lymphocyte panning: methodological aspects. J. Virol. Methods 26, 105–114.

Nielsen, C., Nielsen, C.M., Petersen, J.L., Gøtzsche, P.C., Pedersen, C., Arendrup, M. and Vestergaard, B.F. (1991) Isolation of HIV from cultures of purified CD4+ Lymphocytes. J. Virol. Methods 35, 15–25.

Nielsen, C.M., Bygbjerg, I.C. and Vestergaard, B.F. (1987) Detection of HIV antigens in eluates from whole blood collected on filterpaper. Lancet I, 566–567.

Nordic Medical Research Council's HIV Therapy Study Group. (1992) Double-blind dose–response study of zidovudine in AIDS and advanced HIV infection. Br. Med. J. 304, 13–17.

Richmann, D.D., Grimes, J.M. and Lagakos, S.W. (1990) Effect of stage of disease and drug dose on zidovudine susceptibility of isolates of human immunodeficiency virus. J. Acquir. Immun. Defic. Syndrom. 3, 743–746.

Rooke, R., Trembley, M., Soudeyns, H., DeStephano, L., Yao, Y.-J., Fanning, M., Tsoukas, C., Gill, J., Ruedy, J., Wainberg, M.A. and the Canadian Zidovudine Multi-Center Study Group. (1989) Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine therapy. AIDS 3, 411–415.

Schnittman, S.M., Greenhouse, J.J., Psallidopoulos, M.C., Baseler, M., Salzman, N.P., Fauci, A.S. and Lane, H.C. (1990) Increased viral burden in CD4+ cells from patients with human immunodeficiency virus (HIV) infection reflects rapidly progressive immunosuppression and clinical disease. Ann. Intern. Med. 113, 438–443.

Sette, P., Tozzi, V., Galgani, S., Tossini, G., Narciso, P., Camporiondo, M.P., Leoni, G.C. and Visco, G. (1989) Low-dose zidovudine for AIDS. Lancet I, 1136–1137.

Tersmette, M., De Goede, R.E.Y., Al, B.J.M., Winkel, I.N., Gruters, R.A., Cuypers, H.T., Huisman, H.G. and Miedema, F. (1988) Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. J. Virol. 62, 2026–2032.

Venet, A., Lu, W., Beldjord, K. and Andrieu, J.-M. (1991) Correlation between CD4 counts and cellular and plasma viral load in HIV-1-seropositive individuals. AIDS 5, 283–288.