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HIV-1 viral load, phenotype, and resistance in a subset of drug-naïve participants from the Delta trial

Francoise Brun-Vézinet, Charles Boucher, Clive Loveday, Diane Descamps, Véronique Fauveau, Jacques Izopet, Don Jeffries, Steve Kaye, Corinne Krzyzanowski, Andrew Nunn, Rob Schuurman, Jean-Marie Seigneurin, Catherine Tamalet, Richard Tedder, Jonathan Weber, Gerrit-Jan Weverling, the National Virology Groups, and the Delta Virology Working Group and Coordinating Committee*

Summary

Background The Delta trial showed that combination therapy (zidovudine plus didanosine and zidovudine plus zalcitabine) substantially lengthened life and reduced disease progression compared with zidovudine monotherapy. We did a nested virological study in three countries (France, the Netherlands, and the UK) to investigate changes in markers for viral load and antiretroviral-drug resistance during therapy.

Methods 240 zidovudine-naïve HIV-1-infected patients were randomly assigned zidovudine only (n=87), zidovudine plus didanosine (n=80), or zidovudine plus zalcitabine (n=73). Viral load in peripheral-blood mononuclear cells and plasma was measured by quantitative culture. Plasma HIV-1 RNA was measured by reverse-transcriptase PCR amplification, and serum p24 antigen by ELISA. Resistance to antiretroviral drugs was measured phenotypically by culture and genotypically by detection and quantification of drug-related point mutations in the *pol* gene. Analyses were done by intention to treat.

Findings The reduction in viral load was greatest 4–12 weeks after the start of therapy and was most pronounced in the combination-therapy study groups (median reductions of RNA at 4 weeks 1.58, 1.28, and 0.49 log₁₀ copies/mL for zidovudine plus didanosine, zidovudine plus zalcitabine, and zidovudine only, respectively). RNA levels at 8 weeks were predictive of disease progression and death after allowance for baseline values.

At 48 weeks, the proportion of participants with phenotypic zidovudine resistance was similar in all three groups: didanosine and zalcitabine resistance were rare; zidovudine genomic resistance correlated with phenotypic resistance ($r=0.54$, $p<0.0001$) and developed earlier in the

combined-therapy groups. However, participants in the zidovudine monotherapy group had higher circulating loads of resistant virus than those in the combined-therapy groups.

Interpretation Combined antiretroviral therapy was more efficient at lowering virus load than monotherapy. Although zidovudine resistance was common in monotherapy and combined-therapy groups, circulating concentrations of resistant virus were substantially lower in the combination groups, which is likely to be a result of the continued antiviral activity of didanosine or zalcitabine.

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See Commentary page 970

Introduction

Three large trials comparing zidovudine monotherapy with combinations of zidovudine plus didanosine or zalcitabine have shown the clinical benefits of combination therapy for HIV-1 patients on both progression to symptomatic disease and survival.^{1–3}

Delta was a randomised double-blind trial comparing zidovudine (600 mg daily) alone with zidovudine plus didanosine (400 mg daily) or zidovudine plus zalcitabine (2.25 mg daily). Participants had AIDS with CD4-cell counts of more than $50 \times 10^6/L$, AIDS-related complex, or asymptomatic HIV-1 infection with CD4-cell counts of less than $350 \times 10^6/L$. The two combined-therapy regimens lengthened life and delayed disease progression compared with zidovudine monotherapy, particularly among participants who had not received zidovudine previously (Delta 1); there was less direct evidence of benefit among participants who had received zidovudine for at least 3 months previously.

We did a prospective virology substudy of zidovudine-naïve HIV-1-infected participants entering the Delta-1 trial. We measured changes in viral load, CD4-cell count, genotypic and phenotypic drug resistance, and assayed and analysed syncytium-inducing phenotype in relation to drug therapy and clinical outcome.

Participants and methods

Participants

We enrolled 240 participants from the Delta-1 trial who had received no previous antiretroviral therapy. Patients were recruited from 22 centres in France, the Netherlands, and the UK. We selected centres by proximity to a participating laboratory and participants by their willingness to attend centres regularly and to provide samples. We randomly assigned patients zidovudine only (n=87), zidovudine plus didanosine (n=80), or zidovudine plus zalcitabine (n=73). Visits were scheduled at 2 weeks before randomisation, at trial entry, every 4 weeks until week 16, every 8 weeks until week 64, and then every 16 weeks until week 112.

We collected blood samples for real-time measurement of plasma and cellular viraemia; peripheral-blood mononuclear cells, serum, and plasma were stored at -70°C or in liquid nitrogen. Not all measurements were done at every time point.

Local ethics committee approval was obtained for each centre.

*Listed at end of paper

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Viral assays

We quantified plasma and cellular viraemia by a consensus method.⁴ We monitored viral expression in culture supernatants at day 28 with an HIV-1 p24 antigen assay (Abbott, Dupont, Coulter, or Pasteur). Cultures were classified as positive when the optical density was at least four times the cut-off value. We calculated the mean number of infectious units per 10^6 cells or per mL of plasma from the number of positive cells among the replicates of each dilution, assuming a Poisson distribution of the number of infectious units (related to TCID₅₀ [50% tissue culture infectious dose] by a factor of log₂).⁵

We measured free serum HIV-1 p24 antigen in serum up to week 48 with the Abbott (Abbott Diagnostics, Chicago, IL, USA) or Coulter (Coulter Corporation, Hialeah, FL, USA) kits. Dissociated HIV-1 p24 antigen was quantified with the Coulter immune complex dissociated p24 assay. We confirmed reactive samples by specific antibody neutralisation.

HIV-1 RNA load was measured in 200 μ L plasma containing edetic acid by a reverse-transcriptase PCR assay⁶ (Amplicor Monitor, Roche Diagnostic Systems) from a single batch of kits. Positive and negative kit controls were replaced on each plate by three standards containing 0, 15 000, and 150 000 HIV-1 (Clade B) copies/mL, quantified by electron microscopy.

In the point-mutation assay for genotypic resistance, HIV-1 RNA from 100 μ L plasma or serum was captured onto latex particles coated with a mixture of polyclonal and monoclonal antibodies to gp120, and was reverse transcribed.⁷ For samples with low HIV-1 RNA levels, we did another extraction from a larger volume of plasma or serum by a silica-capture method.⁸ The cDNA was amplified in a nested PCR and the proportion of mutant sequences at codons 41, 65, 67, 69, 70, 74, 75, 184, 215, and 219 in the reverse-transcriptase genome was measured in a quantitative assay. Minimum mutant detection was 4% for codon 215 and 2% for the other codons.

We analysed phenotypic susceptibility of the cellular isolate from samples taken at weeks 0 and 48 in a peripheral-blood mononuclear-cell assay.⁹ Baseline and 48-week isolate pairs were compared in the same assay run. Decreased susceptibility to didanosine and zalcitabine was defined as at least a five-fold increase in IC₅₀ values between week 0 and week 48. For zidovudine, IC₅₀ of 0.5 μ mol/L or more was defined as decreased susceptibility.

We investigated the ability of the cellular viral isolates to induce syncytia in MT2 cells by a common protocol, in real time in the Netherlands and the UK, and on cryopreserved peripheral-blood mononuclear cells in France.¹⁰ In brief, a co-culture of 1×10^6 /L peripheral-blood mononuclear cells with 1×10^6 /L MT2 cells was made in a 25 cm² culture flask. We monitored cultures visually twice a week for 3 weeks for development of syncytia, and measured HIV-1 p24 antigen in the culture supernatant. Viral strains were syncytium-inducing (SI) if syncytia were seen in the MT2 co-culture at any time and if p24 antigen was detected. Strains were non-syncytium-inducing (NSI) if no syncytia were seen, independently of p24-antigen production.

Statistics

Cellular viraemia, HIV-1 RNA load, and immune complex dissociated p24 antigen titres were expressed as log₁₀ with undetectable concentrations set to log₁₀ of the cut-off of the assay. Plasma viraemia, MT2 assays, and free p24-antigen detection were classified as positive or negative. We used non-parametric tests to study the associations between the different viral variables and between viral and clinical variables at baseline. We summarised longitudinal changes in viral-load variables for each participant by the average change to 48 weeks and to 96 weeks, measured by area under the viral-load/time curve per unit of time, minus the baseline value, and compared the three treatment groups with the Kruskal-Wallis test.

For each treatment comparison (zidovudine monotherapy *vs* each combination), differences in RNA levels and in the proportion of participants with RNA levels below the limit of detection were estimated by the generalised estimating equations approach by means of independent working correlations between observations within participants.¹¹ We also did a global Wald test

(on 13 degrees of freedom) of the differences between groups at the 14 assessment time points. To test for the significance of differences at any particular time, we used the sequential multiple testing described by Marcus and colleagues.¹²

The ability of CD4-cell count and viral variables to predict clinical outcome was investigated by means of proportional hazards models, stratified by treatment group. We did univariate analyses for each variable and multivariate analyses with plasma HIV-1 RNA and CD4-cell count incorporated, in addition to other variables.

During the study and analysis, virologists were unaware which therapy the participants had received. All analyses compared treatment groups as randomised by intention to treat.

Quality assurance

Blood samples obtained from four participants with AIDS were coded and transferred to laboratories in each of the three countries within 6 h of collection for analysis. The three laboratories ranked the plasma samples by mean log₁₀ number of infectious units per mL plasma in the same order (0.02, 0.76, 1.4, 2.6). The quantitative cellular-viraemia titres of blood samples were closer (mean log₁₀ number of infectious units per 10^6 cells 1.3, 1.3, 2.4, 2.8) and the ranking of lower values differed slightly between the three laboratories. The SD of the mean titres between countries was 0.67 for plasma and 0.62 for cellular viraemia.

15 samples collected at 24 weeks were analysed by automated sequencing of *pol* (ABI, Applied Biosystems, Paris, France, and Vistra, Amersham International, Little Chalfont, UK). A comparison of results of the point-mutation assay with those of sequencing for 112 codons showed concordance between the methods in 97 instances. A novel silent mutation at codon T215Y/F (ACC to ACT) resulted in a false-positive point mutation result that was also found in the pretreatment sample. The 14 with apparent discrepancies arose because sequencing could not detect minor populations (<25%), or because interpretation in the sequencing software was ambiguous.

We also compared three control standards (150 000 copies/mL [5.17 log₁₀], 15 000 copies/mL [4.17 log₁₀], and negative plasma) to assess differences in laboratory HIV-1 RNA assays between countries.¹³

Results

We recruited 240 participants—126 from France, 48 from the Netherlands, and 66 from the UK. Baseline virological and immunological characteristics were similar in all groups (table 1). The characteristics were broadly similar to those of the remaining 1884 participants in Delta.

We found no significant differences in plasma HIV-1 RNA loads between participants with SI and NSI phenotypes ($p=0.6$). SI phenotype and clinical stage at entry were significantly associated ($p=0.05$); only 13% of participants with AIDS had the SI phenotype compared with 34% of both asymptomatic participants and those with AIDS-related complex. Individuals with SI phenotypes generally had lower CD4-cell counts (mean 170×10^6 /L) than those without SI phenotypes (mean 225×10^6 /L, $p=0.003$).

CD4-cell counts were negatively correlated with viral load, irrespective of whether load was quantified by culture (cellular viraemia, $r=-0.25$, $p<0.0001$, plasma viraemia, $r=-0.42$, $p<0.0001$), by PCR (plasma HIV-1 RNA, $r=-0.38$, $p<0.0001$), or by p24 antigen (immune complex dissociated p24 antigen, $r=-0.23$, $p<0.0006$). The four measures of viral load correlated significantly with each other and with disease stage. Participants with advanced disease had significantly higher RNA concentrations (mean plasma log₁₀ copies/mL: asymptomatic 4.44; AIDS-related complex 4.77; AIDS 5.19).

The narrow distribution of susceptibility to zidovudine resistance had a median IC₅₀ value of 0.013 μ mol for the

	Zidovudine alone (n=87)	Zidovudine plus didanosine (n=80)	Zidovudine plus zalcitabine (n=73)	All patients (n=240)*
Clinical disease stage				
Asymptomatic	46 (53%)	39 (49%)	42 (58%)	127 (53%)
AIDS-related complex	25 (29%)	27 (34%)	19 (26%)	71 (30%)
AIDS	16 (18%)	14 (18%)	12 (16%)	42 (17%)
CD4-cell count mean (SD)	207 (113)	210 (107)	205 (112)	207 (110)
p24 antigenaemia				
Positive	30 (36%)	28 (37%)	22 (32%)	80 (35%)
ICD				
Positive	56 (67%)	53 (70%)	41 (60%)	150 (66%)
Log ₁₀ pmol/mL median (IQR)	1.49 (1.14–2.27)	1.69 (1.20–3.14)	1.52 (1.13–3.11)	1.54 (1.19–2.25)
HIV-1 RNA				
Positive	80 (96%)	73 (97%)	70 (100%)	223 (98%)
Log ₁₀ copies/mL median (IQR)	4.77 (4.18–5.29)	4.76 (4.15–5.07)	4.68 (4.05–5.15)	4.71 (4.12–5.17)
Plasma culture				
Positive	60 (72%)	52 (68%)	56 (77%)	168 (72%)
Cellular culture				
Positive	79 (95%)	71 (93%)	69 (94%)	219 (94%)
NIU log ₁₀ median (IQR)	1.52 (0.91–2.04)	1.39 (0.39–2.04)	1.64 (0.86–2.12)	1.50 (0.67–2.06)
Biological phenotype				
SI/total	20/64 (31%)	15/56 (27%)	16/49 (33%)	51/169 (30%)

NIU=number of infected units.

*Participants tested for p24 antigen (n=228), HIV-1 RNA (n=228), plasma culture (n=232), cellular culture (n=232), and biological phenotype (n=169).

Table 1: Baseline clinical, virological, and immunological characteristics of 240 participants

103 isolates tested (table 2). Virus isolates from two participants showed borderline phenotypic resistance ($IC_{50} > 0.05 \mu\text{mol}$) to zidovudine, but none to didanosine or zalcitabine. We found no mutations in the reverse-transcriptase gene associated with didanosine or zalcitabine resistance at baseline, but found point mutations known to be associated with zidovudine resistance in plasma from 11 (6%) of 196 participants. We found the K70R mutation alone in ten participants (median rate 8% [range 4–70]). The T215Y/F mutation was detected in one participant (rate 14%) and no mutations of M41L were detected.

The median duration of taking assigned treatment (masked or open label) was 21 months for zidovudine, 17 months for zidovudine plus didanosine, and 23 months for zidovudine plus zalcitabine. At 48 weeks, the number of participants who could be assessed and who were still receiving assigned treatment was 66 (77%) for zidovudine, 53 (69%) zidovudine plus didanosine, and 54 (76%) for zidovudine plus zalcitabine, falling to 44 (61%), 32 (51%), and 41 (72%), respectively, by 96 weeks.

Viral load was significantly reduced after starting antiretroviral therapy (figure 1). The maximum decrease in HIV-1 RNA was between 4 and 12 weeks. The reduction was most pronounced in the two combined-treatment groups, therapy with median reductions at 4 weeks of 1.58 log₁₀ copies/mL for zidovudine plus didanosine and 1.28

for zidovudine plus zalcitabine, compared with 0.49 for zidovudine alone. At 24 weeks, the median decreases were 1.1, 1.1, and 0.3 log₁₀ copies/mL, respectively. Differences were significant between each combined-therapy group compared with monotherapy to 80 weeks. The global comparison of the zidovudine plus didanosine and zidovudine plus zalcitabine groups was significant in favour of the zidovudine plus didanosine group ($p < 0.05$) but not at any one time point after adjustment for multiple testing. During the first 96 weeks, HIV-1 RNA was below the cut-off point of the assay more often in the combined-therapy groups than in the monotherapy group. A global comparison of the combined therapy groups was significant in favour of the zidovudine plus didanosine group ($p < 0.05$); after 12 weeks the differences at individual weeks were not significant. At 24 weeks, 4% of participants in zidovudine group had undetectable concentrations of HIV-1 RNA compared with 22% of the zidovudine plus didanosine group and 18% of the zidovudine plus zalcitabine group.

An analysis limited to the small number of participants with baseline HIV-1 RNA concentrations of more than 5.0 log₁₀ copies/mL showed no significant difference ($p = 0.13$) between the reduction in load at 4 weeks, in the zidovudine plus didanosine group ($n = 20$, 1.9 log₁₀ copies/mL) compared with the zidovudine plus zalcitabine group ($n = 19$, 1.3 log₁₀ copies/mL).

Drug	Baseline All participants	Week 48		
		Zidovudine	Zidovudine plus didanosine	Zidovudine plus zalcitabine
Zidovudine				
Number of observations	103	27	14	11
IC_{50} mmol (median [IQR])	0.013 (0.01–0.02)	0.055 (0.016–0.21)	0.057 (0.017–0.42)	0.035 (0.018–1.15)
More than five-fold increase of IC_{50}	0	11 (41%)	6 (43%)	6 (55%)
$IC_{50} > 0.05$ mmol	2 (2%)	14 (52%)	7 (50%)	5 (45%)
Didanosine				
Number of observations	56	13	14	..
IC_{50} mmol (median [IQR])	0.215 (0.093–0.475)	0.28 (0.13–0.67)	0.33 (0.12–0.98)	..
More than five-fold increase of IC_{50}	0	0	1 (7%)	..
Zalcitabine				
Number of observations	46	13	..	10
IC_{50} mmol (median [IQR])	0.010 (0.005–0.0275)	0.009 (0.007–0.011)	..	0.013 (0.007–0.019)
More than five-fold increase of IC_{50}	0	0	..	0

Table 2: Phenotypic measurements of antiretroviral resistance for virus isolates at baseline and 48 weeks

Figure 1: Changes in indicators of HIV-1 infection
 A=change in HIV-1 RNA (median IQR); B=% below assay cut-off (95% CI); C=change in cellular viraemia from baseline (median IQR); D=change in dissociated p24 antigen from baseline (median IQR); E=change in CD4-cell counts (median IQR). Numbers of patients per group available from investigators.
 *Different time-scale used.

The highest proportions of patients negative on plasma culture occurred at 4 weeks in all three groups (44 [58%] in the zidovudine group, 57 [88%] in the didanosine group, and 44 [71%] in the zalcitabine). The highest proportion with undetectable free p24 antigen were 53 (73%) at week 4, 60 (88%) at week 12, and 51 (85%) at week 12, respectively (data not shown). Maximum rises in CD4-cell counts of 32 cells for the zidovudine group, 60 cells for the zidovudine plus didanosine group, and 51 cells for the zidovudine plus zalcitabine group occurred at 8, 8, and 12 weeks, respectively (figure 1).

When we compared changes in viral load, measured by differences during the first 48 and 96 weeks in area under the curve from baseline for plasma viral RNA, the differences between the two combined-therapy groups and monotherapy were highly significant ($p < 0.0001$ each), but there was no evidence of a difference between the combined-therapy groups.

Changes in area under the curve for quantitative cellular viraemia were also significantly greater in the two combined-therapy groups compared with zidovudine alone at 48 and 96 weeks, but at 96 weeks the change in area under the curve for the zidovudine plus zalcitabine group

was significantly greater than for the zidovudine plus didanosine group ($p = 0.03$). By contrast to RNA, for dissociated p24 antigenaemia there was no difference between changes during 48 weeks between the zidovudine plus zalcitabine and monotherapy only groups ($p > 0.5$) but the differences between the combined-therapy groups and between the zidovudine plus didanosine and monotherapy groups were significant ($p = 0.04$ and $p = 0.001$).

Cox's proportional-hazards analyses showed an association between all baseline markers of viral load, CD4-cell counts, death, and clinical progression or death. Multivariate analyses identified HIV-1 RNA load and CD4-cell counts as the only independently significant variables (data not shown). To assess the prognostic value of the HIV-1 RNA load at weeks 4 and 8, each were included in turn in a model with baseline HIV-1 RNA load and CD4-cell count based on a total of 209 and 200 participants with available data, respectively. After adjustment for baseline HIV-1 RNA load and CD4-cell count, we could not find an association between HIV-1 RNA load and death at week 4 ($p = 0.1$), but we did find an association with progression to AIDS, severe AIDS, or death (hazard ratio 2.0, $p = 0.002$). HIV-1 RNA load at week 8 was associated with the two endpoints; hazard ratio 1.9 ($p = 0.04$) for death and 2.2 ($p = 0.001$) for AIDS, severe AIDS, or death. After adjusting for baseline CD4-cell count neither the week 4 nor the week 8 CD4-cell count were of prognostic value for either endpoint.

Genomic zidovudine resistance was assayed to find the proportion of HIV-1 RNA sequences detectable in plasma that carried the M41L, D67N, K70R, T215Y/F, and K219E mutations. The median proportion of resistant mutations at codons 41, 70, and 215 are shown in figure 2. The highest frequency of D67N occurred at week 80 in 56% of participants (median proportion 7%) and of K219E at week 112 in 23% of participants (median 0%).

Zidovudine monotherapy was associated with rapid generation of viruses carrying the K70R mutation. The median proportion was 67% by 24 weeks and until 48 weeks and decreased after this time to about 40%. The frequency of the T215Y/F mutation increased rapidly after 24 weeks, with median frequencies of more than 90% at 80

Figure 2: **Percentage mutant sequences (median IQR) at codons M41L, K70R, and T215Y/F in plasma RNA**

Prevalence is expressed as percentage of genome and codon carrying drug-resistant mutation, shown by point mutation assay. Numbers of patients per group available from investigators.

and 112 weeks; M41L mutation accumulated much more gradually. By contrast, the accumulation of resistance mutations in the combined-therapy groups was more rapid, with high frequencies of T215Y/F by 48 weeks. This mutation was not preceded in either combined-therapy group by K70R mutant populations, which remained in the minority. We found a further difference in the evolution of genetic resistance between the two combined-therapy groups. In the zidovudine plus didanosine group, T215Y/F development was followed closely by the M41L mutation; in the zidovudine plus zalcitabine groups the T215Y/F genotype was present as a monotypic mutant population at 48 weeks (92%) in the virtual absence of M41L (2%), which increased to a high frequency by 112 weeks.

To find out the contribution of the resistant virus to the overall viral load detected over time, we plotted the viral load and percentage of mutant virus at codons M41L,

Figure 3: **Median absolute HIV-1 RNA load for the total and mutant populations at codons 41, 70, and 215**

Numbers of patients per group available from investigators.

K70R, and T215Y/F together (figure 3). Although the frequency of genomic resistance was lower in the monotherapy group, the overall rate of virus replication was higher and the load of mutant virus was substantially greater in this group than in the combined-therapy groups.

The proportion of isolates thought to be phenotypically resistant to zidovudine at 48 weeks ($IC_{50} \geq 0.05 \mu\text{mol}$) was similar in all three groups (table 2). One of 14 isolates from the participants in the zidovudine plus didanosine group showed resistance to didanosine and none of ten in the zidovudine plus zalcitabine group showed resistance to zalcitabine. HIV-1 RNA viral load at 48 weeks was, on average, $0.9 \log_{10}$ copies/mL higher and the CD4-cell count $51 \text{ cells} \times 10^6/\text{L}$ lower among participants for whom the virus could be isolated for phenotypic-resistance analysis than in those for whom it could not, but there was no evidence to suggest that the genotypic resistance pattern differed between the two groups of participants.

To assess the significance of the mutations, we calculated a single measure of genomic resistance for codons 41, 70, and 215 in plasma RNA (derived by adding the percentage of resistant mutants for each of the three codons), and this correlated with the zidovudine phenotypic resistance at 48 weeks ($r=0.54$, $p<0.0001$).

Mutations associated with resistance to didanosine and zalcitabine were seldom detected and generally late in the treatment period. The T69D mutation was seen alone in three participants between 48 and 112 weeks, two in the zidovudine plus didanosine group (64% and 78% mutant) and one in the monotherapy only group (25%). The L74V mutation was seen alone in three participants, two in the

monotherapy group (20% and 93%), one in the zidovudine plus zalcitabine group (98%). The V75T mutation alone was seen between 48 and 112 weeks in four participants, three in the monotherapy group (9%, 53%, and 62%) and one in the zidovudine plus zalcitabine group (41%). In one individual in the zidovudine plus didanosine group, T69D and L74V were present simultaneously as minority species at 112 weeks (44% and 14%, respectively). Four of the six participants in the monotherapy group in whom we found mutations at codons 69, 74, or 75, suggesting resistance to didanosine or zalcitabine, had started therapy with either drug before the mutation was found.

Discussion

We investigated changes in markers for viral load and antiretroviral-drug resistance during therapy and the relation between virological indices and disease progression within an antiretroviral-drug-naïve population in the Delta study. In that study, mortality and disease progression were substantially reduced in the two combined-therapy groups compared with zidovudine alone.¹ In our virology substudy, based on an intention-to-treat analysis, the reduction of viral load was greater and more sustained in the combined-therapy groups than in the monotherapy group and seemed greater in the zidovudine plus didanosine group, an observation that mirrors the clinical outcome of the main study in which there was a trend in favour of zidovudine plus didanosine delaying progression.

After adjusting for the effect of therapy, the baseline HIV-1 RNA load was, as in other studies,¹⁴ the most significant virological predictor of clinical progression; the baseline CD4-cell count was also an independent predictor. For a given concentration of baseline HIV-1 RNA viral load, a 1.0 log₁₀ difference at week 8 was associated with a two-fold difference in the risk of death, and of clinical progression or death. Similarly the HIV-1 RNA load at week 4 was predictive of progression to AIDS, or severe AIDS or death, but not of death alone. The changes in CD4-cell count at week 4 and week 8 were not clinically predictive after adjustment for baseline CD4-cell count, which suggests a dissociation between the predictive values of cell count and viral load and implies that therapy-related CD4-cell-count changes may reflect altered trafficking rather than cellular regeneration.

We found that the drug combinations that led to the greatest viral-load suppression and clinical benefit also seemed to lead to the more rapid generation of viruses bearing genetic markers for zidovudine resistance. At baseline, only a very small proportion of participants carried mutations associated with zidovudine resistance; we could not find out whether these mutations were the result of previous exposure to zidovudine, transmission of variants already mutated, or natural polymorphisms.¹⁵ In the zidovudine group, the previously reported pattern of appearance of mutations was seen with rapid occurrence of the K70R substitution followed by a concomitant rise in the T215Y/F mutation and reciprocal loss of the K70R mutant.¹⁶ The M41L mutation evolved more slowly. By contrast, in both combined-therapy groups the initial emergence of K70R was not seen, and M41L and T215Y/F mutations evolved more rapidly.

The results at 48 weeks of the phenotypic analysis showed similar percentages of participants with decreased susceptibility to zidovudine in all three groups; there was also a very low frequency of reduced viral susceptibility to

didanosine and zalcitabine. The isolation rate among participants assigned combined therapy was much lower than among those on monotherapy, which suggests the need for caution when comparing phenotypic resistance by treatment group. Genotypic results were, however, very similar, irrespective of whether the virus could be isolated. Genotypic and phenotypic data showed that at enrolment nearly all participants carried virus populations naïve to zidovudine.

The point mutation assay, like the line-probe assay¹⁷ uses sequences amplified from plasma. The percentage of sequences at a given codon rather than the amount of nucleic acid governs the signal of such an assay. This fact is important in the context of measuring drug resistance during therapy. Therefore, although the percentage prevalence of zidovudine resistance is lower among participants in the monotherapy group (figure 2), we found that with viral load and prevalence of mutation measures at any time point, the absolute concentration of virus bearing zidovudine-resistant mutations was higher in the monotherapy group than in the combined-therapy groups (figure 3). We suggest that measurement of antiretroviral resistance used as a solitary indicator for therapy change has little place in the management of patients on the combined therapies used in Delta. Measurement of viral RNA load and its trend with time on therapy are likely to remain the best measures of evolving resistance to antiviral treatment and therapy failure.

The emergence of resistance to zidovudine in the combined-therapy groups does not seem to indicate failure of antiviral treatment and may be associated with continued drug efficacy. Two factors may account for this: the mutated reverse transcriptase could confer lower replicative potential upon the virus population; or selection for zidovudine resistance may impose a genetic background that constrains coincidental evolution of didanosine and zalcitabine resistance. This effect could explain the rarity of didanosine and zalcitabine resistance by any assay in this study; these drugs may have maintained an antiviral effect despite zidovudine resistance. Didanosine and zalcitabine also seem to impose different selective pressures in the presence of zidovudine in naïve patients since the pattern of evolution of the markers M41L and T215 Y/F differed in the two combined-therapy groups. Evolution of zidovudine resistance during therapy with zidovudine plus didanosine seems to be by means of coincident selection of strains carrying the two mutations genetically linked on the same molecule. But, during therapy with zidovudine plus zalcitabine, the mutations appear sequentially with T215Y/F appearing long before M41L (figure 2). Single-molecule sequencing studies will be needed to define accurately the mutational routes to resistance. This finding is particularly important since a proportional genotypic assay, such as the point mutation assay used in this study, cannot differentiate between mutations carried on the same genome which lead to aminoacid changes in a single reverse-transcriptase protein and are synergistic in effect, and mutations carried on separate genomes. Only those combinations of mutations carried on the same molecule are likely to exert a synergistic selection pressure *in vivo*.

Few large trials comparing zidovudine plus didanosine and zidovudine plus zalcitabine with zidovudine monotherapy have included analysis of viral load and resistance.^{18,19} The virology substudy of 391 participants in trial ACTG175 (216 zidovudine-naïve patients) assayed viral load at only five time points (two at baseline).

Participants in ACTG175 had less advanced disease (85% asymptomatic), higher baseline CD4-cell counts (mean $372 \times 10^6/L$), and 13% of participants in the zidovudine-naïve group had virological evidence of previous zidovudine experience.¹⁹ Higher baseline HIV-1 RNA load and detection of SI phenotype were associated with clinical progression but baseline CD4-cell count was not correlated with clinical progression after adjusting for HIV-1 RNA load. The observation that the predictive value of CD4-cell counts for clinical progression is only apparent at values below 320×10^6 cells/ L^{14} may explain the latter difference. Similar changes have been described in two other short-term, virological-endpoint trials studying combinations of two nucleoside reverse-transcriptase inhibitors.^{20,21} The Wellcome Resistance Study Collaboration Group²² followed a similar protocol to Delta 1 (with half the didanosine dose) and also found only a low frequency of both resistant mutations and isolates with decreased susceptibility to didanosine and zalcitabine in the combined-therapy groups. There were no differences found in the evolution of zidovudine mutations at codon 215 in the three treatment groups. This difference from Delta virology results may be explained by their use of differential PCR,²³ a method that cannot measure changes in the proportion of viruses carrying resistant mutations. The Wellcome group did not examine codons 41 and 70.

Phenotypic and genotypic resistance to didanosine and zalcitabine occurred infrequently in the combined-therapy groups. Although zidovudine resistance seemed to develop more rapidly in the combined-therapy groups, plasma HIV-1 RNA loads remained substantially lower. Our substudy confirms the prognostic significance of viral load in clinical practice both on and off treatment. However, to assess whether HIV-1 RNA can be used as an alternative to clinical endpoints for assessing therapeutic efficacy needs a much larger study.

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Contributors

Francoise Brun-Vézinet, Charles Boucher, Clive Loveday, Don Jeffries, Richard Tedder, and Jonathan Weber designed the study, coordinated the protocols in France, the Netherlands, and the UK, respectively, and discussed and advised on data analysis. Diane Descamps supervised the virological work, in France, Rob Schuurman, in the Netherlands, and Steve Kaye, in the UK, and all three discussed the results. Corinne Kryzanowski, Andrew Nunn, and Gerrit-Jan Weverling were involved in the statistical analyses. Véronique Faveau, Jacques Izopet, Jean-Marie Seigneurin, and Catherine Tamalet did the virological assays in France. All investigators, except Véronique Fareau, Jaques Izopet, Jean-Marie Seigneurin, and Catherine Tamalet, contributed to the writing of the paper.

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Case-control study of phyto-oestrogens and breast cancer

David Ingram, Katherine Sanders, Marlene Kolybaba, Derrick Lopez

Summary

Background Phyto-oestrogens are a group of naturally occurring chemicals derived from plants; they have a structure similar to oestrogen, and form part of our diet. They also have potentially anticarcinogenic biological activity. We did a case-control study to assess the association between phyto-oestrogen intake (as measured by urinary excretion) and the risk of breast cancer.

Methods Women with newly diagnosed early breast cancer were interviewed by means of questionnaires, and a 72 h urine collection and blood sample were taken before any treatment started. Controls were randomly selected from the electoral roll after matching for age and area of residence. 144 pairs were included for analysis. The urine samples were assayed for the isoflavonic phyto-oestrogens daidzein, genistein, and equol, and the lignans enterodiol, enterolactone, and matairesinol.

Findings After adjustment for age at menarche, parity, alcohol intake, and total fat intake, high excretion of both equol and enterolactone was associated with a substantial reduction in breast-cancer risk, with significant trends

through the quartiles: equal odds ratios were 1.00, 0.45 (95% CI 0.20, 1.02), 0.52 (0.23, 1.17), and 0.27 (0.10, 0.69)—trend $p=0.009$ —and enterolactone odds ratios were 1.00, 0.91 (0.41, 1.98), 0.65 (0.29, 1.44), 0.36 (0.15, 0.86)—trend $p=0.013$. For most other phytoestrogens there was a reduction in risk, but it did not reach significance. Difficulties with the genistein assay precluded analysis of that substance.

Interpretation There is a substantial reduction in breast-cancer risk among women with a high intake (as measured by excretion) of phyto-oestrogens—particularly the isoflavonic phyto-oestrogen equol and the lignan enterolactone. These findings could be important in the prevention of breast cancer.

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See Commentary page 971

Introduction

There is strong epidemiological evidence that diet has a role in the development of breast cancer. This evidence initially came from population and migration studies, the subsequent cohort and case-control studies in human beings, and from animal experiments. The bulk of this research is based on the hypothesis that a diet rich in fat predisposes a woman to breast cancer. The results of large cohort studies, however, do not support this hypothesis,^{1,2} and interest has moved to other dietary factors.

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