Chapter III
In vitro dicentric and translocation dose response curves for Co-60 gamma rays by the FISH assay

3.1. Introduction:

Numerous biological indicators have been enlisted till date as markers of ionizing radiation exposure in humans (1-8). Historically, the use of cytogenetics in biodosimetry has focused on dicentric chromosomes that are easy to observe using conventional methods (9). The dicentric chromosome (DC) is widely used due its specificity, sensitivity and radiation dose dependency for acute exposures (10-11). The analysis of DC is considered to be the gold standard for biological dosimetry. However, DC are unstable with time after exposure and thus are limited in their utility for many important applications, such as for biological dosimetry more than a couple of months after exposure or for those who may have received chronic or multiple exposures over a long period of time (12). When reliable dose estimates are unavailable for past exposures, the alternative would be to analyze stable chromosomal aberrations such as translocations (TL) (13-17). It is well established that TL can serve as good indicators for both immediate and retrospective biodosimetry (18-19).

The advent of the Fluorescence *In Situ* Hybridization (FISH) assay has reformed the field of biodosimetry. FISH with DNA probes is a well-accepted method of detecting and quantifying induced chromosomal rearrangements. The reasons for this include the ability to detect exchanges (DC and TL), the increased speed of analysis compared with conventional approaches, a greater sensitivity for detecting subtle rearrangements and the increased specificity for quantifying aberrations in the chromosome(s) of choice. The frequency of TL measured using FISH in human blood lymphocytes of individuals exposed to ionizing radiation is considerered as a promising assay for biological dosimetry.

In FISH, TL yields detected in the painted chromosomes are extrapolated into the genomic TL frequency taking into account the percentage of DNA of the labelled chromosomes. This relies on the assumption that radiation induces chromosome damage in a random manner and that larger chromosomes have a greater probability of being impacted (20).

The existence of both, stable biomarkers – TL, alongside unstable biomarkers – DC (each possessing inherent positive and negative characteristics under various circumstances) demands that laboratories performing biodosimetry be equipped with both aspects (11).

In this regard, it was of interest to compare radiation induced DC and TL frequencies obtained by FISH. Experiments were undertaken to determine DC and TL yields in cultured peripheral blood lymphocytes exposed to varying doses of Co-60 gamma rays in order to construct dose-response curves. The DC and TL yields obtained by FISH were checked against each other. This is particularly timely now as TL are becoming more generally employed in dose reconstruction.

3.2. Methodology

Blood irradiation, cell culture, FISH with WCP 1 and 3 were performed as detailed in Sections 2.1.1, 2.3.1, 2.3.1.5. Aberration scoring, statistical analysis and dose response construction was done as mentioned in Section 2.3.1.9. All statistical tests ('t' test, correlation and regression analysis) were performed using the online statistical software - Instat Graphpad.

3.3. Results

The data obtained for DC and TL induced in human lymphocytes by Co-60 gamma rays and as observed by FISH shows a dose dependent increase in the respective aberration yields. Complex aberrations were observed after 2 Gy doses.

Frequencies of DC and TL measured by FISH were obtained and are listed in Tables 3.1 and 3.2 respectively. The frequencies of aberrations (DC and TL) obtained by FISH have been fitted to the linear quadratic equation $y = c + \alpha D + \beta D^2$ by an iteratively weighted (reciprocal sample mean variance n/σ^2) least-squares approximation (Figure 3.1).

The coefficients for DC and TL dose-response curves have been tabulated in Table 3.3. Yields obtained for both incomplete and complete TL classified according to the PAINT nomenclature are tabulated in Table 3.4.

A comparison was made between DC and TL frequencies acquired by FISH (regression analysis). This is depicted in Figure 3.2. Though TL yields were slightly higher when compared to DC yields for the same doses obtained by FISH, a good correlation was obtained ($r^2 = 0.9972$). Some chromosomal aberrations seen by FISH are shown in Figure 3.3.

Table 3.1: Frequency and distribution of dicentrics involving WCP 1 and 3 in peripheral blood lymphocytes exposed *in vitro* to 0.8 Gy/min Co-60 gamma radiation.

Dose (Gy)	CS	GE	DC	DC / GE ± SE	DC Distribution				u	σ^2/y
					0	1	2	3		
0.0	1230	336.03	1	0.003 ± 0.003	1229	1	-	-	-	1.008
0.10	856	233.86	2	0.009 ± 0.006	854	2	-	-	0.28	1.019
0.25	502	137.14	2	0.015 ± 0.010	500	2	-	-	0.37	1.032
0.50	539	147.25	3	0.020 ± 0.012	536	3	-	-	0.43	1.041
1.00	420	114.74	7	0.061 ± 0.023	413	7	-	-	0.90	1.111
2.00	394	107.64	14	0.130 ± 0.035	380	14	-	-	1.72	1.227
3.00	303	82.78	27	0.326 ± 0.063	278	23	2	-	4.63	1.710
4.00	326	89.06	55	0.618 ± 0.083	275	47	4	-	8.01	2.195

CS - Cells Scored **GE – Genomic Equivalent** **DC – Dicentrics**
u – Dispersion Index **SE – Standard Error** **σ^2/y – Relative Variance**

Table 3.2: Frequency and distribution of translocations involving WCP 1 and 3 in peripheral blood lymphocytes exposed *in vitro* to 0.8 Gy/min Co-60 gamma radiation.

Dose (Gy)	CS	GE	TL	TL / GE ± SE	TL Distribution				u	σ^2/y
					0	1	2	3		
0.0	1230	336.03	1	0.003 ± 0.003	1229	1	-	-	-	1.008
0.10	856	233.86	1	0.004 ± 0.004	855	1	-	-	-	1.012
0.25	502	137.14	2	0.015 ± 0.010	500	2	-	-	0.38	1.033
0.50	539	147.25	5	0.034 ± 0.015	534	5	-	-	0.63	1.066
1.00	420	114.74	6	0.052 ± 0.021	414	6	-	-	0.83	1.101
2.00	394	107.64	13	0.121 ± 0.034	381	13	-	-	1.67	1.221
3.00	303	82.78	32	0.387 ± 0.068	276	23	3	1	6.92	2.072
4.00	326	89.06	66	0.741 ± 0.091	269	49	7	1	10.76	2.621

CS - Cells Scored **GE – Genomic Equivalent** **TL – Translocations**
u – Dispersion Index **SE – Standard Error** **σ^2/y – Relative Variance**

Figure 3.1: *In vitro* DC and TL dose response curves obtained by FISH using WCP 1 and 3 for peripheral blood lymphocytes exposed *in vitro* to different doses of Co-60 gamma radiation.

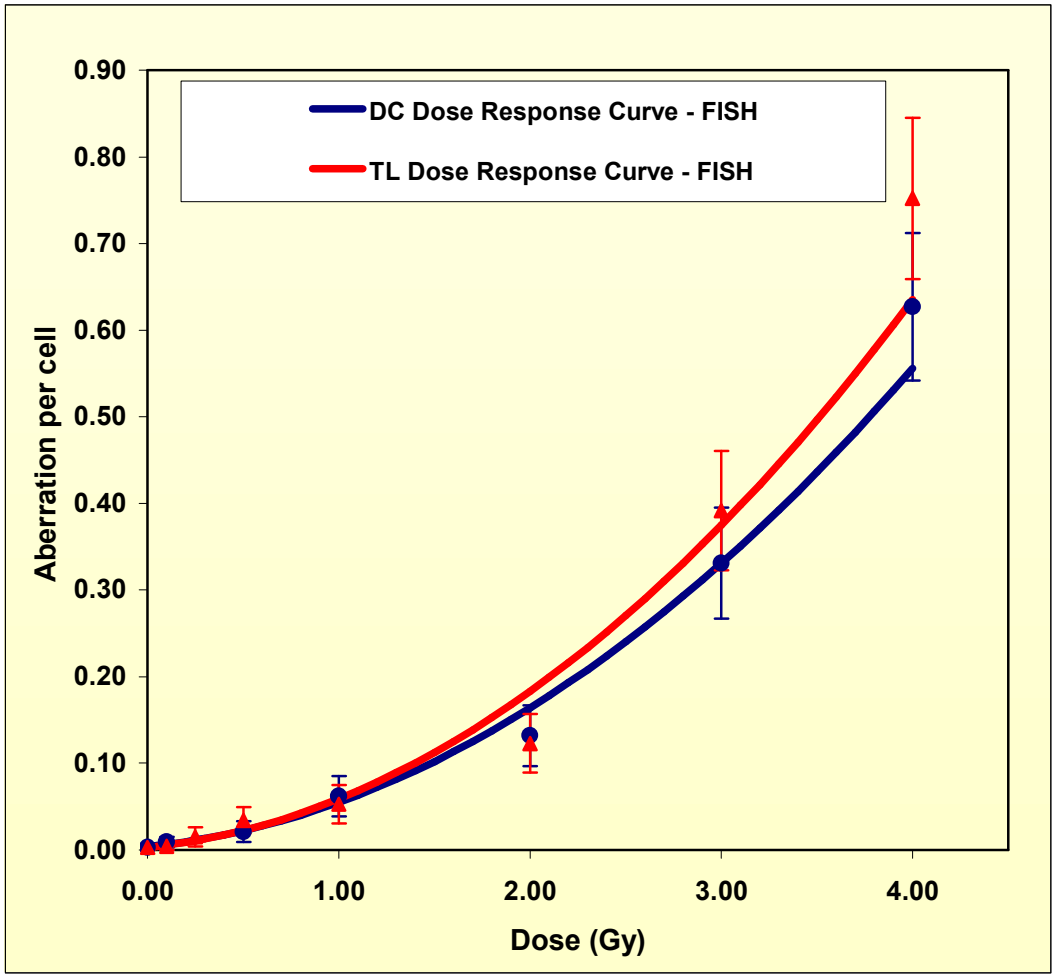


Table 3.3

Dicentric and translocation dose-response coefficients (linear-α, quadratic-β and constant-c) obtained for Co-60 gamma rays.

Aberration	α coefficient ± SE	β coefficient ± SE	c ± SE
Dicentrics (FISH)	0.022 ± 0.011	0.029 ± 0.005	0.004 ± 0.002
Translocations (FISH)	0.022 ± 0.018	0.034 ± 0.008	0.003 ± 0.002

Figure 3.2: Comparison of *in vitro* DC and TL yields obtained by FISH using WCP 1 and 3 in peripheral blood lymphocytes exposed *in vitro* to different doses of Co-60 gamma radiation.

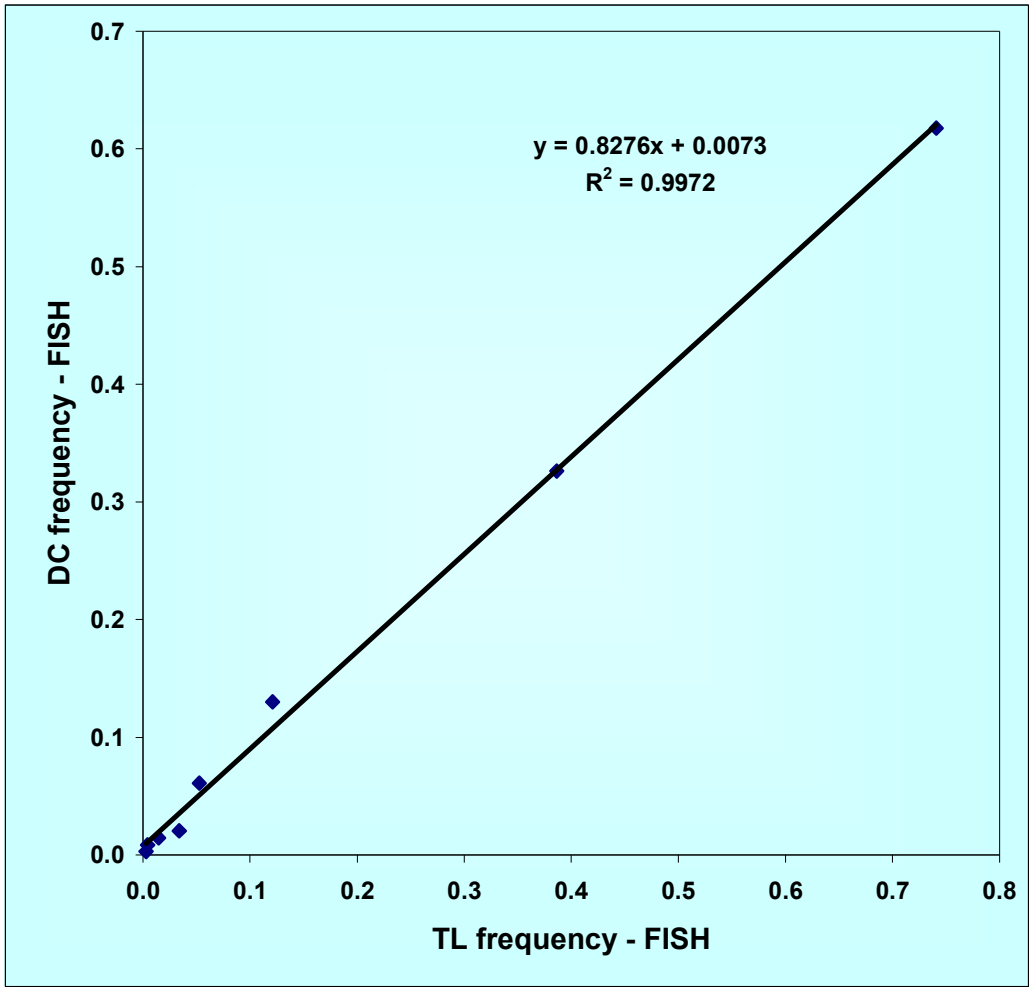


Figure 3.2: Chromosomal aberrations observed by FISH using WCP 1 and 3 in peripheral blood lymphocytes exposed *in vitro* to different doses of Co-60 gamma radiation.

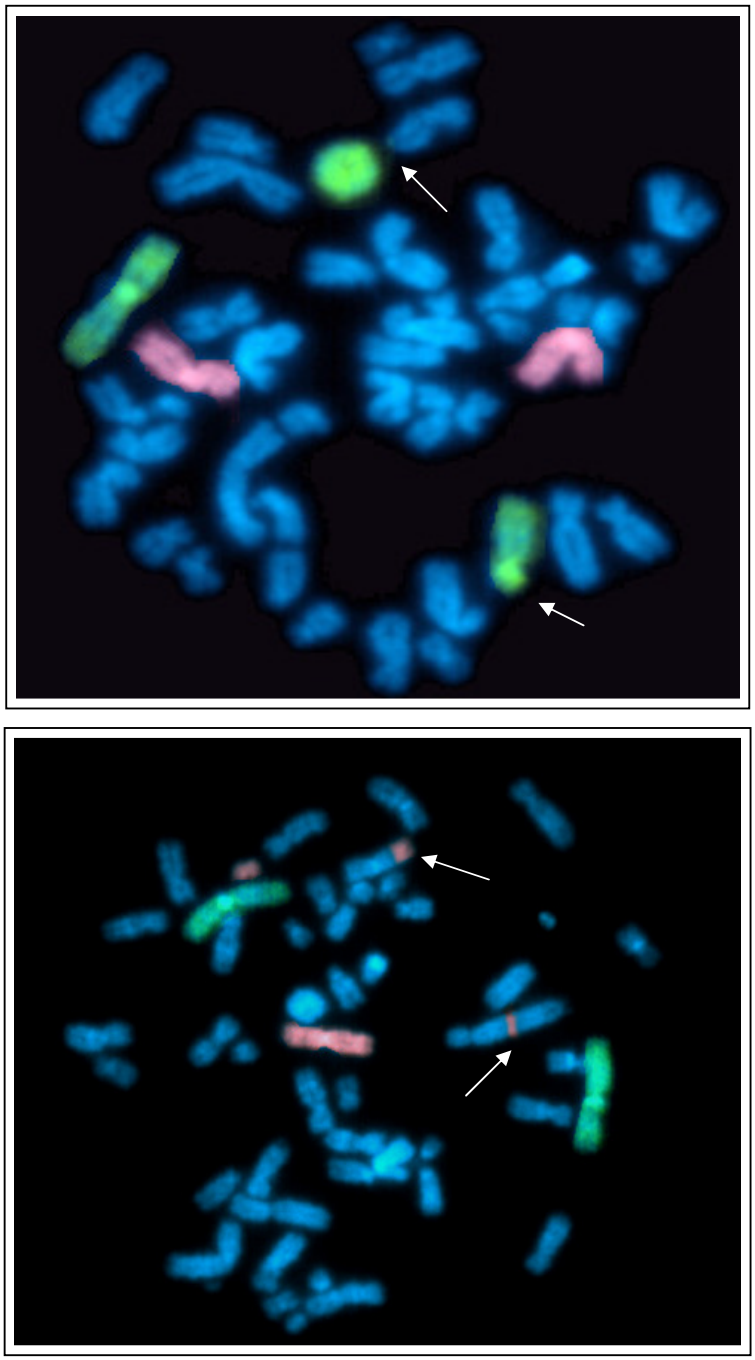


Table 3.4: Translocations involving WCP 1 and WCP 3 in peripheral blood lymphocytes exposed *in vitro to* 0.8Gy/min Co-60 gamma radiation as categorized by the PAINT nomenclature.

Dose (Gy)	CS	GE	Incomplete TL							Complete TL				Insertions		Total TL	TL and insertions
			Ab	Ba	Ac	Ca	Bc	Cb	Total	Ab+Ba	Ac+Ca	Bc+Cb	Total	Aba	Bab		
Ctrl	1230	336.03	-	-	1	-	-	-	1	-	-	-	0	-	-	1	1
0.1	856	233.86	-	-	1	-	-	-	1	-	-	-	0	-	-	1	1
0.25	502	137.14	1	-	1	-	-	-	2	-	-	-	0	-	-	2	2
0.5	539	147.25	-	-	2	-	-	-	2	2	1	-	3	-	-	5	5
1	420	114.74	3	-	1	-	-	-	4	2	-	-	2	-	-	6	6
2	394	107.64	3	2	1	1	-	-	7	6	-	-	6	-	-	13	13
3	303	82.78	5	2	4	3	1	-	15	9	4	1	14	2	-	31	33
4	326	89.06	20	4	15	6	2	1	48	15	7	2	24	2	-	72	74

CS – Cells Scored GE – Genomic Equivalents TL - Translocations

t(Ab): translocation consisting of the centromeric piece from the counterstained chromosome and a non-centromeric piece from the painted chromosome 1.
t(Ba): translocation containing a centromere of the painted chromosome 1 and counterstained non centromeric region.
t(Ac): translocation comprising of the centromeric piece from the counterstained chromosome and a non-centromeric piece from the painted chromosome 3.
t(Ca): translocation bearing the centromere of the painted chromosome 3 and counterstained non centromeric region.
t(Bc): translocation involving the centromeric region of chromosome 1 and the non centromeric region of chromosome 3.
t(Cb): translocation of a non-centromeric region of chromosome 1 onto a centromere bearing region of chromosome 3.
ins(Aba): non-centromeric region of chromosome 1 inserted into a counterstained chromosome.
ins(Bab): non- centromeric counterstained region inserted into painted chromosome 1.

3.4. Discussion

Dose response curves are necessary if a biomarker is to be used for dose assessment. To be able to use FISH analysis for dose assessment and also to compare DC yields with TL yields for various doses of radiation, laboratories establish their own *in vitro* dose response curves using different radiation qualities.

Hence, FISH was employed to measure frequencies of DC and TL in human lymphocytes induced by Co-60 gamma rays. The dose response curves for Co-60 using DC and TL as endpoints follow a linear quadratic model as reported in literature (21-25). Also, it has been shown that the coefficients of dose-response curves for the induction of DC and TL obtained by FISH are similar (23, 26).

In the acute linear-quadratic dose-response curve for low-LET radiation, the quadratic term is highly time-dependent, so that when the irradiation time increases, the quadratic term approaches zero. Therefore, the calculated dose is very dependent on the value and reliability of the α coefficient. In cases studied by FISH (TL analysis) and suspected of having received relatively low doses at low dose rates, the linear component (α) of the dose-response curve becomes very important. It is thus necessary to stress on the linear part of the calibration curves. This is particularly helpful because there are indications, that the α coefficient obtained using chronic exposure at body temperature is not different from that reported for acute dose response (27).

From Table 3.3, it is clear that the α coefficients are very similar for the aberrations investigated. This is as expected, because they are barely influenced by heavily damaged cells or cells with complex exchanges (26). The alpha coefficients of the dose-response curves for TL in stable cells or in total cells do not differ (28). The β coefficient for TL obtained in our study, is slightly higher when compared to that for DC. With two different colours, it is easier to detect complex exchanges between the painted chromosomes than with a single colour. Complex exchanges detected by FISH at doses above 2 Gy could contribute to the elevated β term. However, this

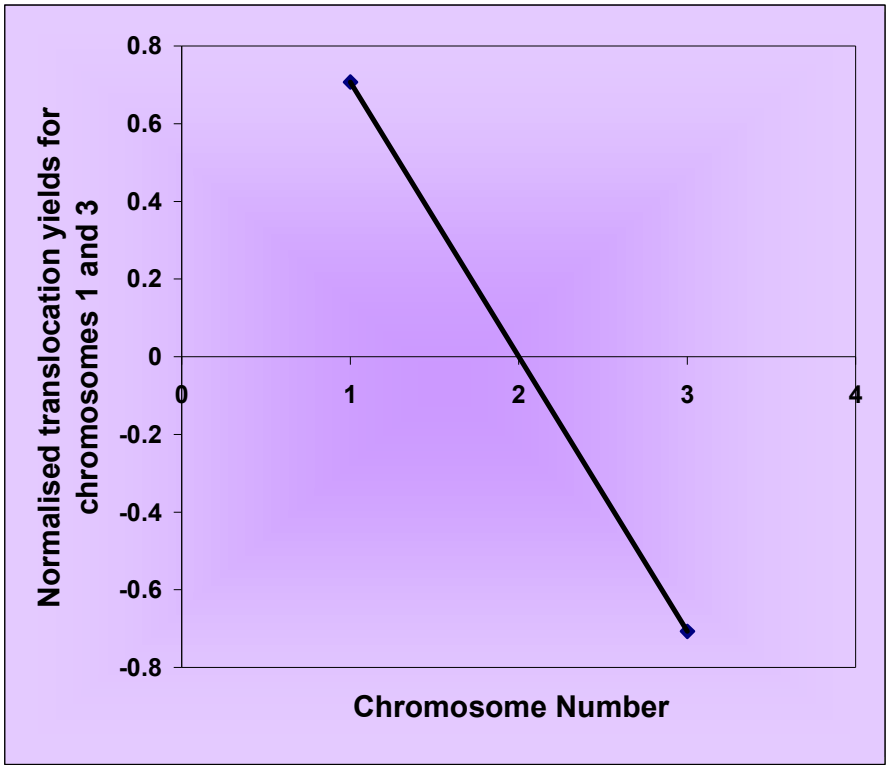
difference is not significant and the β coefficients for the dose-response curves (TL and DC by FISH) are almost the same.

During the early years of TL analysis, several reports indicated that TL were induced far more often than DC (21, 29-30). The introduction of centromere labelling has evidently diminished this discrepancy and reduced the TL-DC ratio closer to 1 (22, 23, 31, 32). In our study, on analysis of TL and DC frequencies by FISH, we did not observe any significant difference at low doses (below 1 Gy). Though, TL yields seem to be slightly higher than DC yields at higher doses, the 't' test did not reveal any significant difference ($p = 0.8622$) and a good correlation was seen between TL and DC scored by FISH ($r^2 = 0.9972$).

According to classical theories of aberration formation, the ratio of radiation-induced symmetrical exchanges (reciprocal TL) to asymmetrical exchanges (DC) should be 1:1 (33). Higher TL frequencies have been observed in some studies (30, 34-35), while a close correlation between DC and reciprocal TL has been indicated by others (32). This difference was attributed to the scoring difficulty by FISH due to non-distinction between DC and TL, as the centromeres are not very prominent (34). This shift may also be dependent upon the structure of the chromosomes, position of the centromere and the nature of the chromatin at the time of irradiation (36). Natarajan *et al* (37) suggest that the formation of DC and TL may be the result of different mechanisms. In the present study, we used DAPI as a counterstain to view the unpainted chromosomes. As DAPI has the property of staining the centromeres and making them distinctive, DC and TL frequencies obtained by FISH did not show any significant difference. However, simultaneous use of centromeric and whole chromosome probes will certainly identify DC without any discrimination (23, 38-39).

In our study, the observed aberrations were categorized using the PAINT system of classification (40). Normalised values for TL involving Chromosome 1 and 3 were checked against each other. Chromosome 1 was seen to be more involved in aberration formation than chromosome 3 (Figure 3.4).

Figure 3.4: *In vitro* normalized translocation yields obtained by FISH using WCP 1 (Spectrum Green) and WCP 3 (Spectrum Orange) from peripheral blood lymphocytes exposed *in vitro* to different doses of Co-60 gamma radiation.



We also found a higher frequency of incomplete TL (61%) over complete TL (39%). This is in accordance with some published reports (41-42). Tucker *et al* (43) attribute this difference to the presence of a specific type of TL – t(Ab) and t(Ac) where the painted region (b or c) translocated to a DAPI counterstained chromosome (A) is readily visible as against the non-painted translocated counterpart (a) involved. The other reason could be that the counterstained region is below the limit of detection of the technique. The percentage of incomplete exchanges for both TL and DC reported in human lymphocytes exposed to low-LET radiation range from 10% to 40% (32, 41, 44). When a telomeric PNA probe is used to detect all telomeres, the percentage of true incomplete exchanges falls to approximately 5%. (38-39). These results confirm the idea that most incomplete exchanges are not identified as complete exchanges by FISH analysis because of the minimal detectable size for painted and unpainted segments (11.1 and 14.6 Mb, respectively) (45). In another study, equal frequencies of

complete DC and TL, where both members of the exchanges were seen, were observed in the chromosome painting analysis at all doses, resulting in similar calibration curves (46).

As such our results indicate that DC and TL yields can be easily measured by FISH and do not show any significant difference. The dose response coefficients obtained matched well with those reported in literature.

3.5. Conclusion

Chromosome staining using fluorescent *in situ* hybridization has substantially changed the rate at which structural aberrations can be detected and scored. This has been achieved by using FISH to stain selected regions of one or more chromosomes so that exchanges of material between a stained chromosome and an unstained chromosome can be recognized immediately, even by relatively untrained observers.

Measurement of the frequency of structural aberrations such as DC and TL can now be accomplished rapidly and accurately using FISH. The aberration signatures are sufficiently distinct that the aberrations can be scored almost as rapidly as metaphase chromosomes can be found. In fact, metaphase spread finding is now the rate limiting step in aberrations analysis; a limitation which can be removed by application of automated metaphase finding. DC chromosome frequency analysis seems to be the method of choice for dosimetry immediately after exposure while TL frequency analysis seems to be the method of choice for analysis of chronic exposure or at long times after exposure.

Detection of TL by FISH is rapid and easier when compared to conventional techniques because of the accuracy, ease of detection and analysis of a large number of cells thus making it the technique of choice for biodosimetry. However, one does need to validate if DC and TL yields obtained by FISH are equivalent to those obtained by conventional techniques such as DC enumeration by FPG staining and TL analysis by G banding. Hence the experiments in the ensuing sections served to validate the accuracy of FISH based results.

3.6. References

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