MITOCHONDRIAL DNA HETEROPLASMY IN RADIATION INDUCED MYELODYSPLASIA AND LEUKAEMIA

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Donation

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I declare that this thesis is my own work.
It is being submitted for the
Magister Technologiae (Medical Technology)
to the Cape Technikon, Cape Town.
It has not been submitted before for any degree or examination
at any other Technikon or tertiary institution.
The work was carried out at the Department of Haematological
Pathology, Tygerberg Hospital, Cape Town.
The opinions and conclusions drawn are not necessarily
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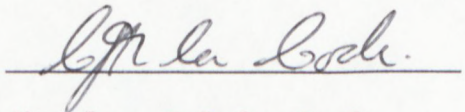

Charles J.R.la Cock

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Date

I dedicate this work to my wife Amanda

and my son and two daugthers

Rentzke,

Marguerite and Izelle

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SUMMARY

Haematological defects observed in clonal deletions of mtDNA and the inhibition of mitochondrial function by benzene and chloramphenicol, suggest a role for mtDNA in the pathogenesis of radiation - induced preleukaemia (MDS). The fact that leukaemia cells contain abnormal mitochondria and abnormally structured mtDNA, makes it reasonable to assume mtDNA mutations could be central to the pathogenesis of both MDS and leukaemia. It was decided to examine MDS patients for the presence of mtDNA length mutations (dimers and cocantameres). Such topological forms have already been reported in the literature in association with human leukaemia. These steric considerations suggest that mtDNA dimers are probably non-functional due to supercoiling. Thus, it was felt that a progressive accumulation of non-functional dimers in the haematopoietic compartment could account for many of the clinical features associated with MDS. Transmission electron microscopy was used to examine haematopoietic mtDNA in the bone marrow of six patients with MDS. Abnormal mtDNA dimer formation was found in all instances. The proportional number of these dimers were found to roughly correlate with the Myeloid/ Erythroid cell ratio in the bone marrow, and it appeared likely that the dimers were generated in the myeloid compartment during early MDS.

Controlled radiation studies were performed on 20 Wistar rats in an attempt to elucidate the approximate time when abnormal mtDNA dimer formation occurred, following fractionated gamma or gamma-neutron irradiation.

Gamma-irradiated rats demonstrated abnormal mtDNA dimer formation

at the time hypoplastic marrow recovery was first observed. The lack of any discernable further hypocellular damage caused by approximatly 60% marrow exposure to 4,0 Gy rad gamma rays, suggests this period of recovery was associated with an acquired radio-resistance in the marrow progenitor cells. Erythropoiesis was least affected in this study.

In contrast, rats exposed to fractionated doses of mixed gamma-neutron radiation, showed a severe decrease in both erythropoiesis and granulopoiesis. This was probably due to the higher RBE (relative biological effect) for cell killing associated with the neutron component of the test radiation.

This population also appeared to develop an acquired radio-resistance after their second fractionated dose. This was evidenced by the continued recovery of marrow cellularity during the time the 3d fractionated dose was administered.

Unlike the gamma-irradiated population, the gamma-neutron irradiated rats showed simultaneous recovery of both erythropoiesis and granulopoiesis. This bileanage recovery continued until Week 12 of the experiment when erythropoiesis abruptly decreased and the myeloid compartment began hyperplastic over-expansion. The hyperplasia was accompanied by asynchronous myeloid maturation, and this culminated in the appearance of myelodysplasia which was thought to be a case of myeloproliferative disease.

Abnormal mtDNA dimers and initially cocantameres, were initially present in low numbers in the gamma-neutron population at 2 weeks after the first radiation insult. The dimers initially increased in number and were accompanied by the development of a population which appeared to be deleted dimers during the period of marrow

recovery. At the time of myeloid hyperplasia, the haematopoietic compartment contained a mixture of normal mtDNA, dimers, deleted dimers, and a small number of mtDNA cocantameres which had by now reappeared. Overall, mtDNA dimers were less in number than the normal mtDNA molecules. This was in contrast with the gamma-irradiated rat population which did not exhibit mtDNA dimers until well into the period of marrow regeneration. Overall, the number of dimers tended to increase with time. This correlated with the slow recovery of marrow cellularity with ineffective haematopoiesis as evidenced by the continuing peripheral cytopenia with histological adequate marrow reserve.

Astronauts participating in Long Duration Manned Space Missions (LDMSM) will be exposed to an increased radiation environment consisting of radiation qualities that contain poorly understood biological effects at low and very low doses. It is likely that it will be necessary to screen participating astronauts during LDMSM, for biological markers of increased leukaemogenic risk.

OPSOMMING

Soortgelyke hematologiese defekte wat waargeneem is in klonale delesies van mtDNA en met die inhibisie van mitochondriale funksie deur benzeen en chloramphenicol, dui moontlik op die rol van mtDNA in die patogenese van bestraling- geinduseerde preleukemie (MDS). Die feit dat leukemiese selle abnormale mitochondria en abnormaal gestruktureerde mtDNA bevat, maak dat dit moontlik kan wees dat mtDNA mutasies die kern is van die patogenese van beide MDS en leukemie. Ons het besluit om MDS pasiente te ondersoek vir die teenwoordigheid van mtDNA lengte mutasies (dimere en kokantamere). Sulke topologiese vorme is soos in die literatuur gerapporteer, verbind aan menslike leukemie. Hierdie inligting dui dat mtDNA dimere moontlik nie-funksioneel is as gevolg van "supercoiling". Daarom bestaan die moontlikheid dat die progressiewe ophoping van nie-funksionele dimere in die hematopoietiese kompartement moontlik die oorsaak kan wees van die vele kliniese eienskappe wat geassosieer word met MDS. Transmissie elektron mikroskopie is gebruik vir die ondersoek van die hematopoietiese mtDNA in die beenmurg van ses pasiente met MDS. Abnormale mtDNA dimeer formasie was gevind in al die gevalle. Die proporsionele hoeveelhede van die dimere wat verkry is korreleer rofweg met die Mieloiede/Eritroiede sel verhouding in die beenmurg, en dit wil voorkom asof die dimere gevorm word in die mieloiede kompartement gedurende vroeë stadiums van MDS. Gekontroleerde bestralings studies is uitgevoer op 20 Wistar rotte in 'n poging om die tydperk vas te stel wanneer abnormale mtDNA formasie na die toediening van gefraksioneerde gamma of gamma-neutron bestraling begin. Gamma-bestraalde rotte het getoon

dat abnormale mtDNA formasie reeds gevorm was met die eerste waarneming van hipoplastiese beenmurg herstel. Die afwesigheid van enige verdere betekenisvolle hiposellulêre skade van die beenmurg met die tweede blootstelling aan 4,0 Gy rad gamma strale, dui daarop dat hierdie periode van herstel geassosieer kan word met 'n verworwe bestralings-weerstandigheid in die beenmurg voorloperselle. Eritropoiese was die minste geaffekteer in hierdie studie.

In kontras, het rotte wat blootgestel was aan gefraksioneerde dosisse van gemengde gamma-neutron bestraling, 'n erge verlaging aangetoon in beide eritropoiese en granulopoiese. Dit was moontlik as gevolg van die hoër RBE vir seldood, geassosieer met die neutronkomponent van die toetsbestraling. Hierdie populasie het ook die ontwikkeling getoon van 'n verworwe bestralings - weerstand na die tweede gefraksioneerde dosis-bestraling. Dit is bewys deur die herhaalde herstel van die beenmurg sellulariteit, selfs nadat die beenmurg vir die derde maal blootgestel was aan gefraksioneerde dosis-bestraling. Teenoorgesteld van die gamma-bestraalde populasie, het die gamma-neutron bestraalde rotte herhaalde herstel getoon van beide eritopoiese en granulopoiese. Hierdie bilinîere herstel het aangehou tot week 12 van die eksperiment, toe eritropoiese skielik verminder het en die mieloide kompartement met hiperplastiese oorkompensasie begin het. Die hiperplasie was gelyktydig met asinchroniese mieloiede maturasie, en dit het voorgekom in die vorm van mielodisplasie wat verskyn het as 'n geval van mielo- proliferatiewe siekte. Abnormale mtDNA dimere en konkantamere, was aanvanklik aanwesig in lae hoeveelhede in die gamma-neutron populasie na twee weke vanaf hul eerste bestraling. Die dimere het aanvanklik gestyg in

kwantiteit wat gelyktydig met die ontwikkeling van 'n populasie voorgekom het, of hulle dimere is met dilesies, gedurende die periode van beenmurg herstel.

Met die aanvang van mieloiede hiperplasie, het die hematopoïetiese kompartement 'n mengsel van normale mtDNA dimere, dimere met dilesies en 'n klein hoeveelheid van mtDNA konkantamere wat nou verskyn het, bevat. Van die hele groep was die mtDNA dimere minder in aantal as die normale mtDNA molekules. Dit was in kontras met die gamma-bestraalde rotpopulasie wat nie die mtDNA dimere aangetoon het tot baie laat in die staduim van beenmurg regenerasie nie. Die hoeveelheid dimere was geneig om te styg oor 'n tydperk. Dit het gekorreleer met die stadige herstel van die beenmurg sellulariteit met oneffektiewe hematopoïese soos aangetoon deur die volgehoue perifere sitopenie met histologies voldoende beenmurg reserwe.

Ruimtevaarders wat deelneem aan lang bemande ruimte sendings sal blootgestel word aan 'n verhoogde radioaktiewe omgewing. Dit bestaan uit radioaktiewe kenmerke waarvan min nog biologies verstaan word, veral die effek van lae en baie lae dosis bestraling. Dit is moontlik dat dit nodig sal wees om ruimtevaarders te monitor gedurende lang ruimtereise vir biologiese merkers wat op verhoogde leukemiese risikosal aandui.

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ABREVIATIONS

LDMSM	:	Long Duration Manned Space Missions
MDS	:	Myeloid Dysplastic Syndrome
RBE	:	Relative Biological Effect
NCRP	:	National Commitee of Radiation Protocol
GCR	:	Galactic Cosmic Radiation
ALU	:	Adenine Lysine Urasil
FAB	:	French American British
ALIP	:	Abnormal localization of immature precursors
RARS	:	Refractory anaemia with ring sideroblast
RAEB	:	Refractory anaemia with excess blast
RAEB-T	:	Refractory anaemia with excess blast in transformation
RA	:	Refractory anaemia
CMML	:	Chronic Myelo Monocytic Leukaemia
CAP	:	Chloramphenicol
HZETRN	:	Langley Research Centre Galactic Cosmic Ray Transport Code
CAM	:	Computerized Anatomical Man
LET	:	Linear energy transfer
TE	:	Tris EDTA
TEK	:	Tris EDTA Kaluim(Potassuim)
EM	:	Electron Microscopy
ANLL	:	Acute non-lymphocytic leukaemia
ANAE	:	Alpha naphtol acetate esterase

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CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

At present, the pathogenic mechanisms involved in the Myelodysplastic Syndromes (MDS), and its transformation into acute leukaemia, are unknown. The importance of normal mitochondrial function in hematopoiesis is illustrated by the haematological defects which result from deletions of mitochondrial DNA, (mtDNA) (Firkin,F.C., *et al*. 1979) in the bone marrow (Pearson's Syndrome) (Pearson,H.A., *et al*. 1979), and the suppression of mitochondrial protein synthesis by the antibiotic Chloramphenicol (Adel,A. Yanis,1973). Similar defects are observed in patients suffering from MDS, or preleukaemia, and abnormal dimers of mtDNA have been observed as a feature of acute leukaemia (Wallace,C.D.,1989). This suggests a causal relationship between the clinical features of MDS, the mitochondria of the leucocytes in the bone marrow, and the early pathogenesis of acute leukaemia.

Acute and chronic radiation exposures may give rise to a number of specific haematological defects which are collectively termed "preleukaemia", or the myelodysplastic syndromes (MDS). These preleukaemic states are irreversible and may antedate the onset of acute non-lymphocytic leukaemia by a variable period of months. It is also histologically indistinguishable from primary "de-novo" myelodysplastic syndrome.

The precise relationship between leukaemia risk and low-dose radiation exposure, is unclear. Estimates have been derived from the extrapolation of high-dose human exposure. However the fundamental flaw in these studies is that, the cellular response to chronic low-dose radiation now appears to be fundamentally

different from the response to medium/high-doses.

To address this problem, a study was performed in order to obtain a biological marker which might signal the development of enhanced leukaemogenic risk in an individual. Such a marker would have to be of significance to both MDS and leukaemia. Mitochondrial inhibitors or deletions of the organelle's DNA (mtDNA) may cause clinical symptoms similar to MDS as abnormal topological forms of mtDNA have been found in leukaemia cells. Therefore , mtDNA was assessed as a possible marker linking the pathogenesis of MDS with that of later developing leukaemia. Electron microscopy analysis of a series of patients revealed that mtDNA length mutations are characteristic of human MDS. Subsequent radiation experiments were performed and gamma, or gamma-neutron irradiated rats were shown to exhibit abnormal dimers of haematopoietic mtDNA during early MDS development. This was associated with the development of an acquired radio-resistance in the bone marrow progenitor cells. The irradiated rat population also developed ineffective haematopoiesis with increased numbers of apoptotic megakaryocytes in the marrow. A subsequent retrospective study confirmed this to be a previously uncharacterized feature of human MDS with a possible prognostic significance.

The mechanism for abnormal mtDNA dimer formation in radiation-induced MDS is unknown, but association with the induction of radio-resistance in the marrow progenitor cells indicates that enhanced DNA repair (error-prone) mechanisms may be involved. This suggests a therapeutic role for protease inhibitors in the clinical management of radiation-induced MDS which inhibits its

transformation into leukaemia.

Thus, the bone marrow may be particularly sensitive to the neoplastic effects of low-dose neutron radiation.

The following question may thus be posed:

What about space missions?

Acute and chronic exposure of humans to ionising radiation during space missions is generally recognised as one of the most important factors in mission design. Long-duration space missions will expose astronauts to a mixed quality radiation environment composed of protons, neutrons and heavy element nuclei.

The induction of preleukaemia or leukaemia in a single crew member would therefore have profound effects on the success of such a mission.

This thesis describes the search for a possible biological marker for enhanced leukaemogenic risk in individuals following excess radiation exposure. An extensive literature review established that the onset of radiation-induced leukaemia in humans and Beagle dogs, is antedated by days to months by the onset of specific haematological defects collectively termed "preleukaemia" or the secondary myelodysplastic syndrome (MDS). Secondary MDS is a disorder characterised by refractory peripheral blood cytopenias in conjunction with the finding of abnormal haematopoietic progenitor cells in the bone marrow. Secondary MDS is histologically indistinguishable from that found in human patients presenting with primary "de-novo" MDS, and it is likely to be a fundamental pathogenic process that is common to both groups. Therefore, the initial experiments in this study

were directed towards both primary and secondary cases of human MDS. These findings were further expanded in an animal model of secondary MDS.

1.1 OBJECTIVES

The objectives of this study were the following:

a) to determine whether abnormal forms of mtDNA can be isolated and identified in samples of myelodysplastic bone marrow;

b) to histologically characterize and compare a standard animal (Wistar rat) model of preleukaemia with human MDS using the current FAB classification scheme;

c) to use the histologically characterized animal model of preleukaemia to determine if topological abnormal forms of mtDNA appear as an early feature of MDS, or as a later feature associated with the transformation to acute leukaemia, and

d) to utilize the above data to outline a theoretical pathogenesis which links the onset and clinical features of MDS with the later development of acute leukaemia.

CHAPTER 2.

LITERATURE REVIEW.

2.0 INTRODUCTION

2.1 CREW EXPOSURE ESTIMATES FOR THE SPACE RADIATION ENVIRONMENT

Our studies suggest that it will be necessary to periodically screen astronauts during outer space journeys, for any development of abnormal leukaemogenic potential resulting from constant exposure to the space radiation environment. Bone marrow biopsies could be taken from individual crew members during these missions and while these might demonstrate the presence of overt myelodysplastic syndrome (MDS), this would probably be technically impractical and insensitive for the assessment of enhanced leukaemia risk in the individual astronaut. Clearly, what is required is some form of early marker for radiation- induced leukaemia, which could be screened for by a simple in-flight assay. For the purpose of this study it was decided to examine a series of human MDS patients for a biochemical feature common also in early acute leukaemia.

It is known that long-duration space missions outside the earth's magnetosphere, will expose astronauts to Galactic Cosmic Radiation (GCR) which is composed of protons, neutrons, and the nuclei of heavy elements moving at relative velocities (Lownsend L.W. *et al*, 1992). The biological risk of GCR was first identified during the Apollo program when light flashes were observed in the eyes of the Apollo astronauts. It was traced back to heavy-ion interactions with the retina, vitreous humour and optic nerve (Lownsend L.W. *et al*,1992). However, the radiation environment was not well characterised at that time and the biological effect of heavy ions was poorly understood. Present radiobiological research on heavy ions are continuing to elucidate these phenomena. The

Relative Biological Effect (RBE) value of this type of radiation is still uncertain and there is no universally accepted application methodology available to evaluate the health risk for this type of exposure. It is known that a 90 day tour aboard a space station, can cause radiation up to ± 13.5 cGy to the bone marrow of a crew member (Nachtwey D.S.,1984) and calculations by Letaw, *et al.* (1987), describe possible biological effects of irradiation by the heavy ion nuclei and protons of GCR. The radiation dose to unshielded astronauts exposed to GCR will be dominated by heavy ions and protons, whilst cosmic ray ion nuclei deposit energy in tissue at 676 times the concentration of cosmic ray protons (Letaw J.R. *et al*, 1987). Consequently, astronauts participating in long-duration interplanetary missions would be expected to receive a minimum of 15 to 35 cGy/y from GCR alone (Letaw J.R. *et al*, 1987). Thus, it is evident from the studies mentioned that a significant fraction of the dose-equivalent exposure to GCR will be composed of heavy ions, protons, and < p(20)/Be neutrons derived from the interaction of primary particles with the spacecraft structure.

The biological effects of this mixed radiation environment however are still poorly understood and urgent studies are required to enable spacecraft designers and flight-medical specialists to plan the safest mission profiles and procedures for radiological safety and protection.

2.2 RADIATION INDUCED DNA DAMAGE AND MUTATION

Studies on the mutational effects of ionising radiation have largely focused on the induction of visible mutations in 7 loci of the

Mouse Specific Locus Test, and in animal and human cell cultures (Sankaranarayanan K.,1993). Radiation induced DNA strand breaks and base oxidations and the mis-repair of this damage is known to result in cellular DNA mutation. Most (up to 90%), of the mutations induced by ionising radiation, appear in the form of nucleotide deletions. Few or no point mutations are observed in mammalian somatic cells (Sankaranarayanan K., 1991). This is probably a result of the random spatial distribution of energy from ionising radiation which is unlikely to induce DNA breaks with the same site specificity on both strands of the molecule. Deletional mutations probably arise from non-homologous or unequal recombinational repair between different single-strand breakpoints in chromatin areas, high in alanine-leucine-uracil- sequences (ALU) or guanine-cytosine (GC) base repeats.

Experimental studies (Sankaranarayanan K.,1993) suggest that the induction of mutations by ionising radiation, appears to follow a complex dose-response with enhanced mutagenesis at low to medium dose exposure, but a lower response due to cell killing at higher doses. There is overwhelming evidence (Sankaranarayanan K.,1991) that enhanced rates of mutation are associated with an increased propensity for neoplastic cellular transformation. In this respect, the bone marrow is among the tissues most susceptible to the carcinogenic effects of ionising radiation. This has been most clearly demonstrated by the increased incidence of acute leukaemia observed in irradiated human populations (Roesch W.C. *et al*,1987). The relationship between leukaemia risk and radiation dose are poorly defined owing to statistical limitations and uncertain

dosimetry within irradiated population groups. Early studies on Japanese A-bomb survivors suggest a linear regression dose-response curve for medium to high-dose exposures (Rotblat J., 1988). The leukaemogenic risk of low-dose radiation has been calculated from this high-dose data. Later estimates of the low-dose risk have utilised revised dosimetry and a linear-quadratic model of the dose-response curve ($E=\alpha D+\beta D^2$) (Rotblat J., 1988).

The DS86 dosimetry study (Roesch W.C. *et al*, 1987) suggests the neutron component at Hiroshima was disproportionately small. This has prompted the National Research Council in the UK to suggest that the current leukaemogenic risk of low-dose radiation should be raised by a factor of four (Roesch W.C. *et al*,1987). Follow-up studies on DS86 dosimetry by the Radiation Effects Research Foundation have used this revised data to assess the upward curvature of the linear -quadratic model rather than estimating the best fitting dose - response curve. The extent of the upward curvature is expressed as a factor by which linear risk estimates can be divided to achieve an appropriate estimate of the leukaemia risk at a lower dose (Roesch W.C. *et al*, 1987). These results suggest that further inconsistencies exist in the extrapolation of linear-quadratic data to estimate low-dose exposure effects. The main effect of ionising radiation on DNA is to induce strand breaks. This effect leads to cell death or chromosomal aberration after medium to high dose exposures (Fukushi O.F. *et al*,1988). A number of studies associate chromosomal abnormalities with malignant change (Rowley J.D. *et al*,1981). Such aberrations are commonly found in leukaemia. Data on experimental neoplasia suggest that these chromosome abnormalities are not entirely random, yet efforts to

quantify leukaemia risk with radiation dose, using this biological endpoint, have been unsuccessful (Rowley J.D. *et al*,1981). This is probably the result of the complex relationship between the radiation quality and the dose required to produce gross chromosomal aberrations (Rotblat J.,1988). However, these aberrations are still commonly employed as a" method" of biological dosimetry for medium and high-dose exposures. Chromosomal aberration dosimetry is insensitive to low-dose exposures, yet chronic low dose-rate irradiation is a known leukaemogen. This suggests that mutation-induction may be more important than DNA strand breaks for low-dose leukaemogenesis.

Current studies indicate that the cellular response to chronic low-dose radiation is fundamentally different from that of acute medium/high dose exposure (Roesch W.C. *et al*, 1987). Human lymphocytes exposed to chronic low-dose x-rays, demonstrate an acquired adaptation which makes the cells less susceptible to further chromosome damage when exposed to a subsequent higher dose of x-rays (Countenay V.D.,1969). This adaptive response is attributable to the activity of an enhanced chromosome-break repair system which requires 4-6 hours to become fully operational. This response may persist for up to 3 cell cycles after a single exposure to a radiation dose as small as 0.5cGy, and can be negated by the inhibition of poly (ADP-ribosyl) synthetase enzyme activity, normally induced in cells following DNA strand breakage (Olivieri G. *et al*, 1984).

2.3 HAEMATOPOIETIC RESPONSE TO CHRONIC LOW-DOSE/LOW ENERGY TRANSFER RADIATION

Haematopoietic progenitor cells in the bone marrow appear to be capable of an adaptive response to chronic low-dose radiation. This response is evidenced by an increased ability of the cells to repair sublethal DNA damage, with an increase in both radio-resistance and the capacity to continuously proliferate in the face of an accumulating total radiation dose (Seed T.M. *et al*, 1986). Associated with this enhanced ability to repair radiation damage, is a loss of normal DNA fidelity.

Error-prone DNA repair systems have been identified in both prokaryotes and eukaryotes (Seed T.M. *et al*, 1986). These repair systems seem to mediate an increased rate of mutation and neoplastic transformation in mammalian cells (D'Ambrosio S.M. *et al*, 1978). The induction of such error-prone DNA repair activity in the bone marrow, could manifest itself as an acquired radio-resistance that may be coupled to progressive DNA mutation in the bone marrow progenitor cells. Support for this concept is provided by *in-vitro* correlates which show that chronic low-dose irradiation acts as a strong selective pressure for the outgrowth of highly radio-resistant subclones in cultures of normally radio-sensitive L 5178y lymphoid cells (Yau T.M. *et al*, 1979). These subclones exhibit a pronounced radio-resistance which is associated with an enhanced capacity for DNA repair, a broad resistance to other physical/chemical cytotoxic agents, as an increased mutation rate and as an increased tendency for proliferative clonal expansion.

Beagle dogs subjected to continuous low daily doses of x-rays, develop an initial phase of hypoplastic marrow suppression followed by partial recovery and the development of acquired radio-resistance in their progenitor cells. Following recovery, a latent period develops which lasts for a variable period of time and may be followed by the onset of leukaemia if the animal survives the initial period of peripheral cytopenia (Seed T.M. *et al*, 1987).

Studies (Countenay V.D.,1969) have shown that very-low dose-rate x-rays (1mGy/hr) appear to have a higher mutagenic effect than low dose-rate (5cGy/hr) exposures. Lymphoid L 5178y cells exposed to constant very-low dose-rate rays (6.3mGy/hr) in culture, show an unexpected high rate of induced mutation which is not observed at higher dose-rates. Unlike the mechanism associated with constant low dose-rate irradiation, very-low dose-rate exposures are not associated with the development of acquired radio-resistance (Fukushi O.F. *et al*, 1988). In general, for sparsely ionising radiation such as x-rays, a lowering of the dose-rate by extension of the exposure time, results in a lower biological effect (such as cell killing). The enhanced ability of very-low dose-rate x-rays to induce mutations in the nuclear DNA of haematological cells, is in direct contrast with low-dose extrapolations made from high dose-rate data (Fukushi O.F. *et al*, 1988). This "inverse dose-rate" phenomenon could be an important factor for leukaemia induction in astronauts during long-duration missions.

2.4 NEOPLASTIC TRANSFORMATION AND THE INVERSE DOSE RATE EFFECT OF NEUTRONS

Considerable evidence has accumulated,by studies both *in vivo* and *in vitro,* to suggest that irradiation with neutrons at low dose-rates or in multiple fractions, results in an enhanced transformational and mutational effect on cells, which is greater than that observed with acute high dose exposures (Hall E.J. *et al*,1991). This "inverse dose-rate" effect is in contrast to the usual effect of sparing at low dose-rates and is dependant on the dose, dose-rate, and quality of radiation:

i) The effect is not primarily observed with linear energy transfer(LET) radiation.

ii) The effect is maximal for fission neutrons at dose-rates below 1cGy/min.

iii) Mono-energetic neutrons have significantly less effect than fission neutrons.

iv) The effect is most prominent for doses around the 20cGy range.

The enhanced rate of mutation and neoplastic transformation which results from low dose-rate irradiation by neutrons, is primarily observed in exponentially growing cell populations. There is also a great variation between the growth fractions of different normal tissues. However, the high cell turnover rates which characterise normal haematopoietic tissue, suggest that the bone marrow progenitor cells may be especially vulnerable to enhanced mutation/ transformation events during low dose-rate, neutron irradiation (Hall E.J. *et al*,1991).

2.5 RADIATION-INDUCED MYELODYSPLASIA AND SECONDARY LEUKAEMIA

Human exposure to ionising radiation may produce distinct morphological changes in the bone marrow which vary with respect to the radiation quality, dose, dose-rate and age at the time of exposure (Seed T.M. *et al*,1981; Ruutu T.,1986). Both acute high-dose and chronic low-dose exposure regimes may give rise to specific haematological defects which are collectively termed "preleukaemia", or the myelodysplastic syndromes (MDS). The myelodysplastic state reflects an irreversible and progressive clonal alteration to the haematopoietic stem cell population which results in progenitor cells that initially mature, but are structurally and functionally defective. Abnormal stem cells gradually replace the normal population to cause ineffective haematopoiesis and a continuous decrease in the number of circulating red cells, platelets, and leukocytes (Countenay V.D.,1969). The development of MDS may be followed by the onset of acute leukaemia after a variable period of time. The passage of time or multiple dose radiation exposures have a cumulative effect on both the risk for MDS and the rate of its progression into acute leukaemia (Ihle J.N.,1978). An increased rate of mutation seems to be a characteristic of the haematopoietic stem cells in myelodysplasia. This is evidenced by the progressively dysplastic cell maturation with time. Enhanced mutation rates could also play a role in the progression of this dysplasia to blocked differentiation of acute leukaemia.It is therefore reasonable to propose that physical agents which increase the mutation rate of haematopoietic cells, could also increase the risk of MDS and secondary leukaemia (Galton D.A.G.,1984).

2.6 HISTOLOGY OF MYELODYSPLASTIC SYNDROMES

The morphologic features of dysplasia in the haematopoietic elements of patients with spontaneous primary MDS (non-radiation induced) have been well-described and illustrated by the French American British Co-operative Group (Bennett,J.M. *et al* ,1985), based on cytologic features as outlined in Table 1. A number of reported cases however, have blood or marrow findings that cause problems in classification. In MDS, there is almost always disruption of the normal marrow architecture. The most common manifestation is the abnormal localisation of immature granulocytic cells, which are located centrally rather than subjacent to the endosteum, a situation known as abnormal localisation of immature precursors (or ALIP) (Scoazec J. *et al*,1985).

In contrast, erythroid precursors and megakaryocytes often migrate toward the bony trabeculae (Roth D.G. *et al*,1980). Some authors (Saarni M.I. *et al*, 1973) have reported that the finding of ALIP indicates an adverse prognosis and that the abnormal localisation of immature precursors helps to confirm an MDS marrow disorder. In MDS, there is evidence of dysmorphogenesis in all three haematopoietic cell lines in both the peripheral blood and bone marrow (Greenberg P.L. *et al*, 1979). Peripheral blood findings indicate abnormal erythrocytes and microcytes, basophilic stippling, nucleated red blood cells, and Howell-Jolly bodies. A characteristic finding is a dimorphic red blood cell image, with a combination of normocytic or microcytic erythrocytes and macrocytes.
In the bone marrow the erythroid series typically shows megaloblastic maturation. Multinucleated precursors may be seen as well as cells

with irregular nuclear contours, nuclear fragmentation and defective

TABLE 1

THE FRENCH AMERICAN BRITISH SUBTYPES OF MYELODYSPLASTIC SYNDROMES

Type	Peripheral Blood	Bone Marrow	Approximate median survival (months)
Refractory anaemia(RA)	<1% blast cells; reticulocyto-penia, macro-cytosis or normochromic/ normocytic	Usually erythroid hyperplasia with dyserythro-poiesis; <5% blast cells	50
RA with ring sideroblasts	<1% blast cells; dimorphic red cell morphology	As in RA, but type III sideroblast >15% of erythroid precursors	50
RA with excess blast(RAEB)	>5% blast cells; cytopenias in 2 or 3 cell lines	5-20% blast cells; 2 or 3 cell lines showing dyspoiesis	11
RAEB in trans-formation (RAEB-t)	5-29% blast cells, or any Auer rods	>20-30% blast cells; any Auer rods; otherwise as RAEB	5
Chronic myelomono-cytic leukaemia (CMML)	<5% blast cells; >1x10^9 monocytes/L	1-20% blast cells; monocytosis	11

haemaglobinization. Ringed sideroblasts are a characteristic of refractory anaemia, which is further evidence of abnormal red cell maturation. The sideroblasts are the result of iron accumulation in the mitochondria of the erythroid cells.

Leucocyte abnormalities in the peripheral blood include the presence of immature as well as abnormal granulocytes that may show defective granulation. Hyposegmented nuclear forms are common (pseudo-Pelger-Huët anomaly). Granulocytic precursors in the marrow may show abnormalities in nuclear segmentation and irregular cytoplasmic basophilia. Abnormal staining of the azurophilic granules is common. More mature precursors may show decreased secondary granules. Granulocytes and marrow precursors may show abnormalities in cytochemical staining such as a deficiency of myeloperoxidase and either decreased or increased leucocyte alkaline phosphatase (LAP).

Platelet abnormalities include giant forms as well as circulating megakaryocyte fragments. Megakaryocytes in the bone marrow may exhibit hypo- or hypersegmented nuclei. Micromegakaryocytes may be seen as well, either in large or small mononuclear forms. Some megakaryocytes may show cytoplasmic vacuolisation (Ruutu T.,1986).

2.7 HYPOCELLULAR MYELODYSPLASTIC SYNDROMES

Although most patients with MDS have hypercellular or normocellular bone marrow, a minority, perhaps 5-10%, exhibit a hypocellular marrow (Silverstein M.N., 1974). Specimens from such patients are challenging, as it may be difficult to distinguish between aplastic anaemia and MDS. The diagnosis of hypocellular MDS should be considered when the marrow is less than 30% cellular, and when there is abnormal localisation of immature precursors(ALIP), which is evidence of dysplasia or disturbed marrow architecture (Saarni M.I., 1973). Patients with a hypocellular bone marrow who develop acute myeloid leukaemia (AML), appear to demonstrate at

first a more cellular marrow, so that hypocellular MDS does not show a specific relationship to hypocellular acute leukaemia. Whether any prognostic significance can be attached to this type of MDS is not clear. Some reports indicate a higher incidence of leukaemia transformation and a worse prognosis (Silverstein M.N.,1974).

2.8 MYELODYSPLASTIC SYNDROME WITH MARROW FIBROSIS

Sultan and co-workers (Storniolo A.M. *et al*,1990) were the first to report the association between MDS and fibrosis when, in 1981, they described 8 patients who showed clear evidence of trilineage dysplasia, marrow fibrosis, and an acute clinical course. Since then, several reports have appeared that confirmed the entity of MDS with fibrosis (Groopman J.E.,1980). A marked increase in marrow reticulin fibres has been reported in up to 10% of patients with MDS, and this has been reported in all F.A.B. subtypes of MDS (Groopman ,1980). Megakaryocytic dysplasia and hyperplasia are invariably present, and this is thought to be largely responsible for the fibrosis (Streuli R.A. *et al*,1980). In most reported cases, patients with MDS and fibrosis demonstrated a poorer prognosis than patients with MDS and no fibrosis (Streuli R.A. *et al*, 1980).

2.9 UNCLASSIFIABLE MYELODYSPLASTIC SYNDROME

In some studies (Pitcock J.A. *et al*,1962), as many as 10% of cases of MDS have been reported to be unclassifiable by the FAB system as outlined in Table 1. The most common reason for this is the presence of severe multilineage dysplasia, with fewer than 5% marrow blast cells and no monocytosis. Because of the trilineage dysplasia, such cases do not fit well into the refractory anaemia or

refractory anaemia with ringed sideroblast categories, and there are insufficient numbers of blast cells present for considering this as refractory anaemia with excess blasts. Michels and co-workers(1985) reported on 16 patients and found a median survival of only 16 months,and therefore they proposed a new category - (refractory anaemia with dysplasia), for such cases.

2.10 PROGRESSION TO ACUTE LEUKAEMIA

Most studies have reported a less than 50% incidence of transformation of the dysmyelopoietic syndromes to acute non-lymphocytic leukaemia or mixed lineage leukaemia (Vallespi T. *et al*, 1985). Transformation is most common in patients with refractory anaemia with excess blast (RAEB) and refractory anaemia with excess blast in transformation (RAEB-T). Occasionally there may be a progression of refractory anaemia (RA), refractory anaemia with ring sideroblast (RARS), or chronic myelomonocytic leukaemia (CMML) to refractory anaemia with excess blast (RAEB) and then to acute leukaemia (Vallespi T. *et al*,1985). A study by Pierre in 1978 reported an overall incidence of 29% of MDS transformation to acute myeloid leukaemia (AML), ranging from 11.8% in RARS to 75% in RAEB-T. The development of AML is most likely to occur in patients who have chromosomal abnormalities (Pierre R.V.,1978). Proliferation to a more complex karyotype is likely to occur in association with the development of acute leukaemia (Pierre R.V.,1978).

Three patterns of progression of MDS have been described (Hamblin T.J. *et al*, 1987). Some patients may show an increase in the number

of blasts plus a peripheral cytopenia. These patients may die from the complications of bone marrow failure, such as infection or haemorrhaging. Such cases usually do not demonstrate karyotypic transformation. Other cases may exhibit an abrupt transformation to leukaemia that is often associated with a change in karyotype. The third pattern, most common in RA and RARS, but also seen in CMML, is a more stable disease not associated with an increase in blast cells.

Prolonged courses of low-dose x-radiation produce a high incidence of myeloid leukaemia. Studies have shown that there is a 4-phase preclinical sequence in the induction of this type of leukaemia: suppression; recovery; accommodation and the development of MDS (Seed T.M. *et al*,1986). Within this sequence, there is an early critical event characterised by the acquisition of increased radio-resistance to x-rays by a 15% fraction of the myeloid progenitor cells. Following initial radiation exposure, the marrow may show serious atrophy,(Fibrinoid Myelitis), with stromed oedema and the loss of normal cellular elements (Seed T.M. *et al*, 1981). The erythroblasts show maturational arrest with megaloblastic changes. The type, intensity and duration of radiation exposure, together with the age of the irradiated individual, will determine the severity of the marrow damage. The damaged marrow may follow one of several patterns: complete recovery; early post-radiation change without complete restitution; or the development of MDS with or without complete restitution; or the development of MDS with or without subsequent leukaemia as a later event. These possible sequelae are demonstrated in Figure 1 (Ruutu T., 1986). Repeated

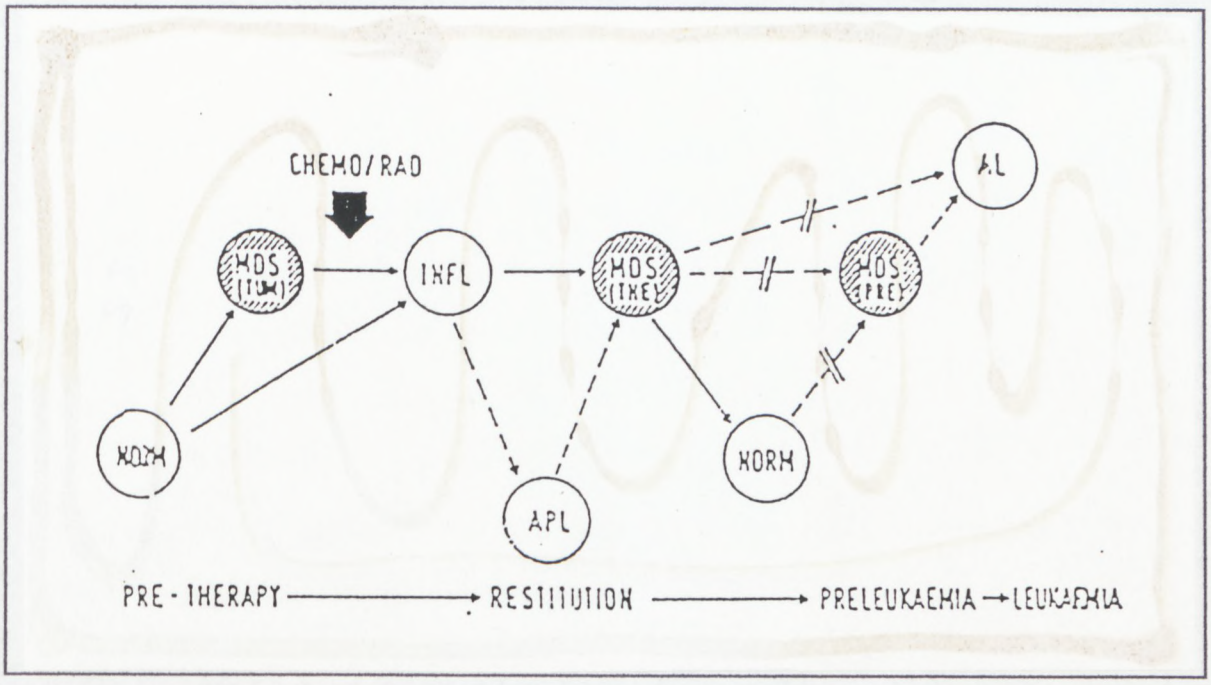

Figure 1 (Ruutu,1986)

INF = inflammatory change in the bone marrow which may develop to a therapy-induced myelodysplasia, directly or via an aplastic phase

APL = aplasia

MDS (THE) = therapy-induced MDS; the possible subsequent, later preleukaemic and leukaemic phases may proceed from a residual MDS or from a reconstituted normal bone marrow

NORM = normal

MDS (PRE) = preleukaemic phase

AL = acute leukaemia

marrow injury usually leads to permanent hypocellularity with dysplastic changes identical to those seen in primary idiopathic MDS. In cases where MDS is followed by leukaemia, the bone marrow histology is indistinguishable from that of "spontaneous" primary MDS.

As yet, there are no studies which completely correlate the early bone marrow reactions to radiation with the occurrence of later leukaemia. Virtually all individuals exposed to sufficient radiation will develop hypoplastic/dysplastic changes in their marrow, yet only some will develop later chromosomal defects and leukaemia. At present, there is no early marker for this ill-defined complication.

Previous studies (Ihle J.N.,1978) suggests that a 2 year manned mission to the planet Mars may entail the chronic exposure of astronauts to a 2,0 Gy total dose of galactic and solar radiation whose components affect the bone marrow. Kinetic studies indicate that only 79 days would be required between the induction of leukaemic change in a single cell of the bone marrow, and the clonal proliferation of this cell into diagnosable leukaemia (Cronkite E.P.,1968).

The induction of radiation-induced leukaemia in even a single crew member would have profound effects on the successful probability of such a mission. Clues to the early pathogenesis of MDS and a possible marker for MDS transformation were deduced from a review of the effect of the nitrobenzene antibiotic, Chloramphenicol (CAP), on the bone marrow (Manyan D.R. *et al*,1972). Mitochondrial protein synthesis is selectively blocked by CAP via its action on the large ribosomal subunit of the organelle. This is the only biochemical pathway in mammals known to be sensitive to the drug. The dose-related effects of CAP on the bone marrow leads to ineffective erythropoiesis with maturational delay, reduced ferrochelase

activity, ringed sideroblast formation and refractory peripheral anaemia (Manyan D.R. *et al*, 1972).

Suppression of mitochondrial protein synthesis by CAP is accompanied by the appearance of dysplastic myeloid progenitor cells, which show a decreased colony forming ability on solid agar assay (Manyan D.R. *et al*, 1972). These pathological features are very similar to that observed in radiation i.e. induced MDS. In this respect, it is interesting to note that some human exposures to CAP have been associated with the later development of leukaemia (Kumana C.R., 1988).

Molecular evidence that mitochondrial DNA (mtDNA) may be associated with the clinical features of MDS comes from studies on the rare congenital Pearson's Syndrome (Rotig A. *et al*,1988). Such patients exhibit mtDNA deletions and the resulting haematopoietic defects are virtually identical to those of MDS. Accumulating evidence suggests the pathogenesis of MDS is somehow associated with that of leukaemia. Some of this evidence comes from the generally accepted relationship of benzene as a leukaemogen and its ability to induce myelodysplasia in groups of occupationally exposed workers (Aksoy M. *et al*, 1976).

Benzene undergoes bio-activation within the haematopoietic mitochondria and degrades into a metabolite which can covalently bind to mtDNA and inhibit its transcription (Kalf G.F. *et al*, 1982). This causes a hypoplastic marrow insult which may progress to MDS and the later onset of leukaemia (Aksoy M. *et al*, 1976).

Childhood myelodysplasia related to a mitochondrial cytopathy has been reported and illustrated the difficulty in recognizing such disorders in patients with solely haematological signs (Bader-Meunier B. *et al*, 1994).

Radiotherapy combined with cytotoxic chemotherapy is an important component in managing malignant disease. Molecular studies in the field of cell response to radiation and the phenomena of DNA damage and repair are providing explanations for unexplained radiobiologic observations (Lichter A.S, *et al*, 1995).

To investigate the possible role of mtDNA in the onset and progression of MDS and secondary leukaemia, it was necessary to develop a procedure for screening haematopoietic cells for the presence of point and length mutations in their mtDNA.

A procedure utilising transmission electron microscopy was used for the direct visualisation of possible length mutations of mtDNA.

CHAPTER THREE

MATERIALS AND METHODS

3.0 INTRODUCTION

3.1 LENGTH MUTATIONS OF mtDNA IN MYELODYSPLASTIC SYNDROMES AND LEUKAEMIA

Leukaemia cells exhibit mitochondria which are abnormal in shape, ultrastructure and function (Schumacker H.R. *et al*,1974). Almost one half of the total mtDNA in leukaemia cells may be configured as complex catenated structures or as abnormal monomeric dimers. The number of these dimers has been shown to correlate with either the severity or the remission of the leukaemia (Clayton D.A. *et al*,1969). Steric considerations suggest these mtDNA dimers are probably non-functional and unsuitable for RNA synthesis,due to of supercoiling (Firkin F.C. *et al*,1979). Therefore it is reasonable to suggest that the progressive accumulation of these non-functional dimers in the haematopoietic compartment can account for the clinical features of MDS (Mufti,G.J.& Galton,D.A.G,1992). The finding of abnormal mtDNA dimers in patients with MDS would not only serve to explain the clinical symptoms, but might also serve to link the pathogenesis of MDS to that of secondary leukaemia. In this respect, transmission electron microscopy was utilised in this study to examine a series of specimens from MDS patients for the presence of length mutations and abnormal dimer formation in the mtDNA of the haematopoietic compartment. It has previously been reported that MDS in childhood is related to mitochondrial cytopathy (Bader-Meunier B. *et al*, 1994), and that MDS cannot be considered a truly malignant disorder.

3.2 REAGENTS AND INSTRUMENTATION

i) TEK Buffer: 6,05gm TRIS; 3,36gm EDTA; 15gm KCl. Add to 950ml distilled water , adjust pH to 7,5 and make solution to 1 liter with distilled water.

ii) TEK + 15% sucrose: 900ml TEK + 150gm sucrose , make to 1 liter with TEK buffer.

Autoclave the above reagents.

iii) T.E.: 10mM Tris, 1mM EDTA pH 7,5

iv) May-Grünewald

v) Ficoll-Hypaque

vi) Giemsa

vii) Haematoxylin & Eosin

viii) Prussian Blue

ix) Reticulin (Gordon & Sweets)

x) Periodic Acid Schiffs

xi) Non-idet detergent

xii) TE saturated phenol

xiii) Parlodien film

xiv) Plasmid pBR322 DNA

xv) Formal-saline

xvi) Zenker's acetic

Apparatus

i) Balzer's High Vacuum Coating apparatus

ii) Transmission Electron Microscope

iii) Map reader

iv) Gamma Beam irradiator

v) Coulter cell counter

vi) Ames rotary microtome

3.3 Sample Preparations

Bone marrow aspirates, trephine biopsies and peripheral blood samples were taken from normal, healthy individuals as controls and MDS patients, at the time of diagnosis. Aspirates were taken from the posterior iliac crest. Immediately following aspiration, the bone marrow samples were collected into EDTA anti-coagulant tubes to prevent clotting and allowed to stand for one hour at room temperature, so the cells could separate in layers. The buffy layer was separated from the 3-5ml marrow aspirates, and used for isolating the mitochondria from the mononuclear leucocytes. Mononuclear cells were separated on a Ficoll-Hypaque gradient. Diagnosis of MDS was based on morphologic and cytochemical studies of peripheral blood smears, bone marrow aspirates and trephine biopsy specimens. The F.A.B. Co-operative Group criteria were used to subclassify the specific disorder. Bone marrow and peripheral smears were stained with the May-Grünewald Giemsa technique. The trephine biopsies were processed, embedded in parrafin wax and sections cut with a Reichert Jung 2040 rotary microtome. The sections were stained by Giemsa; Haematoxylin/Eosin; Reticulin and PAS techniques for cytologic examinations.

The EDTA peripheral blood samples were treated the same as the bone marrow and the mononuclear cells isolated to use for isolating the mitochondria.

3.3.1 Mitochondrial Isolation

All reagents and equipment used were autoclaved or cleaned with dichromic acid to prevent contamination. The isolated mononuclear cells from the bone marrow and the peripheral blood were separately homogenised in 5 volumes of TEK buffer (TEK, 50mM Tris, 10mM EDTA 1,5% KCl, pH7.5) for 5 seconds with a motor driven stainless steel homogeniser. The homogenate was transferred to a 50ml polypropylene disposable centrifuge tube, and a 15% sucrose-TEK solution, pH 7,5, underlayered by passing a long-stem Pasteur pipette through the homogenate and allowing gravity feed to form a sharp meniscus. Homogenates were centrifuged (1000 x g) for 10 min at 4°C, the supernatant syphoned off into another 50ml disposable centrifuge tube and underlain by a second 15% sucrose-TEK solution. Centrifugation at 1000 x g was repeated. The supernatant from the second low speed spin was syphoned off and the mitochondria pelleted by centrifugation at 18 000 x g for one hour at 4°C. The supernatant was discarded leaving the pellet behind. This mitochondrial pellet was resuspended in 10 ml TEK buffer as a wash phase and centrifuged again at 19 000 x g for 30 min to form another pellet.

3.3.2 Mitochondrial DNA isolation

The mitochondrial pellet was resuspended in 0.9ml TEK buffer, pH 7,5, and placed in a microfuge tube. For complete lysis of the mitochondria, 9μL of a non-idet detergent was add to the solution and stood for 5-10min. The suspension was then centrifuged at 12 000 x g for 10 min to remove any debris. The non-idet detergent

does not attack nuclear membranes, and any intact nuclei carried over from the low speed spins will pellet at this stage. The supernatant was syphoned off and mixed with an equal volume of TE buffer(Tris EDTA) pH 8,5, saturated with redistilled phenol to precipitate the proteins. The sample was mixed thoroughly, allowed to stand for 5 minutes and centrifuged at 12 000 x g for 10 minutes. A white flocculant protein precipitate separated the phenol phase from the upper aqueous phase. If the aqueous phase was not clear, the phenol extraction step was repeated. The transparent aqueous phase was syphoned off and mtDNA extracted with a 24:1 chloroform : iso-amyl alcohol solution. The aqueous phase was syphoned off and mixed with 2 volumes of cold (4°C) 95% ethanol, and placed at -20°C for two hours to ensure complete precipitation of nucleic acids. mtDNA was pelleted by centrifugation at 12 000 x g for 10 minutes at 4°C, the ethanol decanted, and the pellet allowed to dry at 37°C. Drying was complete when the white precipitate turned translucent. The sample was then rehydrated in 50μl TE buffer and frozen at -70°C.

3.3.3 Electron Microscopy of mtDNA

Isolated mtDNA was thawed and suspended in TE buffer (0.1 M TRIS; 10 mM EDTA; pH 8,5) and 50μl of suspension was placed onto 200/400 mesh copper EM grids covered with Parlodien film from a 3.5% w/v solution of n-pentyl acetate. The suspension was allowed to evaporate on the Parlodien film after which the grids were placed in a Balzers high vacuum coating plant (BAE120 Balzers High Vacuum Corporation, Santa Ana, California). Rotary shadowing was performed using a silver/platinum filament with an

evaporation time of 10-15 seconds at an angle of 7°. Molecules were photographed at x 17 000 using a Philips CM12 transmission electron microscope at 60kV accelerating voltage. Photographic prints were traced onto tracing paper and mtDNA circumferential lengths measured with a Keuffel and Essen 620-300 map reader. Only molecules with neither intra- nor intermolecular overlaps were measured in order to avoid ambiguity. Plasmid pBR322 DNA was obtained from the Department of Biochemistry, University of Cape Town, for use as an internal length standard (Figure 2). Plasmid DNA was added to all the patient specimens and controls before aqueous mounting on the EM grids, in order to allow calibration on the same photomicrograph as the mtDNA of interest.

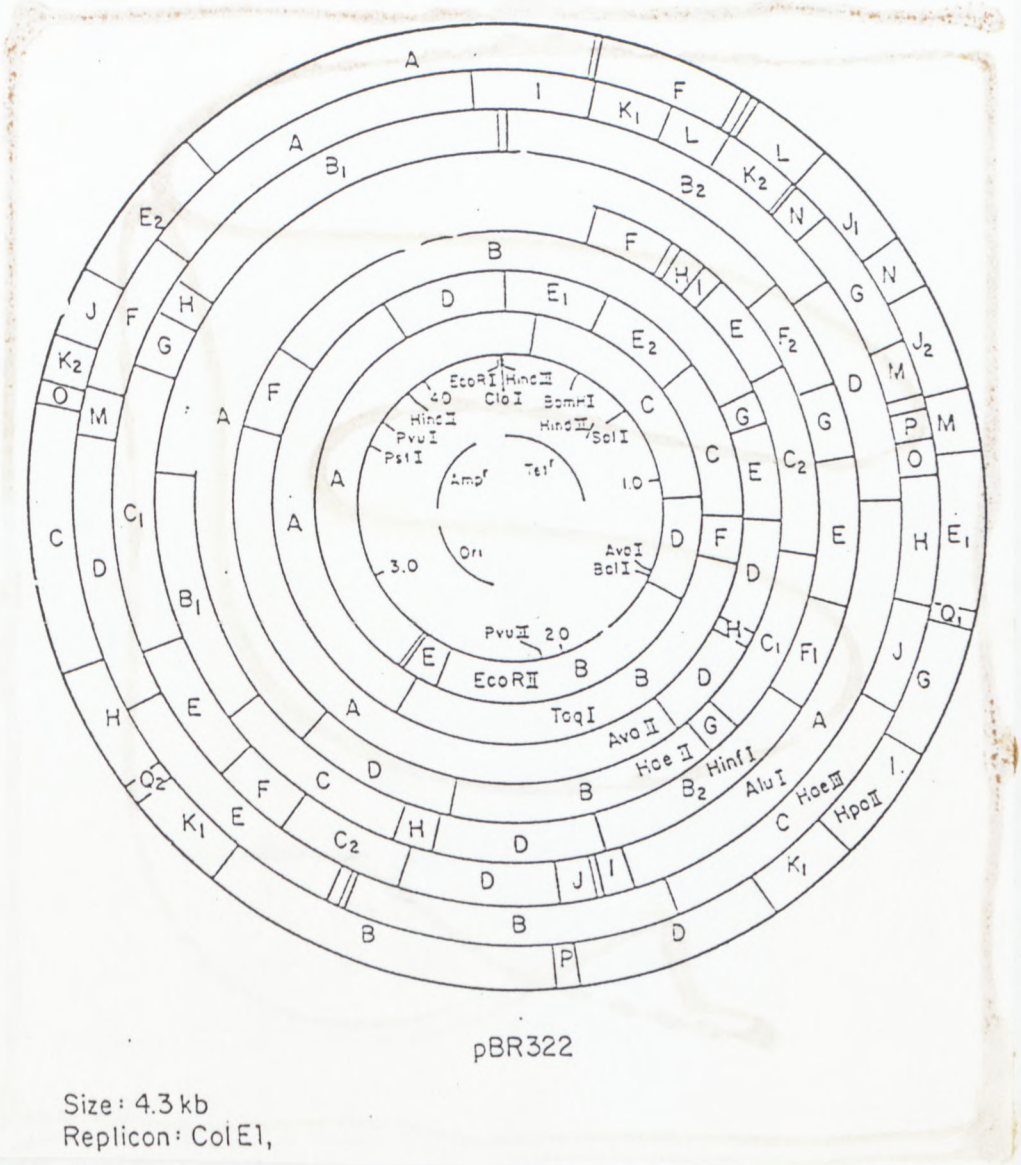

Figure 2

The above figure shows the plasmid pBR322, a plasmid commonly used in recombinant DNA technology, showing cleavage sites of restriction endonucleases which cut the DNA only once.

3.4 mtDNA Length Measurement and Internal Calibration by pBR322 Plasmid

The length of a DNA molecule visualised by transmission electron microscopy, depends on the conditions under which the DNA is mounted on the EM grid (Wetmur J.G. *et al*,1966). A number of factors are involved. The absolute length of double-strand DNA

decreases with increasing ionic strength, and both single and double-strand DNA lengths are subject to uncontrollable variations from grid to grid, even when the grids are mounted under seemingly identical conditions (Wetmur J.G. *et al*,1966). Instrument variations and focusing conditions may also contribute to a lesser extent. However, by measuring DNA molecular lengths relative to an appropriate DNA standard, quantitatively significant results can be obtained. For homogenous DNA samples such as mtDNA, in which all molecules have the same number of nucleotides in the same base sequence, it is an inherent property of the protein film technique that the length of the measured DNA fluctuates around a quantitatively significant mean value with a reproducible standard deviation (Colowick S.P. *et al*,1969). Hence, by using length measurements relative to a homogenous standard (in this case pBR322 plasmid DNA), both the mean value for the length of the unknown DNA and the standard deviation of its length about the mean, become reproducible in significant quantities (Colowick S.P. *et al*,1969). An effective way of obtaining molecular lengths and thus molecular weights of DNA, is to photograph standard DNA on the same grid as the unknown (i.e. pBR322 and patient mtDNA). The intrinsic fluctuations in the length of DNA visualised by TEM, is such that the variance (σ) around the mean is proportional to the mean length (L) of the DNA. Thus, the standard deviation of molecular length L (SD.1) is described by the equation:

$$SD = D \times (L)^{\frac{1}{2}}$$

This holds for molecular weights of between 3×10^4 to 3×10^7. If the unknown DNA lengths are measured in units of the length

of pBR322, then the empirical constant (D) has the value of 0.037. A plot of the SD of length measurements against the square root of the mean length of various standard control DNA molecules is shown in Figure 3 (Colowick J.G. *et al*,1969).

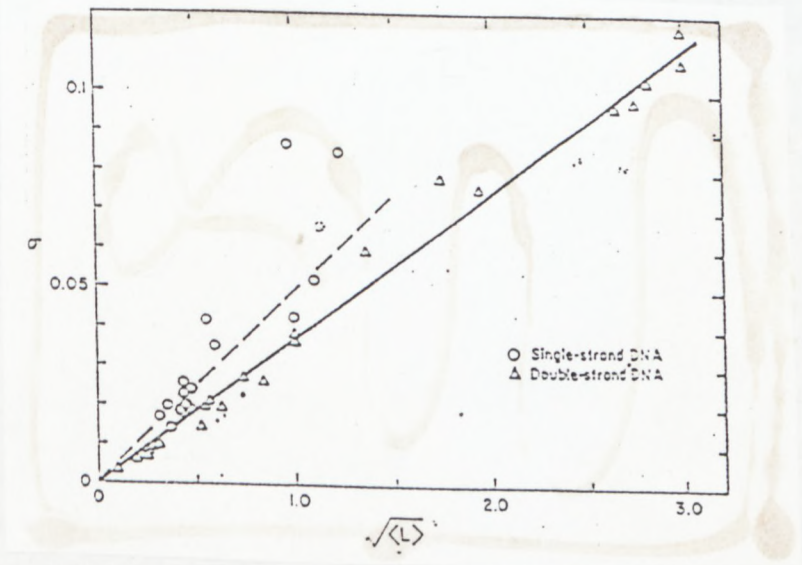

Figure 3 *(Colowick,1969).*

Plot of standard deviation of length measurements for samples of double- and single-strand DNA versus the square root of the mean length (L). Most of the points represent samples of 25 or more molecules. The double strand points include Escherichia coli minicircles, N-phage DNA's, X RF DNA, and various segments of heteroduplexes. Measurements were made on samples mounted by both the aqueous technique and the formamide technique

The data presented in Figure 3 conforms to the equation:

$\sigma = (0.037) \times (L)^{\frac{1}{2}}$

Given the SD.1 (r) equation, the principles of statistics assert that if sets of measurements are made on (n) samples of DNA, the mean lengths (L) of the samples should be normally distributed with a standard deviation of:

$$\frac{SD}{n^{1/2}}$$

where SD is calculated from the SD.1 equation.

Therefore, if contour lengths are measured on na and nb molecules of mtDNA, and calibrating molecules of pBR322 (nc) of known length (Lc), then the standard sample error (Ea) in the measurement of (La) is given by:

$$\frac{Ea}{(La)} = 0.037 \quad \frac{1}{na(La)} + \frac{1}{nc(Lc)}$$

Thus, it is advantageous to use a known long DNA length as a standard (Colowick J.G. *et al*,1969). The variance of length of a sample size (n) is given by s^2, and the quantity ns^2/σ^2 is a stochastic variable with a χ^2 distribution for n-1 degrees of freedom. For reasonable large values of n, the values of s in a sample population, are approximately distributed around σ, with a variance of $\sigma^2/2(n-1)$. The SD of circumferential length for ± 25 mtDNA molecules should lie within 95% probability within the interval 0.70 SD to 1.25 SD, when the mean molecular length pBR322 is used as an internal measuring standard. If the measured s is larger than 1.3 SD, then some other cause of length heterogeneity, such as a true difference in the number of base pairs per molecule, should be suspected.

In this study, patient and control mtDNA, and pBR322 lengths, were measured with the appropriate conversion factor between negative and print size and the mtDNA values expressed in tabular form by use of the equation:

$$\frac{\text{Individual mtDNA lengths measured on negative}}{\text{Mean Length of pBR322 on negative}} = \text{Mean length value of mtDNA molecule}$$

3.5 Experimental Animals

20 Male Wistar rats at 120 ± 5 days old with body weights of 280-323 gm were used. Animals were maintained in individual clean wire cages with free access to food and water, in a group isolated and temperature controlled environment.

3.5.1 Irradiation

A) Ten rats were exposed to 3 fractional doses each of 400 rads of ^{60}Co λ -radiation, this dose was given according to the weight of the animals and to ensure not to overexpose them. (Gamma Beam 150 Irradiator, Groote Schuur Hospital, Cape Town). Dosimetry methods and calculations were performed by the Radiotherapy Department, Groote Schuur Hospital (RSA). Dose rate was 13.20 cGy min using a 30 x 30cm field size. Dose intervals and sacrifice schedules are shown in Table 2

Table 2

Animal irradiation and sacrifice schedule - Gamma irradiation of rat population.

WEEK	PROCEDURE
1	4,0 Gy exposure
2	Nil
3	Sacrifice 1 rat
4	Nil
5	4,0 Gy exposure
6	Nil
7	Sacrifice 2 rats
8	Nil
9	4,0 Gy exposure
10	Nil
11	Sacrifice 2 rats
12	Sacrifice 2 rats
20	Sacrifice 3 rats

B) The different irradiation doses of the animals were calculated to enhance cell damage and possible leukaemia and not to over expose them. Ten rats were exposed to 3 fractionated doses, each of 2,88 Gy of mixed gamma-neutron radiation. Gamma irradiation of 1,98 Gy was by 60 Co radiation from a Gamma Beam 150 Irradiator. Dose rate was 13.20 cGy min(-1) using a 30cm x 30cm field size.

Gamma irradiation was immediately followed (within 45 minutes) by neutron radiation exposure of the rats to 0,90 Gy of p(66)/Be neutrons, generated by cyclotron-directed protons impacted against a standard beryllium metal target with post-impact

attenuation. Dose rate of the neutron irradiation was 6.65 cGy min (-1) with a field size of 30cm x 30cm. Dose intervals and sacrifice schedules were as illustrated in Table 3.

Table 3

Animal irradiation and sacrifice schedule - mixed gamma-neutron irradiation rat population.

WEEK	PROCEDURE
1	2,88 Gy x-Neutron dose #1
2	Nil
3	Sacrifice 2 rats
4	Nil
5	2,88 Gy x-Neutron dose #2
6	Nil
7	Sacrifice 2 rats
8	Nil
9	Nil
10	2,88 Gy x-Neutron dose #3
11	Nil
12	Sacrifice 2 rats
13	Nil
14	Sacrifice 4 rats

3.5.2 Haematology and Histology of Rat tissues

Animals were sacrificed by exsanguination while under sodium pentobarbital (Nembutal, Parke Davis) anaesthesia. Full blood counts were done with a Coulter STKS and marrow counts were performed manually by light microscopy and pathological changes recorded. Only gross pathological changes were recorded to keep the data simple and practical. The bone marrow trephine samples were systematically collected in Zenker's acetic and processed

for histological evaluation.

3.5.2.1 Bone marrow biopsy procedure

Marrow specimens from the pelvis and femurs were sequentially collected from both non-irradiated controls and irradiated test rats on scheduled days, utilising surgical biopsy technique under terminal Nembutal anaesthesia. This technique ensured provision of architecturally intact pieces of marrow for morphological evaluation.

3.5.2.2 Tissue preparation and fixation

Immediately following the excision of bone marrow, the specimen was split longitudinally with a razor blade. One half was quickly immersed in Zenker's acetic fixative. The other half was trimmed and impression smears made from the marrow slices. Smears were fixed and stained by May Grünewald-Giemsa technique, and subsequently used for examination by light microscopy for the determination of marrow cell differential counts. Fixed marrow tissue was paraffin-embedded by standard histological procedure and sectioned at 7 μm on an Ames rotary microtome. May-Grünewald-Giemsa staining was performed before permanent mounting. Separate sections were stained for marrow iron by Prussian-Blue histological technique. Examination of the sections was by light microscopy x 500 with an oil immersion objective.

3.5.2.3 EM Technique

The EM copper grids with samples were stored under vacuum in a glass container. Each grid was carefully screened and photographed at x17,000 using a Philips CM12 transmission electron microscope at a 60kV accellerating voltage.

3.5.2.4 Rat mtDNA Analysis

Normal control and irradiated rats were sacrificed to schedule and haematopoietic mtDNA isolated as described. The rat mtDNA molecules were rotary-shadowed with platinum for electron microscopy, by the same procedure as described on page 31. pBR322 plasmid DNA data and normal control rat mtDNA was used as a standard for derivation of the mean length and mean length values of normal and irradiated rat mtDNA.

CHAPTER 4.

RESULTS

4.0 EXPERIMENTAL RESULTS

4.1 NORMAL mtDNA LENGTH DETERMINATION

4.1.1 Standard pBR322 plasmid Length Measurements

Purified pBR322 plasmids were mounted on two separate EM grids as described(p.31) and visualised by transmission electron microscopy, followed by photography and length measurements. The following conversion factor was used to equate photographic negative and print sizes:

Negative 8cm
Prints 9cm

$\frac{9}{8} = 1.125 \times 17\ 000 = 19\ 125$

$\text{FACTOR} = \frac{17\ 000}{\text{Sw}19\ 125} = 0.888$

A typical resulting electron micrograph is shown in Figure 4 and molecular lengths are outlined in Table 4 and 5

Conversion factor x length measured = length used for graphs

0.888×1.90

$= 1.68$

Figure 4

Electron micrograph of pBR322 plasmid showing its normal closed circular DNA structure (x 17 000)

<u>Measured pBR322 plasmid lengths</u>

Table 4

pBR322 Measured Length (cm)	Conversion (length x 0.888)	Mean pBR322 Length on Negative
1.90	1.68	1.55
1.50	1.33	
1.70	1.50	
2.20	1.95	
1.65	1.46	
1.70	1.50	
1.35	1.19	
2.00	1.77	
1.50	1.33	
2.05	1.82	

Table 5

pBR322 Measured Length	Conversion	Mean pBR322 Length
2.00	1.77	1.57
1.55	1.37	
1.55	1.37	
2.10	1.86	
1.55	1.37	
2.00	1.77	
1.85	1.64	
1.40	1.24	
1.85	1.64	
1.95	1.73	

The above pBR322 lengths is presented graphically in Figure 5

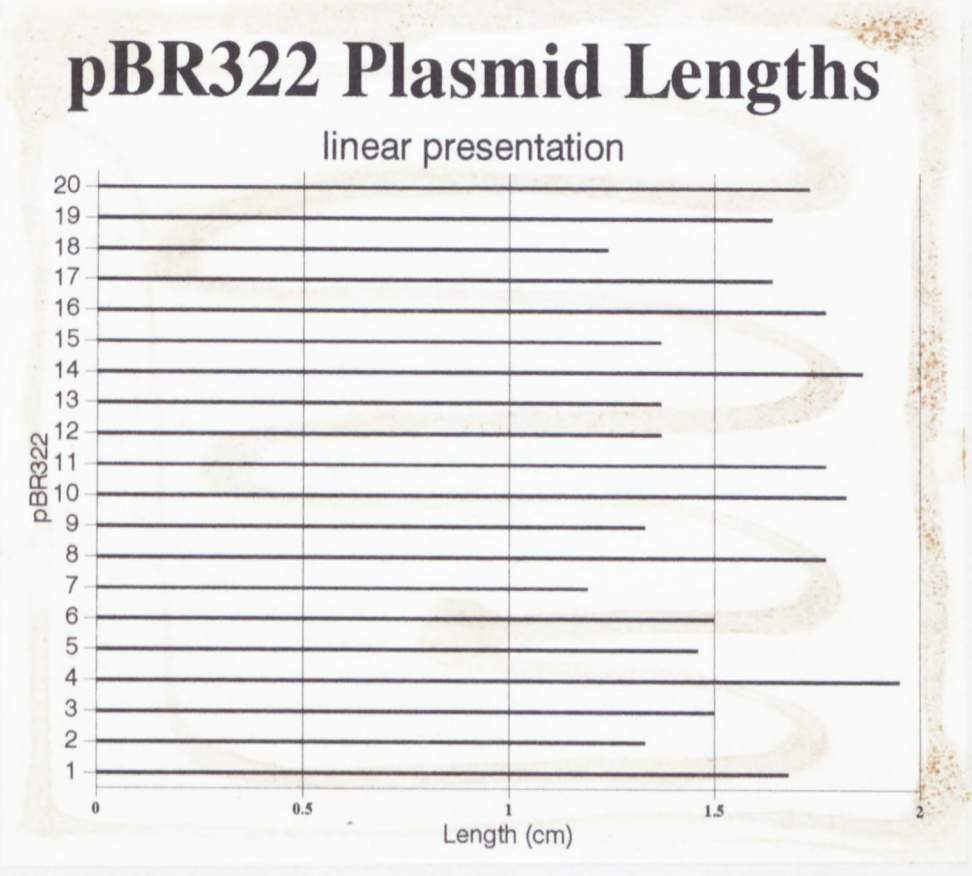

Figure 5
Linear presentation of pBR322 lengths by arbitrarily selecting a point which represents the closed circular molecule as though they were opened at that point.

4.1.2 Normal mtDNA (Control 1 & 2) and pBR322 Length Measurements

Purified haematopoietic mtDNA from two healthy individuals were co-mounted with purified pBR322 onto two separate EM grids and visualised by electron microscopy as described(p.31). The following conversion factor was required to equate negative and print sizes as describe below.

Negative 8cm
Prints 9cm

$$\frac{9}{8} = 1.125 \times 17{,}000 = 19\ 125$$

$$\text{FACTOR} = \frac{17\ 000}{19\ 125} = 0.888$$

A resulting electron micrograph is shown in Figure 6, and molecular lengths are outlined in Tables 6 to 13

4.1.3 Standard pBR322 and Control 1 mtDNA

Table 6

pBR322 Measured Length (cm)	Conversion (0.888 x length)	pBR322 Mean Length
2.60	2.30	1.94
1.20	1.06	
2.85	2.53	
2.35	2.08	
3.20	2.84	
1.80	1.59	
1.55	1.37	
2.50	2.22	
1.30	1.15	
2.25	1.99	
2.50	2.22	
2.20	1.95	

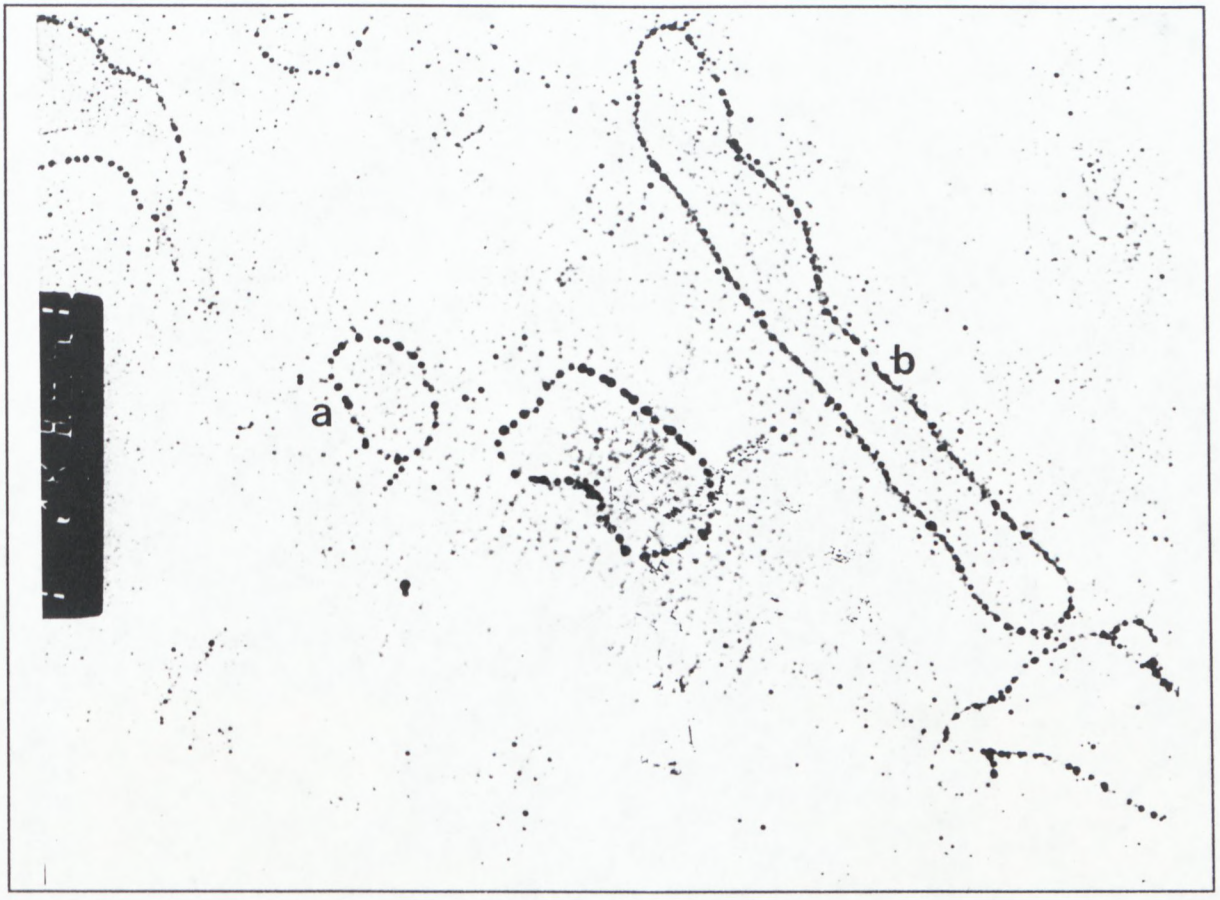

Figure 6

Electron micrograph of pBR322 plasmid and normal mtDNA, both showing a closed circular DNA structure (x 17,000) Silver/platinum shadowing. The (a) shows the plasmids and (b) shows the normal mtDNA.

Table 7

Control 1 mtDNA Length (cm)	Conversion (0.888 x length)	Mean length Value mtDNA (% 1.94)	Average Mean Length Value
4.3	3.80	1.95	2.01
4.4	3.90	2.01	
4.8	4.26	2.19	
4.75	4.20	2.16	
4.4	3.90	2.01	
4.6	4.08	2.10	
3.75	3.33	1.71	
4.3	3.80	1.95	

4.1.4 Standard pBR322 and Control 2 mtDNA

Table 8

pBR322 Measured Length (cm)	Conversion (0.888)	pBR322 Mean Length
2.3	2.04	1.97
2.1	1.86	
2.0	1.77	
2.20	1.95	
2.35	2.08	
2.15	1.90	
2.50	2.22	
2.25	1.99	

Table 9

Control 2 mtDNA Length (cm)	Conversion (0.888)	Mean Length Value mtDNA (% 1.97)	Average Mean Length Value
4.75	4.2	2.10	2.11
4.90	4.35	2.20	
4.86	4.3	2.18	
4.25	3.77	1.91	
4.50	3.99	2.02	
4.95	4.39	2.22	
4.35	3.86	1.95	
4.75	4.21	2.13	
5.30	4.70	2.38	
4.50	3.99	2.02	

Table 10

pBR322 Measured Length (cm)	Conversion (0.888)	pBR322 Mean Length
2.20	1.95	1.80
2.35	2.08	
2.15	1.90	
1.30	1.15	
2.25	1.99	
2.50	2.22	

Table 11

Control 2 mtDNA Length (cm)	Conversion (0.888)	Mean Length Value mtDNA (% 1.80)	Average Mean Length Value
4.75	4.20	2.33	2.03
3.85	3.41	1.89	
3.85	3.41	1.89	

Table 12

pBR322 Measured Length (cm)	Conversion (0.888)	pBR322 Mean Length
1.55	1.37	1.96
2.50	2.22	
2.60	2.30	

Table 13

Control 2 mtDNA Length (cm)	Conversion (0.888)	Mean Length Value mtDNA (% 1.96)	Average Mean Length Value
4.75	4.21	2.14	1.92
4.05	3.95	1.83	
3.70	3.28	1.67	
3.85	3.41	1.73	
4.95	4.39	2.23	

4.1.5 SUMMARY OF CONTROL mtDNA LENGTH MEASUREMENTS

The average mean length and mean length values of mtDNA from 4 healthy individuals (Control 1 & 2) were as follows:

Table 14

Average Mean Length Normal Control 1 & 2 mtDNA	Mean
3.90	3.85
4.10	
3.67	
3.77	

AVERAGE MEAN LENGTH VALUES NORMAL CONTROL 1 & 2 mtDNA
2.01
2.11
2.03
1.92

Individual mean length values for normal mtDNA are displayed as a linear presentation in Figure 7

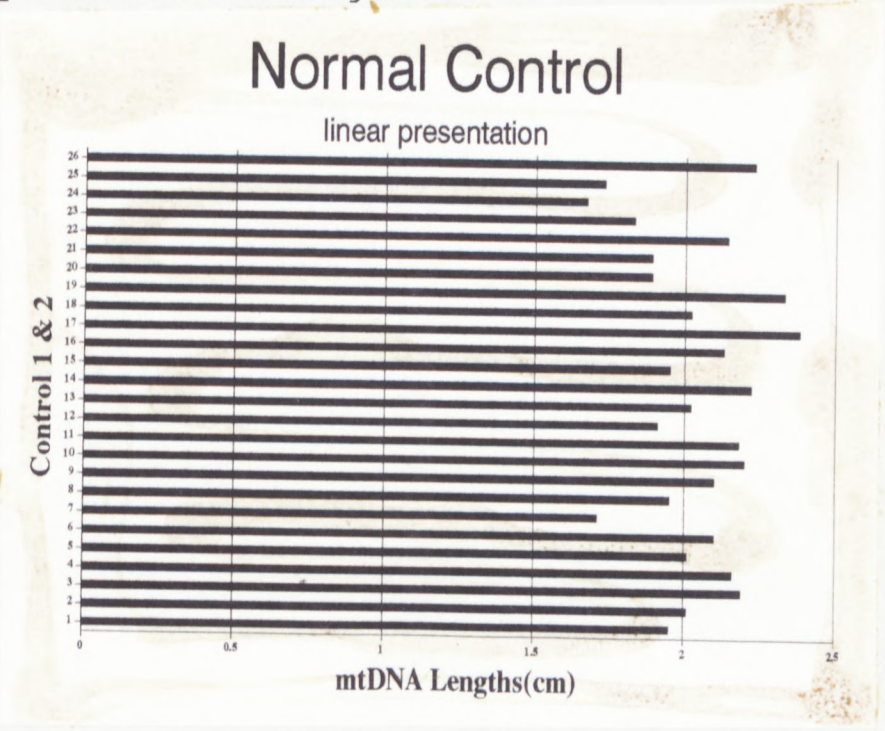

Figure 7
Linear presentation of normal Control 1 & 2 mtDNA lengths expressed as mean length value of DNA length standard pBR322.

4.2 MYELODYSPLASTIC SYNDROME PATIENT RESULTS (mtDNA LENGTHS)

4.2.0 INTRODUCTION

The myelodysplastic syndromes comprise a hetrogeneous group of disorders (RA, RARS, RAEB, RAEB-T and CMML). Although the clinical picture and prognosis vary from case to case, it is thought that MDS patients share a common abnormality in the multipotential stem cell population. This leads to the later formation of abnormal progenitor cells in the 3 primary lineages of the bone marrow. The small number of mtDNA dimers found in the patients with early primary MDS (E,F) suggests abnormal dimer formation may be a critical feature in establishing the severity of MDS at the time of presentation. Dimer formation may also be useful as a marker for the impending development of secondary MDS following ionizing radiation or chemotherapy (alkylating agent) exposure. In this respect, it was decided to conduct a controlled study of radiation-induced myelodysplasia in an animal model, in an attempt to determine precisely what phase of MDS development is associated with abnormal mtDNA dimer formation (as described on p.37)

Six patients (4 male, 2 female) with MDS were examined for possible mtDNA length mutations in their hematopoietic cell compartment. All MDS patients demonstrated a significant mtDNA length heteroplasmy which was not observed in the normal control.

4.2.1 Patient A: 67 year white male.

4.2.2 Peripheral Smear

Pancytopenia with anisopoikilocytosis, pencil cells, peripheral normoblasts, hypogranular neutrophils, Pseudo-Pelger Huet cells, large platelets, peripheral monocytosis.

MYELOGRAM			
Neutrophils	- 6	Blast Cells	- 50
Band Cells	- 2	Eosinophils	- 1
Metamyelocytes	- 2	Lymphocytes	- 6
Myelocytes	- 4	Plasma Cells	- 1
Promyelocytes	- 3	Monocytes	- 18
Iron(Fe)	5/6	Normoblasts	- 7
(Iron is the iron stores in the bone marrow)			

NUCLEATED CELLS OF NORMAL BONE MARROW

Blast cells	0,3-5%	Histiocytes	0,1-2%
Promyelocytes	1-8%	Megakaryocytes	0,03-3%
Myelocytes	5-22%	Pro-erythrocytes	1-8%
Metamyelocytes	13-32%	Normoblasts	7-32%
Neutrophils	7-30%		
Eosinophils	0,5-4%		
Basophils	0,1-0,7%		
Lymphocytes	3-17%		
Plasma cells	0,1-2%		
Monocytes	0,5-5%		

4.2.3 BONE MARROW IRON

Normal marrow stained for iron (Prussian blue stain) demonstrated dark blue granular material scattered throughout the marrow. This is storage iron within the macrophages. As a patient becomes iron deficient it diminishes and then disappears. Normoblasts containing such stainable iron are called sideroblasts.

4.2.4 BONE MARROW BIOPSY

8mm biopsy, good quality, with 100% cellularity. Normal haematopoiesis was suppressed by sheets of primitive cells with prominent nucleoli. Bone trabeculae and reticulin were normal. Marrow reserve was inadequate.

4.2.5 DIAGNOSIS

MDS (CMML FAB Subtype) in transition to acute myeloid leukaemia.

4.2.6 mtDNA length measurements - Patient A

Table 15

pBR322 Measured Length (cm)	Conversion FACTOR 0.888	Mean Length Value
1.55	1.37	1.68
1.50	1.33	
2.0	1.77	
1.35	1.19	
1.90	1.68	
2.20	1.95	
1.65	1.46	
1.95	1.73	
2.25	1.99	
1.90	1.68	
2.30	2.04	
2.0	1.77	
2.0	1.77	
2.05	1.80	
1.85	1.64	

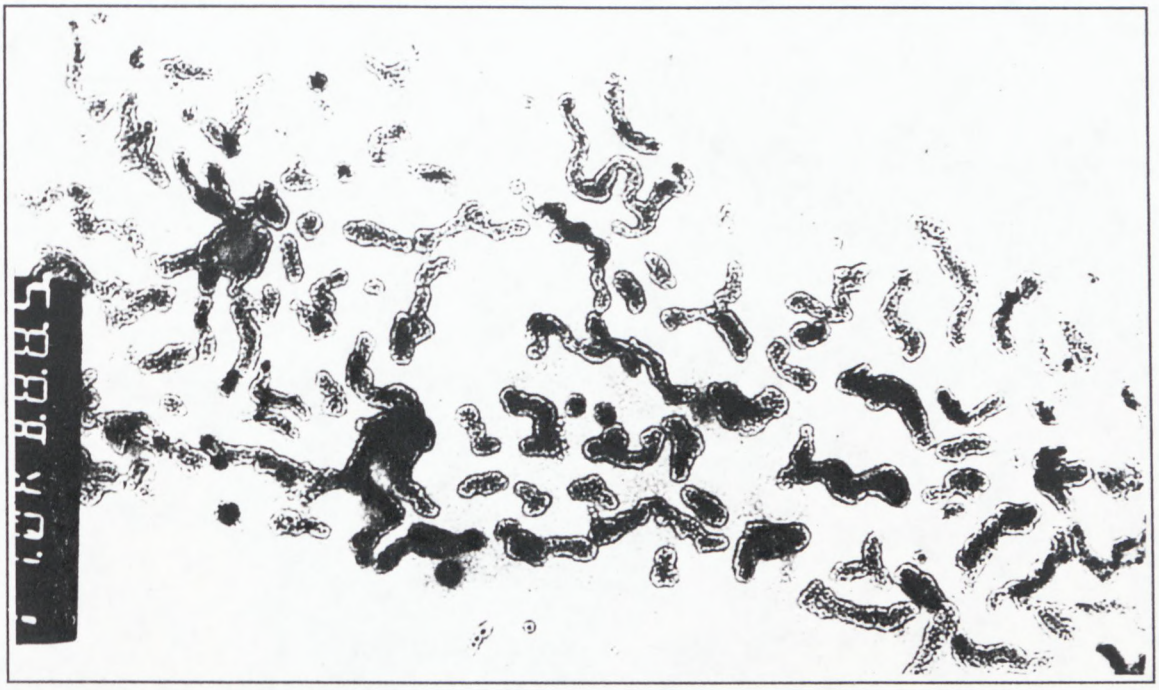

Figure 8

Electron micrograph of mtDNA of MDS patient A showing definite closed circular size heterogeneity with pBR322 plasmid (arrow) as internal length standard (x 17,000)

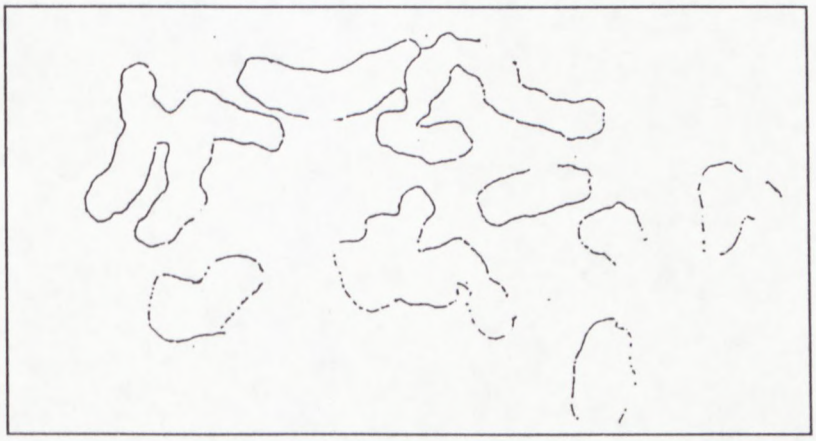

Figure 9

Tracing of photographic enlargement of electron micrograph prepared of MDS patient mtDNA used for length determination.

Table 16

mtDNA Length (cm)	Conversion FACTOR 0.888	Mean Length Value mtDNA (% 1.68)	Average Mean Length Value mtDNA
4.35	3.86	2.29	3.08
13.85	12.29	7.32	
7.45	6.61	3.93	
4.30	3.81	2.26	
4.45	3.95	2.35	
4.20	3.75	2.23	
6.75	5.99	3.57	
7.10	6.30	3.75	
4.40	3.90	2.32	
7.15	6.34	3.77	
4.15	3.68	2.19	
9.0	7.99	4.76	

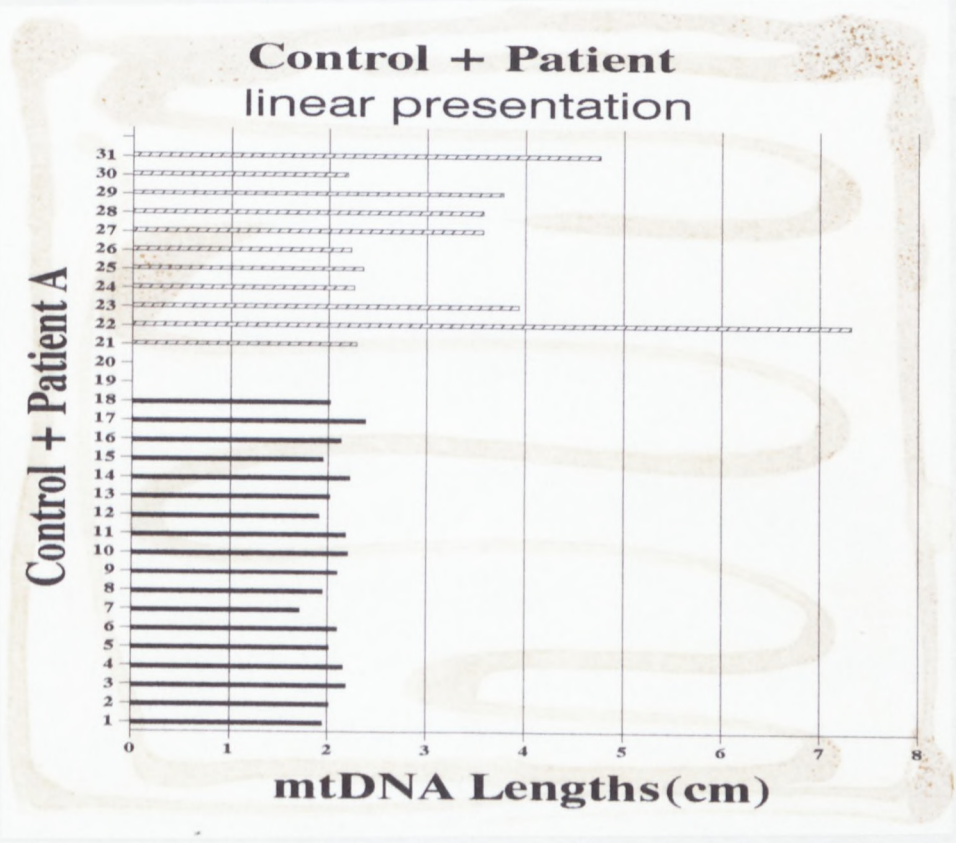

Figure 10

Linear presentation of normal Control 1 & 2 mtDNA lengths and MDS patient A mtDNA lengths expressed as the mean length value of DNA length standard pBR322

4.2.7 Patient B: 71 year white male

4.2.8 Peripheral Smear

Pancytopenia with anisopoikilocytosis targeting, hypogranular neutrophils, with Pseudo-Pelger-Huet cells and decreased platelets

MYELOGRAM			
Neutrophils	- 28	Blast Cells	- 11
Band Cells	- 6	Eosinophils	- 2
Metamyelocytes	- 3	Lymphocytes	- 5
Myelocytes	- 10	Plasma Cells	- 0
Promyelocytes	- 3	Monocytes	- 9
Iron(Fe)	0/6	Normoblasts	- 19

4.2.9 Bone Marrow Biopsy

12mm biopsy, good quality, with 80% hypercellularity. Erythropoiesis was megaloblastic and suppressed. Megakaryocytes were decreased in number with slightly dysplastic features. Granulopoiesis was left shifted with maturational delay and ALIP (Abnormal Localisation of Immature Precursors). Eosinophils were slightly increased. Reticulin was mildly increased. There was no evidence of granuloma of residual myeloma in the available examined material.

4.2.10 DIAGNOSIS

MDS (RAEB FAB Subtype) secondary to previous chemotherapy with alkylating agents for initial myeloma.

4.2.11 Follow-up Bone Marrow Biopsy

7mm biopsy, good quality, with 45% cellularity. Erythropoiesis was megaloblastic and suppressed. Megakaryocytes were decreased in number with overt dysplasia. There were numerous abnormal clusters of blast cells in the intertrabecular spaces. Reticulin was increased. There was no evidence of residual myeloma.

MYELOGRAM			
Neutrophils	- 16	Blast Cells	- 15
Band Cells	- 4	Eosinophils	- 0
Metamyelocytes	- 6	Lymphocytes	- 24
Myelocytes	- 4	Plasma Cells	- 0
Promyelocytes	- 5	Monocytes	- 2
Iron(Fe)	4/6	Normoblasts	- 24

4.2.12 DIAGNOSIS

MDS (RAEB) beginning early transformation into Acute Non Lymphocytic Leukaemia (ANLL).

4.2.13 mtDNA length measurements - Patient B (Biopsy 1)

Table 17

pBR322 Measured Length (cm)	Conversion (0.888)	Mean pBR322 Length on Negative
3.40	2.13	2.14
2.55	2.26	
2.50	2.22	
2.10	1.86	
2.50	2.22	
2.46	2.18	
2.37	2.10	
2.62	2.32	
2.25	1.99	

Table 18

mtDNA Length (cm)	Conversion FACTOR 0.888	Mean Length Value mtDNA (% 2.14)	Average Mean Length Value mtDNA
4.18	4.27	1.99	2.21
7.13	6.33	2.95	
4.3	3.81	1.78	
4.95	4.39	2.05	
6.14	5.45	2.54	
4.63	4.11	1.92	

4.2.14 mtDNA Length Measurements - Patient B (Biopsy #2)

Table 19

pBR322 Measured Length (cm)	Conversion	Mean pBR322 Length
2.10	1.86	1.99
2.20	1.95	
2.40	2.13	
2.25	1.99	
2.50	2.22	
2.00	1.77	
2.10	1.86	
2.50	2.22	
1.85	1.64	
2.55	2.26	

Table 20

mtDNA Length (cm)	Conversion (0.888)	Mean Length Value mtDNA (% 1.99)	Average Mean Length mtDNA
6.10	5.4	2.71	2.50
6.15	5.46	2.74	
4.75	4.2	2.11	
3.55	3.15	1.58	
4.0	3.55	1.78	
10.6	9.41	4.72	
5.35	4.78	2.40	
4.05	3.60	1.80	
6.45	5.72	2.87	

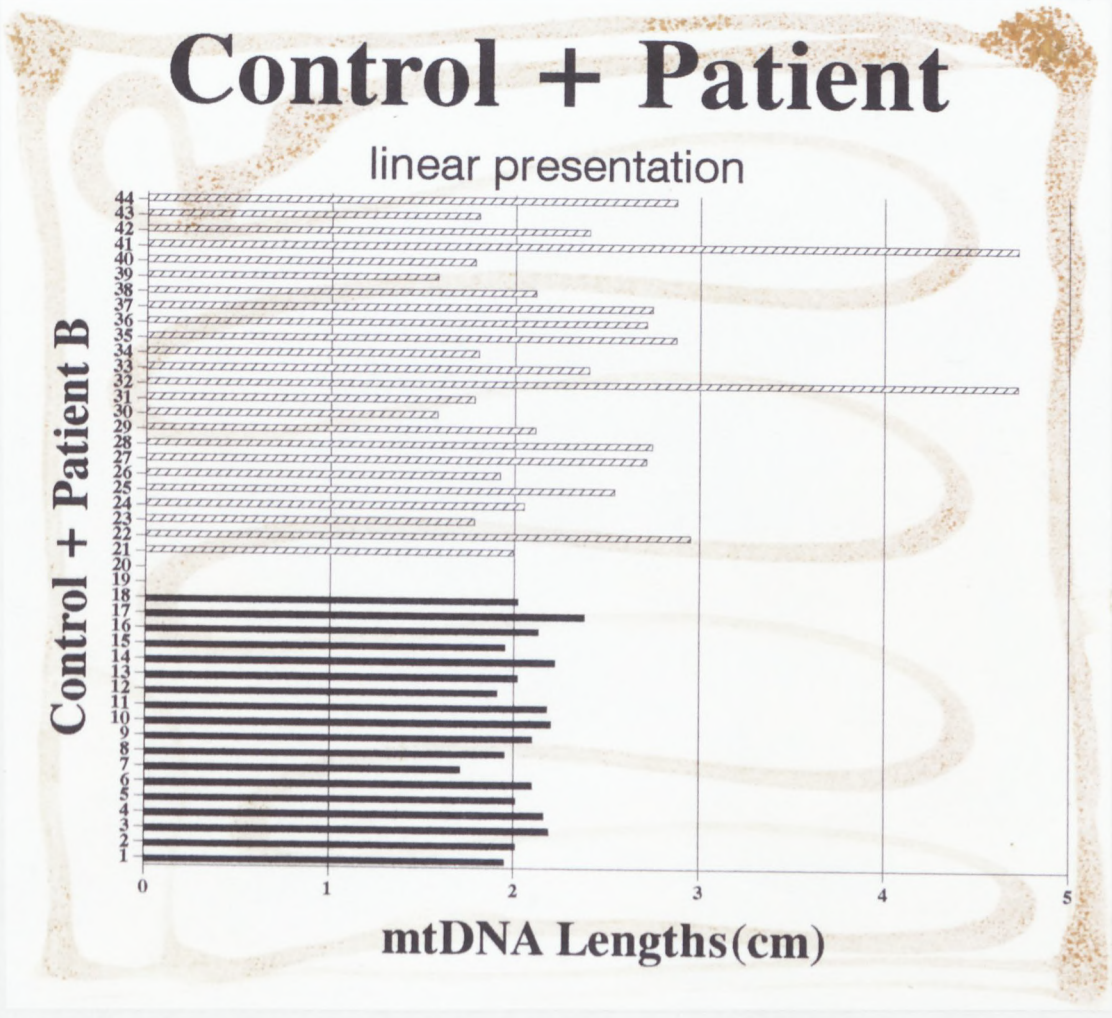

Figure 11

Linear presentation of normal (Control 1 & 2) mtDNA mean length values and MDS patient B mtDNA at the time of diagnosis of secondary MDS (Biopsy #1) and 4 months later at the time of impending transformation into acute leukaemia (Biopsy #2)

4.2.15 Patient B: 44,5 years white male

4.2.16 Peripheral Smear

Pancytopenia with anisopoikilocytosis, neutrophil hypersegmentation, decreased platelets and circulating normoblasts. Occasional Auer Rods were present in some blast cells.

MYELOGRAM			
Neutrophils	- 11	Blast Cells	- 15
Band Cells	- 2	Eosinophils	- 2
Metamyelocytes	- 6	Lymphocytes	- 6
Myelocytes	- 5	Plasma Cells	- 0
Promyelocytes	- 7	Monocytes	- 5
Iron(Fe)	2/6	Normoblasts	- 41

4.2.17 BONE MARROW BIOPSY

8mm biopsy, good quality, with 90% hypercellularity and decreased megaloblastic erythropoiesis. Megakaryocytes were decreased in number with dysplastic features. Granulopoiesis was hyperplastic with gross maturational delay. There were larger areas showing abnormal localisation of immature precursors (ALIP), which were becoming confluent. Bony trabeculae and reticulin were normal.

4.2.18 DIAGNOSIS

MDS (RAEB FAB Subtype), probably secondary to chronic benzene exposure in the printing trade (5 year occupational exposure history).

4.2.19 mtDNA Length Measurements - Patient C

Table 21

pBR322 Measured Length (cm)	Conversion (0.888)	Mean pBR322 Length
1.60	1.42	1.47
1.58	1.40	
1.49	1.32	
2.06	1.82	
1.58	1.40	

Table 22

mtDNA Length (cm)	Conversion (0.888)	Mean Length Value mtDNA (% 1.47)	Average Mean Length mtDNA
7.98	7.08	4.81	4.26
6.95	6.17	4.19	
10.30	9.14	6.21	
8.25	7.32	4.97	
4.77	4.23	2.87	
4.39	3.89	2.64	
4.88	4.33	2.94	
7.13	6.33	4.30	
7.10	6.30	4.28	
10.50	9.32	6.34	
15.85	14.07	9.57	
6.05	5.37	4.73	
4.33	3.84	2.61	
4.25	3.77	2.56	
4.37	3.88	2.63	
4.20	3.72	2.53	

Table 23

pBR322 Measured Length (cm)	Conversion	Mean pBR322 Length
1.68	1.49	1.64
1.61	1.42	
2.23	1.98	
2.11	1.87	
2.36	2.09	
1.45	1.28	
1.53	1.35	

Table 24

mtDNA Length (cm)	Conversion	Mean Length Value mtDNA (% 1.64)	Average Mean Length mtDNA
4.33	3.84	2.34	3.99
4.25	3.77	2.29	
4.37	3.88	2.36	
7.13	6.33	3.85	
7.10	6.30	3.84	
10.50	9.32	5.68	
6.05	5.37	3.27	
10.95	9.72	5.92	
8.23	7.30	4.45	
6.08	5.39	3.28	
7.04	6.25	3.81	
12.58	11.17	6.81	
7.38	6.55	3.99	

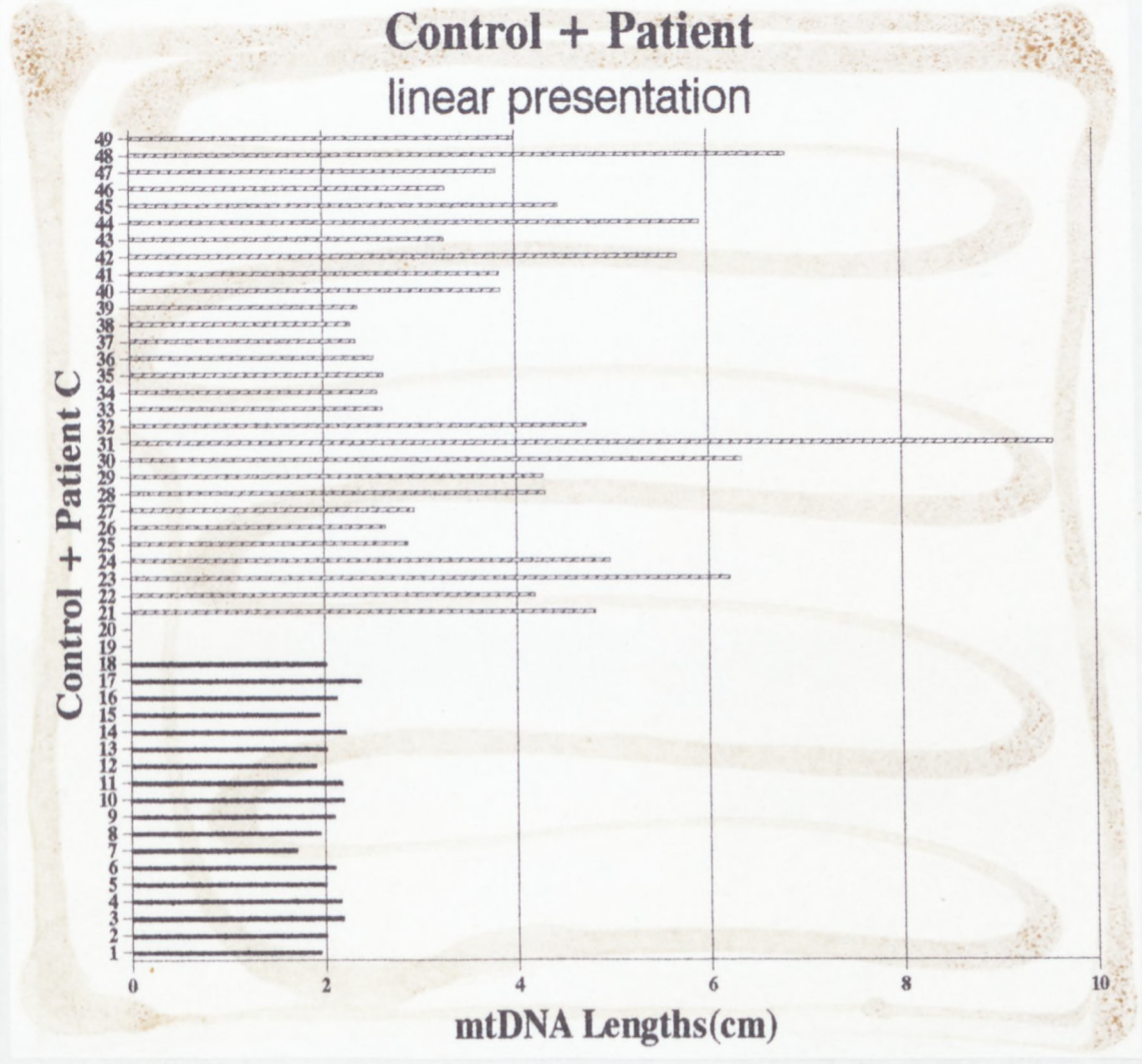

Figure 12

Linear presentation of normal (Control 1 & 2) mtDNA Mean Length Values and Patient C mtDNA

4.2.20 Patient D: 74 year white male

4.2.21 Peripheral smear

Pancytopenia with anisocytosis, ovalocytes, left shifted granulocytes to the myelocyte stage.

MYELOGRAM			
Neutrophils	- 29	Blast Cells	- 1
Band Cells	- 6	Eosinophils	- 2
Metamyelocytes	- 16	Lymphocytes	- 6
Myelocytes	- 23	Plasma Cells	- 1
Promyelocytes	- 5	Monocytes	- 16
Iron(Fe)	4/6	Normoblasts	- 3

4.2.22 BONE MARROW BIOPSY

16mm biopsy, good quality with 90% hypercellularity and severely suppressed megaloblastic erythropoiesis. Megakaryocytes were severely decreased with ++ dysplastic features. There was significant expansion of the myeloid compartment with delayed maturation and abnormal localisation of immature precursors (ALIP). Monocytoid cells,(alpha naftol acetate esterase positive), comprised 16% of the bone marrow cell population. Bony trabeculae were osteopenic and reticulin was slightly increased.

4.2.23 DIAGNOSIS

Five months previously, the patient was diagnosed with MDS (RAEB FAB Subtype). The marrow picture at this stage was consistent with MDS in a transitional state into chronic myelomonocytic

leukaemia (CMML).

4.2.24 mtDNA LENGTH MEASUREMENTS - PATIENT D

Table 25

pBR322 Measured Length (cm)	Conversion (0.888)	Mean pBR322 Length
2.10	1.86	1.74
2.15	1.90	
2.15	1.90	
1.30	1.15	
1.47	1.30	
2.25	1.99	
2.40	2.13	
1.92	1.70	

Table 26

mtDNA Length (cm)	Conversion (0.888)	Mean Length Value mtDNA (% 1.74)	average Mean Length mtDNA
3.60	3.20	1.83	1.78
4.25	3.78	2.17	
2.40	2.13	1.23	
2.65	2.35	1.74	
2.50	2.22	1.27	
2.40	2.13	1.22	
3.55	3.15	1.81	
3.75	3.33	1.91	
4.15	3.69	2.12	
4.85	4.31	2.48	

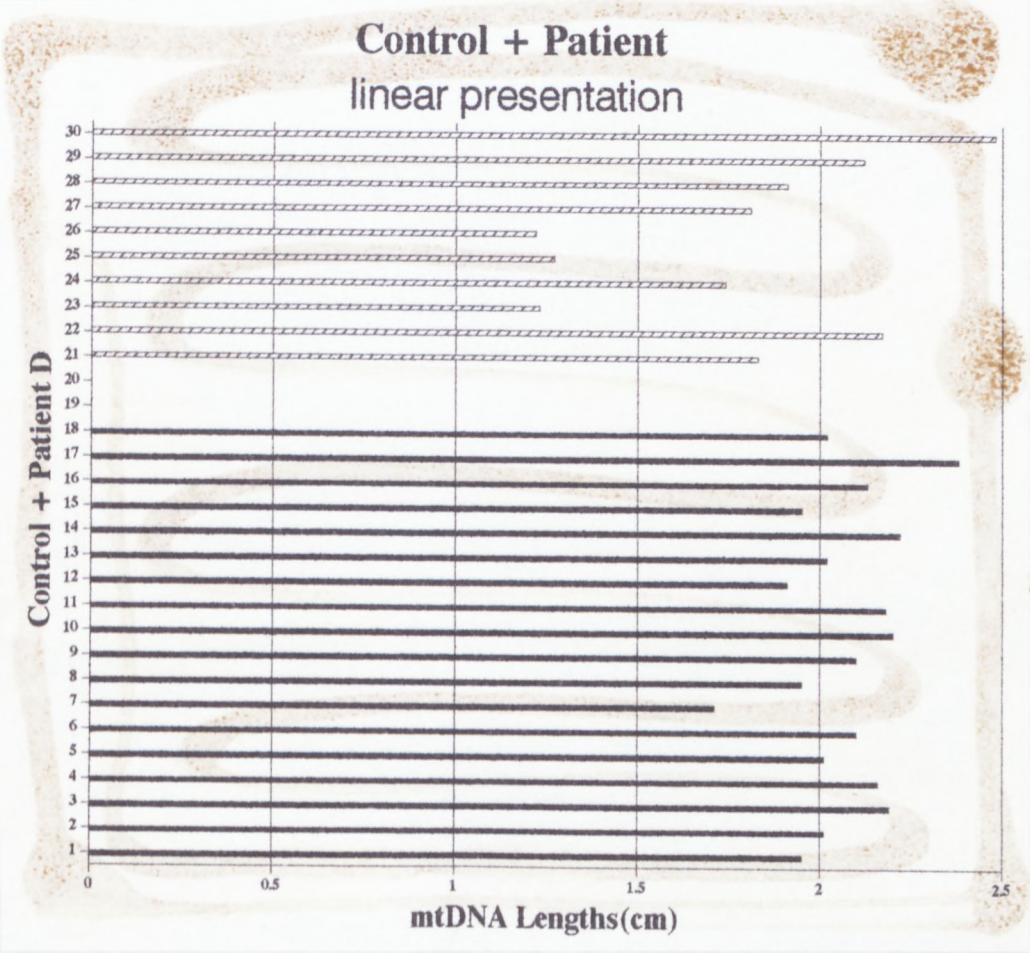

Figure 13

Linear presentation of normal (Control 1 & 2) mtDNA length values and patient D mtDNA

4.2.25 Patient E: 79 year white male

4.2.26 Peripheral smear

Pancytopenia with anisopoikilocytosis, left shifted granulocytes, decreased platelets.

MYELOGRAM			
Neutrophils	- 7	Blast Cells	- 7
Band Cells	- 3	Eosinophils	- 1
Metamyelocytes	- 5	Lymphocytes	- 6
Myelocytes	- 7	Plasma Cells	- 0
Promyelocytes	- 8	Monocytes	- 1
Iron(Fe)	Trace	Normoblasts	- 55

4.2.27 BONE MARROW BIOPSY

9mm biopsy, good quality showing 80-85% hypercellularity with megaloblastic erythropoiesis. Megakaryocytes were decreased in number from the previous biopsy taken 3 weeks previously and ++ dysplasia was found. The granulopoietic compartment was over expanded with maturational delay and ALIP. Blast cells still comprised 7-11% of the bone marrow cell population. Reticulin was (+) increased.

4.2.28 DIAGNOSIS

The picture was consistent with MDS (RAEB FAB Subtype).

4.2.29 mtDNA Length Measurements - Patient E

Table 27

pBR322 Measured Length (cm)	Conversion (0.888)	Mean pBR322 Length
1.55	1.37	1.57
1.50	1.33	
2.0	1.77	
1.65	1.46	
1.90	1.68	
2.30	2.04	
1.85	1.64	
1.65	1.46	
2.0	1.77	
1.35	1.19	

Table 28

mtDNA Length (cm)	Conversion	Mean Length Value mtDNA (% 1.57)	Average Mean Length mtDNA
4.3	3.81	2.42	3.83
4.5	3.99	2.55	
4.85	4.30	2.73	
4.44	3.94	2.50	
4.63	4.11	2.61	
3.75	3.33	2.12	
2.73	2.44	1.55	
8.12	7.21	4.59	
7.78	6.90	4.39	
8.86	7.86	5.0	
9.32	8.27	5.26	
10.31	9.15	5.82	
7.34	6.51	4.14	
7.59	6.65	4.23	
6.0	5.34	3.40	
9.6	8.54	5.43	
11.3	10.05	6.40	

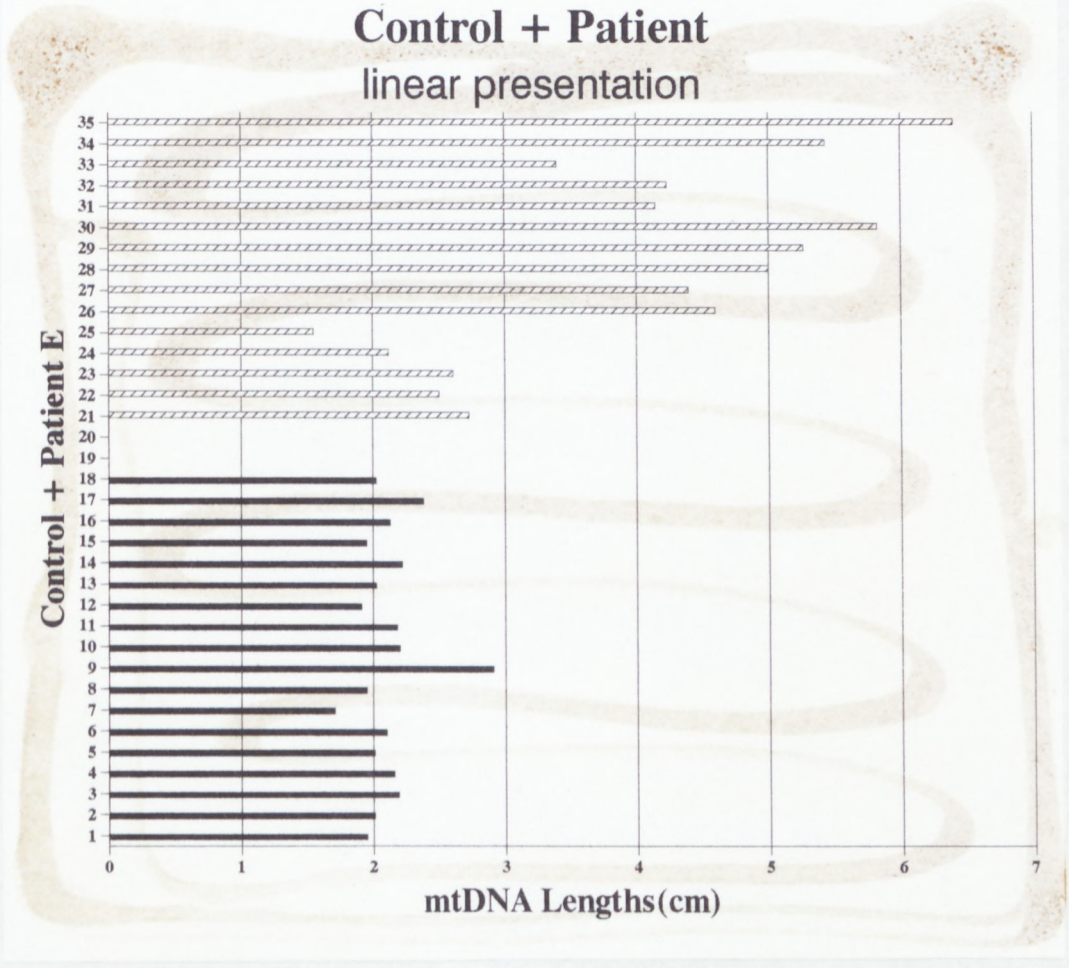

Figure 14

Linear presentation of normal (Control 1 & 2) mtDNA Mean Length values and Patient E mtDNA

4.2.30 Patient F: 65 year white female

4.2.31 Peripheral smear

Pancytopenia with anisopoikilocytosis, round macrocytes, severe neutropenia with occasional circulating blast cells.

MYELOGRAM			
Neutrophils	- 2	Blast Cells	-6/8
Band Cells	- 3	Eosinophils	- 2
Metamyelocytes	- 8	Lymphocytes	- 10
Myelocytes	- 7	Plasma Cells	- 4
Promyelocytes	- 6	Monocytes	- 1
Iron(Fe)	6/6	Normoblasts	- 51

4.2.32 BONE MARROW BIOPSY

9mm biopsy, good quality showing 80% hypercellularity and decreased megaloblastic erythropoiesis. Megakaryocytes were decreased and there was an increase in the number of denuded apoptotic forms. The granulopoietic compartment was over expanded with maturational delay and ALIP. Bony trabeculae were osteopenic and reticulin was (+) increased.

4.2.33 DIAGNOSIS

The picture was consistent with MDS (RAEB FAB Subtype).

4.2.34 mtDNA Length Measurements - Patient F

Table 29

pBR322 Measured Length (cm)	Conversion (0.888)	Mean pBR322 Length
1.80	1.59	1.37
1.30	1.15	
1.37	1.21	
1.53	1.35	
1.47	1.30	
1.30	1.15	
2.13	1.89	
1.61	1.42	
1.53	1.35	

Table 30

mtDNA Length (cm)	Conversion (0.888)	Mean Length Value mtDNA (% 1.37)	Average Mean Length mtDNA
2.3	2.22	1.62	3.09
2.4	2.13	1.55	
2.1	1.86	1.35	
2.30	2.04	1.48	
3.90	3.47	2.53	
4.30	3.82	2.78	
4.75	4.22	3.0	
3.20	2.84	2.07	
5.6	4.98	3.63	
5.10	4.53	3.30	
15.90	14.15	10.32	
5.05	4.49	3.27	
4.60	4.09	2.98	
4.55	4.04	3.32	

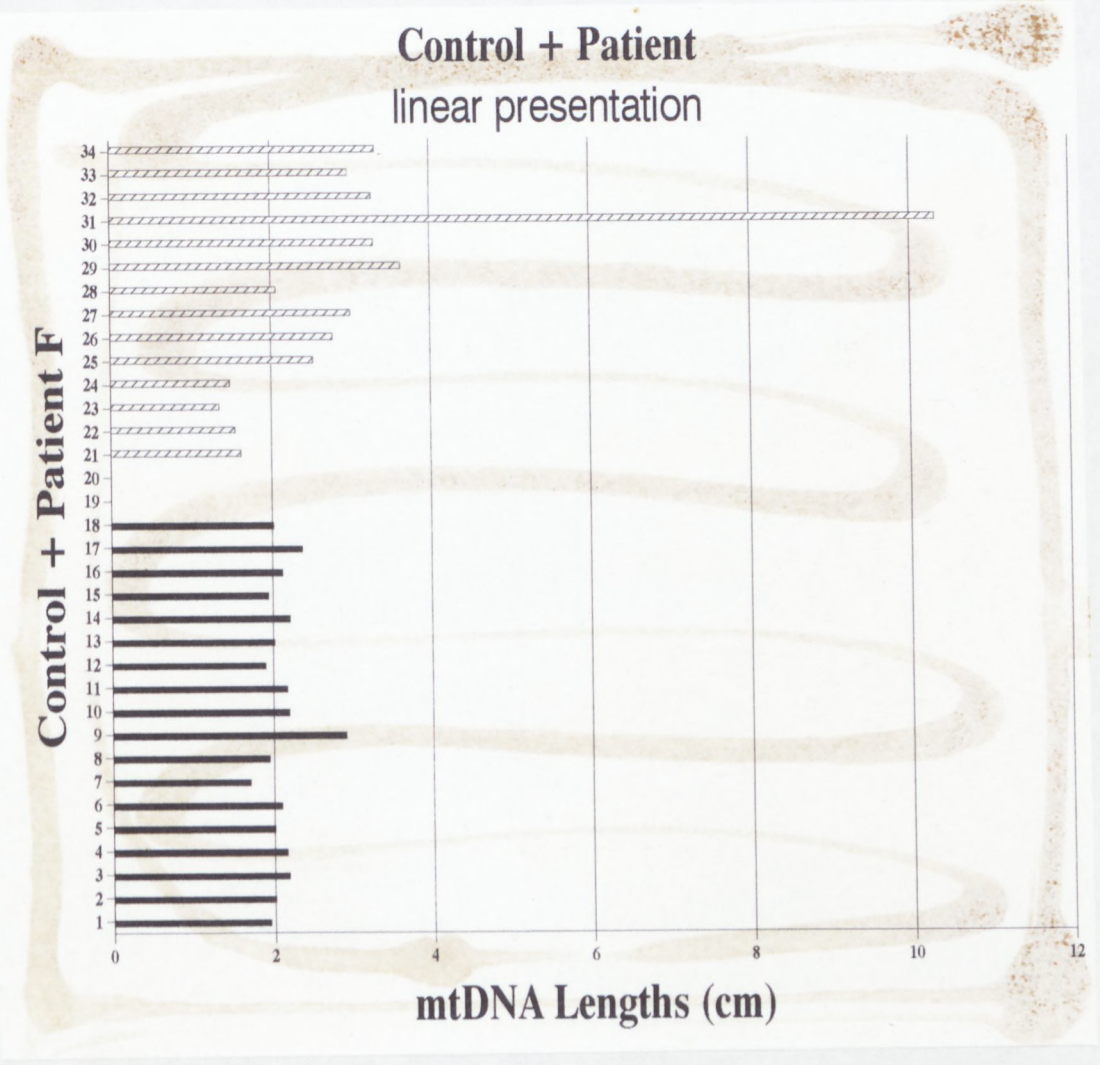

Figure 15

Linear presentation of normal (Control 1 & 2) mtDNA Mean Length value and Patient F mtDNA.

SUMMARY OF MDS PATIENT DATA

Table 31

Patient	Diagnosis	Myelogram
A	CMML transition into AML	50 Blasts 18 Monocytes 12 Myeloid 7 Erythroid
B	RAEB(secondary MDS) Radiation exposure	11 Blasts 28 Myeloid 28 Neutrophils 19 Erythroid
C	RAEB(secondary MDS) Benzene exposure	1 Blast 16 Mono 28 Myeloid 3 Erythroid
D	MDS transition of RAEB into CMML	6 Blasts 13 Myeloid 51 Erythroid
E	Early RAEB	7 Blasts 23 Myeloid 55 Erythroid
F	RA with very early RAEB	8 Blasts 26 Myeloid 31 Erythroid

Patients A and D were considered to be late MDS.

Patients B and C were considered to have firmly established MDS.

Patients E and F were considered to have very early MDS with good histological marrow reserve.

4.3 CONTROLLED RADIATION STUDIES ON INDUCED mtDNA HETEROPLASMY IN AN ANIMAL MODEL OF HUMAN MDS

4.3.0 INTRODUCTION

Human MDS preceding acute radiation-induced leukaemia, is characterised by haematopoietic insufficiency and a time lag for leukaemic transformation. During this time lag, death may occur from infection, anaemia or bleeding, before the manifestation of leukaemia. Thus, it has been difficult to clarify the pathogenic relationship between secondary MDS and leukaemia. Abnormal mtDNA heteroplasmy has been found to be a consistent feature of our studies in 100% of 6 patients with established MDS. Therefore, supportive examination of abnormal mtDNA length changes in an animal model of secondary MDS seemed highly desirable. Both Beagle dogs and rats exposed to adequate levels of ionising radiation, have been found to develop a clinical picture similar to human secondary MDS.(Seed T.M. *et al*,1987). Modification of the total radiation dose, fractionated dose intervals, or number of fractions, all showed effects on the incidence of induced leukaemia(Seed T.M. *et al*,1981;Ruutu T.,1986). In parallel to the situation in human MDS, these animals might have died from causes related to haematopoietic insufficiency before leukaemic transformation. Wistar rats demonstrate mtDNA which is equivocable in both molecular size and genomic structure, to human mtDNA - see Figure 16 which illustrates the genetic and transcription map of rat mtDNA

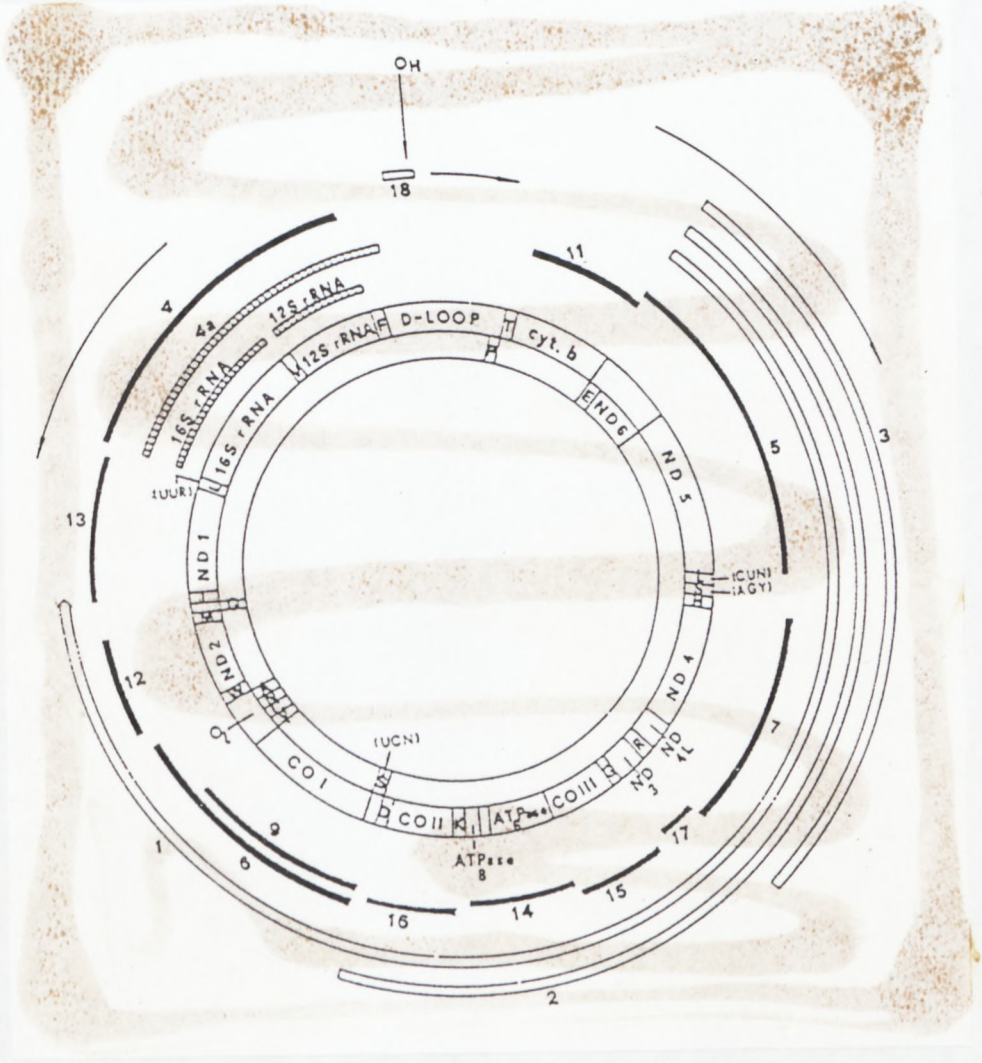

Figure 16

Genetic and transcription map of rat mtDNA. The two inner circles show the positions of the rRNA genes of the protein coding genes, and of tRNA genes as derived from mtDNA sequence. In some cases (mainly in human mtDNA) the positions of rRNA and mRNA genes have also been confirmed by mapping and RNA sequencing experiments of the mitochondrial transcripts.

Controlled rat experiments were performed to determine if gamma irradiation could induce abnormal mtDNA heteroplasmy development, and to determine if this was equatable with the very early stages of rat MDS induction, when defined by the FAB criteria used for humans. Rats were also irradiated with mixed quality radiation containing a significant neutron component, in an attempt to crudely simulate one aspect of the space radiation environment.

In this study, rats were exposed to either 3 periodic doses of 3,0 Gy of γ-radiation, or to 3 doses of mixed 1,98 Gy dose gamma radiation/0,90 Gy dose of p(66)/Be neutrons. Blood counts and marrow biopsies were sequentially performed on control and irradiated rat populations. Haematopoietic mtDNA lengths were measured by electron microscopy (see Fig.18) and equated to the bone marrow findings, using the FAB classification system for MDS and myeloproliferative/leukaemia disease.

4.3.1 EXPERIMENTAL RESULTS OF A NORMAL RAT POPULATION

4.3.2 PERIPHERAL BLOOD COUNTS - Normal Control Rat Haematology

RAT	1	2	3	4	5	6	UNITS
WBC	5.07	4.17	5.75	5.65	3.21	3.49	$x10^3/\mu L$
RBC	6.24	6.14	6.29	6.73	6.25	6.72	$x10^6/\mu L$
HGB	12.6	12.8	12.6	13.6	11.5	13.8	g/dL
HCT	37.5	38.2	39.1	40.4	38.7	41.3	%
MCV	60.1	62.2	62.2	60.0	61.8	61.5	fl
MCH	20.2	20.8	20.0	20.2	18.5	20.5	pg
MCHC	33.6	33.4	32.1	33.6	29.8	33.4	g/dL
PLT	778	800	827	721	779	744	$x10^3/\mu L$

4.3.3 BONE MARROW - CONTROL RATS

MYELOGRAM (Average)			
Neutrophils	- 38	Monocytes	- 3
Myelocytes	- 3	Mast Cells	- 3
Promyelocytes	- 2	Lymphocytes	- 6
Blast Cells	- 1	Plasma Cells	- 4
Iron(Fe)	3/6	Erythroid Cells	- 42

4.3.4 BONE MARROW BIOPSY

80% normocellular marrow with normoblastic erythropoiesis comprising 25-40% of the marrow cell population was observed. Megakaryocytes were normal in number, location and morphology. Granulopoiesis was ± 50% of the marrow cell population with full maturation and differentiation (See Figure 17).

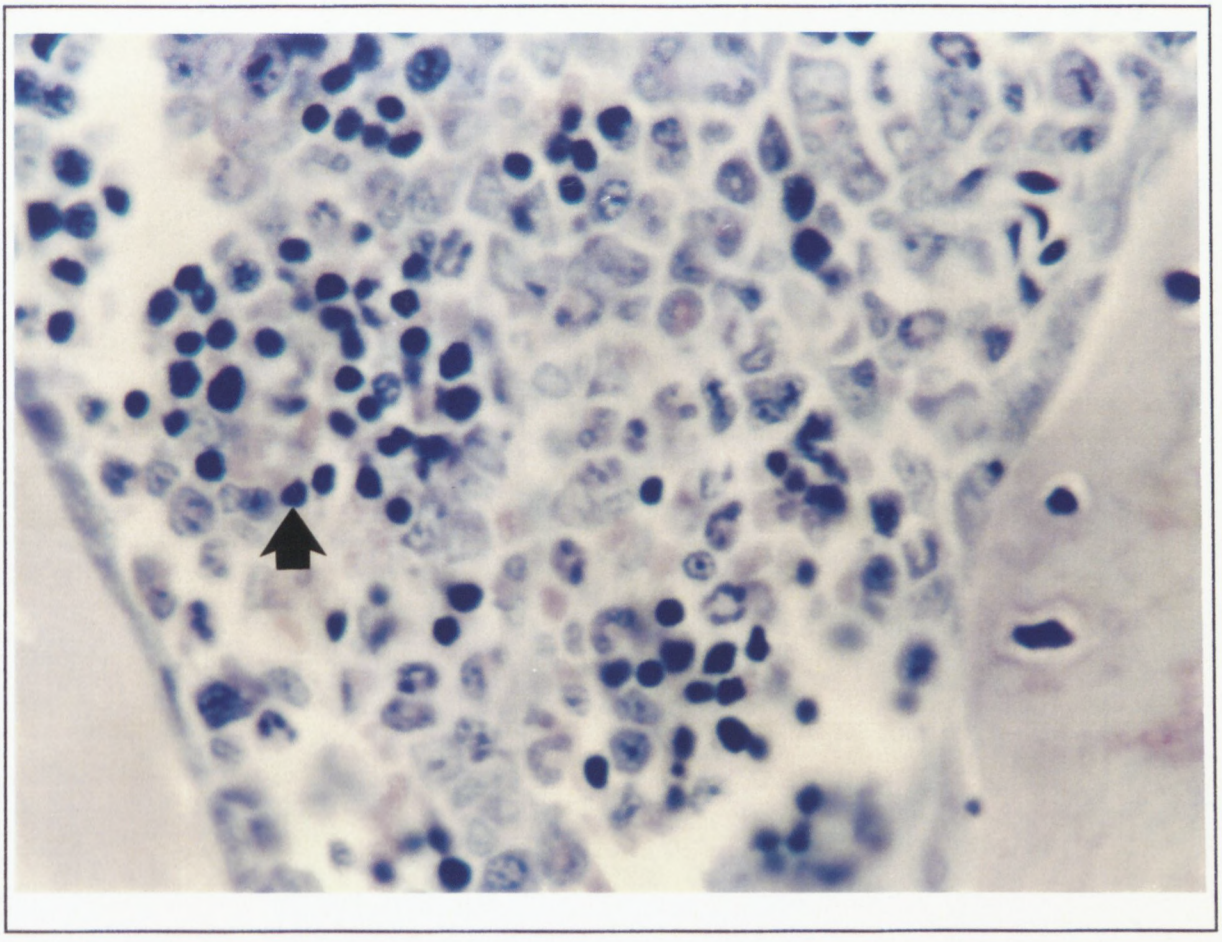

Figure 17

Normal erythron unit, see arrow

4.3.5 DIAGNOSIS

Normocellular marrow with normal haematopoiesis.

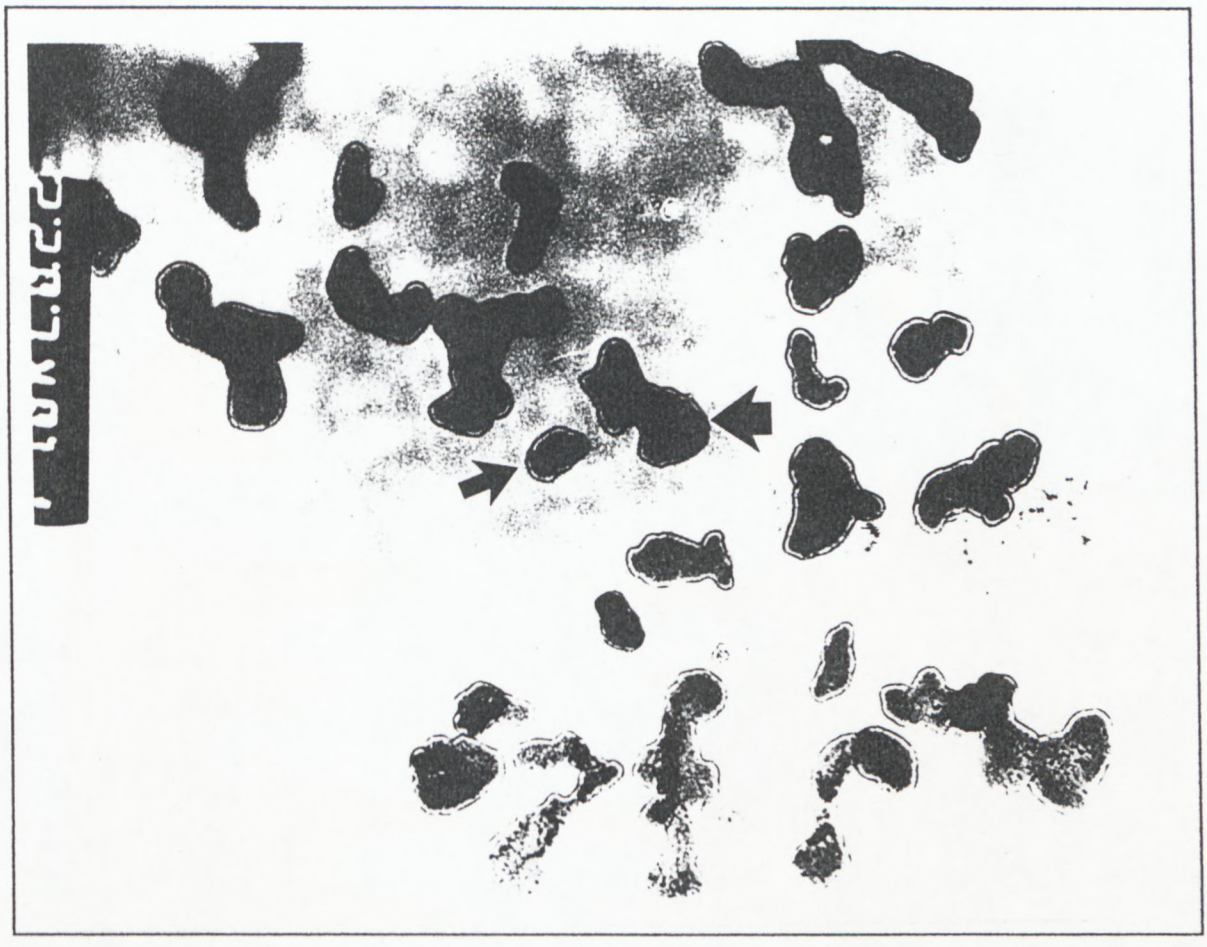

Figure 18

Photomicrograph of normal rat mtDNA with pBR322 internal length standard.

Circular DNA strands measured with small arrow showing pBR322 and big arrow showing normal rat mtDNA.

4.3.6 mtDNA LENGTH MEASUREMENTS - NORMAL RAT POPULATION

Average pBR322 Length 1.21

Table 32

Rat mtDNA Length (cm)	Conversion (factor 0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length Value mtDNA
2.0	1.78	1.47	1.78
2.8	2.49	2.05	
2.3	2.04	1.68	
2.1	1.86	1.53	
2.5	2.22	1.83	
2.6	2.31	1.90	
2.8	2.49	2.05	
2.3	2.04	1.68	
2.7	2.40	1.98	
2.8	2.49	2.05	
1.9	1.69	1.39	
2.2	1.95	1.61	
2.8	2.49	2.05	
1.9	1.69	1.39	
2.0	1.78	1.47	
2.9	2.58	2.13	
3.1	2.75	2.27	
2.8	2.49	1.61	

Table 33

Rat mtDNA Length (cm)	Conversion (factor 0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length Value mtDNA
2.0	1.78	1.47	2.14
2.3	2.04	1.68	
2.8	2.49	2.05	
3.0	2.67	2.20	
3.1	2.75	2.27	
3.4	3.0	2.47	
3.5	3.11	2.48	
2.4	2.13	2.57	
2.9	2.58	1.76	
3.1	2.75	2.13	
3.2	2.84	2.27	

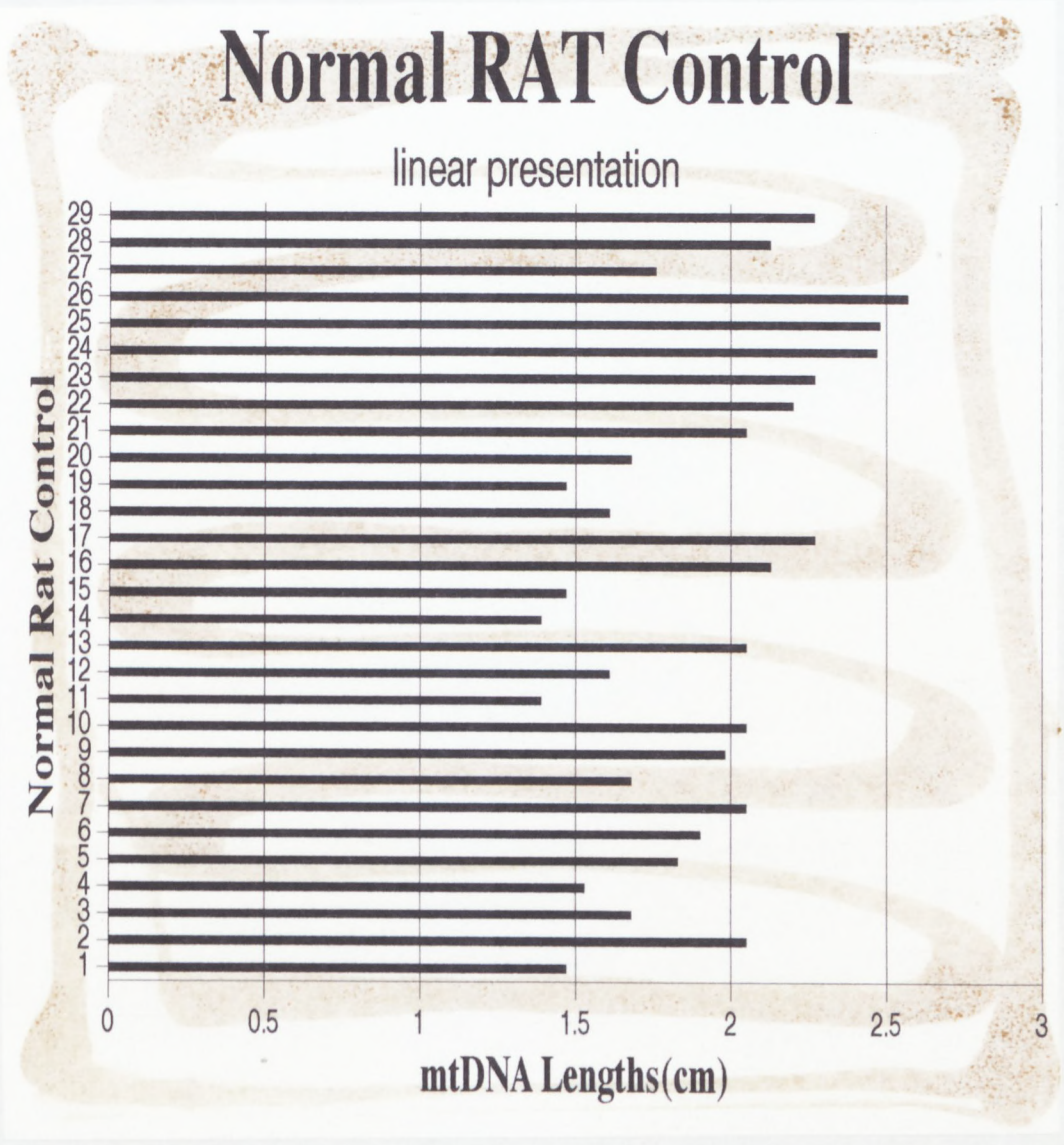

Figure 19

Control normal rat mtDNA lengths: linear presentation

4.4 EXPERIMENTAL RESULTS - GAMMA IRRADIATED RAT POPULATION

Procedure	Rat
2 weeks post-irradiation with 400 rad X-rays	G-1

4.4.1 PERIPHERAL COUNTS - RAT G-1

RAT-G1	PARAMETERS	UNITS
WBC	3.14	$x10^3/\mu L$
RBC	4.89	$x10^6/\mu L$
HGB	10.5	g/dL
HCT	31.5	%
MCV	64.3	fL
MCH	21.6	pg
MCHC	33.5	g/dL
PLT	145	$x10^3/\mu L$

4.4.2 PERIPHERAL SMEAR

Anisocytosis with neutropenia, normochromic anaemia, atypical lymphocytes and low platelet count was seen on the smear.

4.4.3 BONE MARROW SAMPLES RAT G-1

MYELOGRAM

Neutrophils	- 30	Monocytes	- 3
Myelocytes	- 5	Mast cells	- 4
Promyelocytes	- 5	Eosinophils	- 2
Blast Cells	- 2	Lymphocytes	- 10
Iron(Fe)	3/6	Plasma Cells	- 0
		Erythroid Cells	- 29

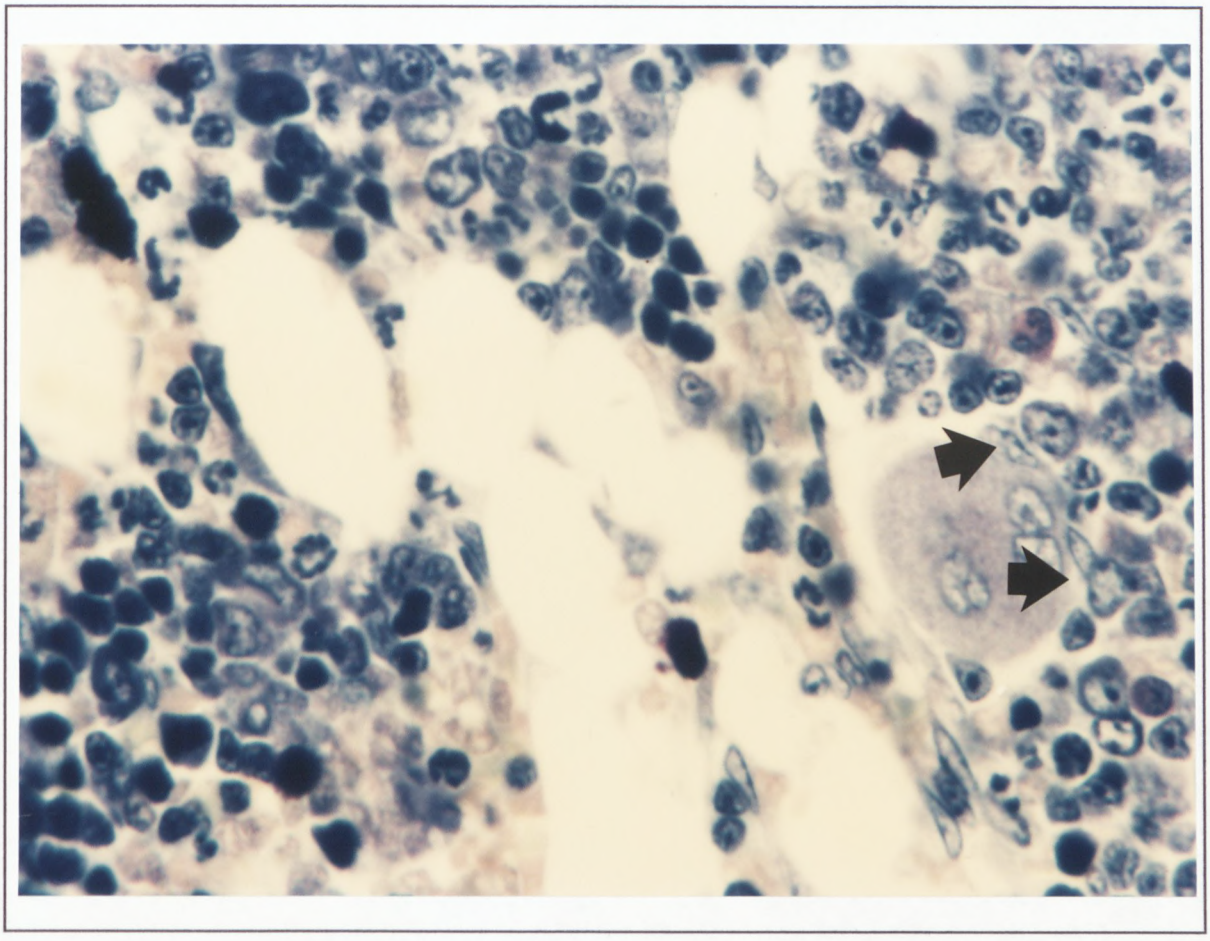

Figure 20

BONE MARROW BIOPSY

75% low normocellular marrow with suppressed normoblastic erythropoiesis comprising 15-29% of the marrow cell population was observed. Megakaryocytes were normal in number, location and morphology. Granulopoiesis comprised 45-55% of the marrow cell population and slight maturation delay was evident (Arrow).

4.4.4 DIAGNOSIS

Low normocellular marrow with suppressed erythropoiesis and slight maturational delay of the myeloid series.

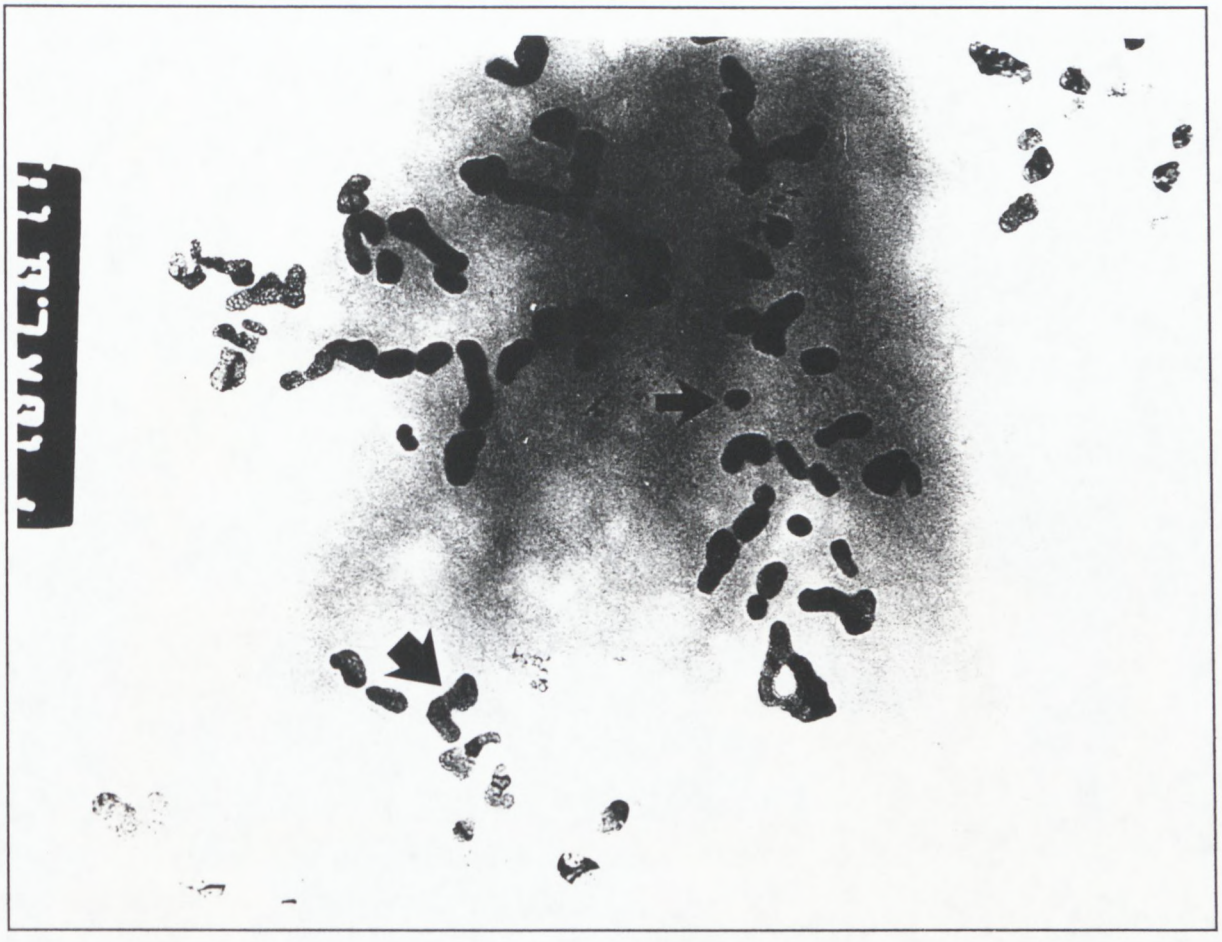

Figure 21

Photomicrograph of Rat G-1 mtDNA with pBR322 plasmid as an internal standard.

DNA strands isolated from mononuclear rat blood cells.

Small arrow show plasmid pBR322 and big arrow show rat mtDNA strands.

4.4.5 mtDNA LENGTH MEASUREMENTS - RAT G-1 mtDNA

Table 34

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length
2.3	2.04	1.68	1.93
2.4	2.13	1.76	
2.4	2.13	1.76	
2.6	2.30	1.90	
3.1	2.75	2.27	
2.9	2.58	2.13	
2.2	1.95	1.61	
2.5	1.86	1.53	
2.7	2.40	1.98	
3.0	2.67	2.20	
2.3	2.04	1.68	
3.2	2.84	2.34	
3.5	3.11	2.48	
2.3	2.04	1.68	
2.8	2.49	2.05	
2.6	2.31	1.90	

Table 35

Rat mtDNA Length (cm)	Conversion	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length Value
3.0	2.67	2.20	2.0
2.9	2.58	2.13	
2.7	2.39	1.97	
2.5	2.22	1.83	
2.4	2.13	1.76	
2.6	2.3	1.90	
3.1	2.75	2.27	

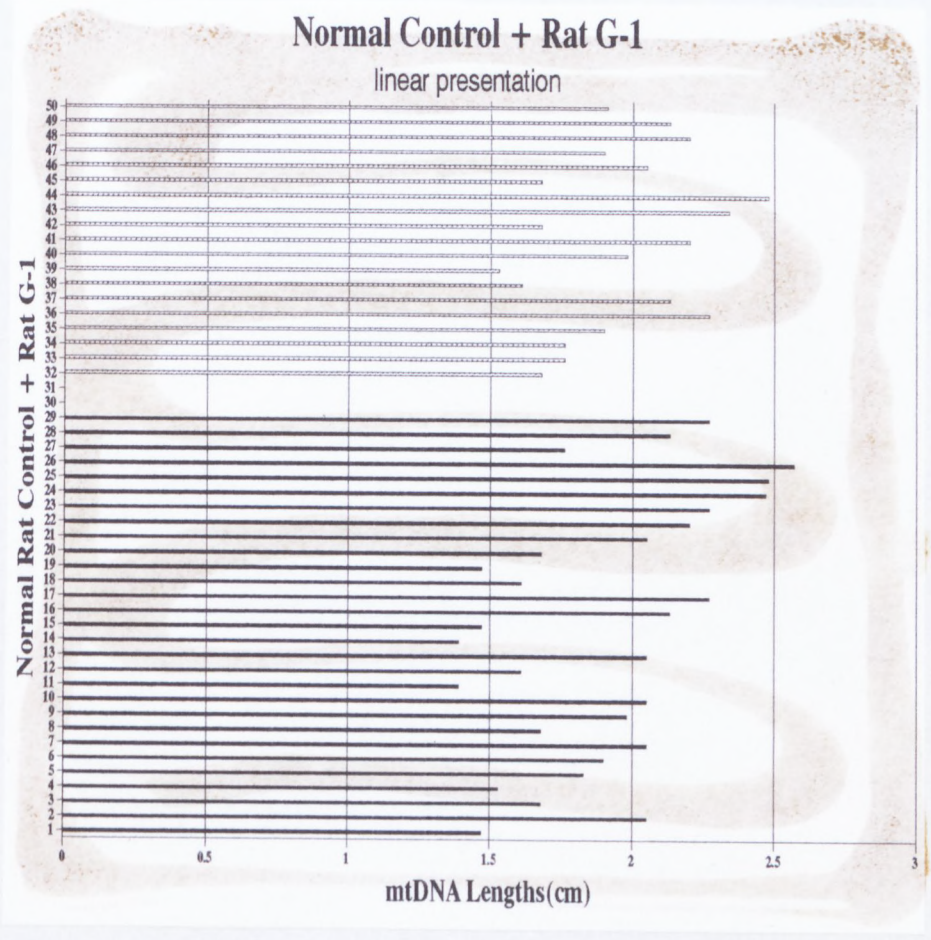

Figure 22

RAT mtDNA MEAN LENGTH VALUES - RAT G-1

Linear presentation of mtDNA lengths measured

4.4.6 RATS G-2; G-3

Procedure	Rat
2 Weeks post-irradiation with #2 400 rad dose X-rays	G-2 G-3

4.4.7 PERIPHERAL COUNTS

RAT	G-2	G-3	UNITS
WBC	1.08	0.91	$x10^3/\mu L$
RBC	5.96	3.79	$x10^6/\mu L$
HGB	13.4	7.7	g/dL
HCT	40.1	25.4	%
MCV	67.3	67.2	fL
MCH	22.4	20.4	pg
MCHC	33.4	30.3	g/dL
PLT	108	65	$x10^3/\mu L$

4.4.8 PERIPHERAL SMEAR

Severe pancytopenia with anisocytosis, neutropenia, oval macrocytes and decreased platelets were observed.

4.4.9 BONE MARROW SAMPLES RAT G-2

MYELOGRAM

Neutrophils	- 13	Monocytes	- 3
Myelocytes	- 1	Mast Cells	- 7
Promyelocytes	- 4	Eosinophils	- 0
Blast Cells	- 2	Lymphocytes	- 9
Iron(Fe)	4/6	Plasma Cells	
		Erythroid Cells	- 63

4.4.10 BONE MARROW SAMPLES RAT G-3

MYELOGRAM			
Neutrophils	- 5	Monocytes	- 4
Myelocytes	- 5	Mast Cells	- 7
Promyelocytes	- 6	Eosinophils	- 1
Blast Cells	- 3	Lymphocytes	- 12
Iron(Fe)	5/6	Plasma Cells	
		Erythroid Cells	- 57

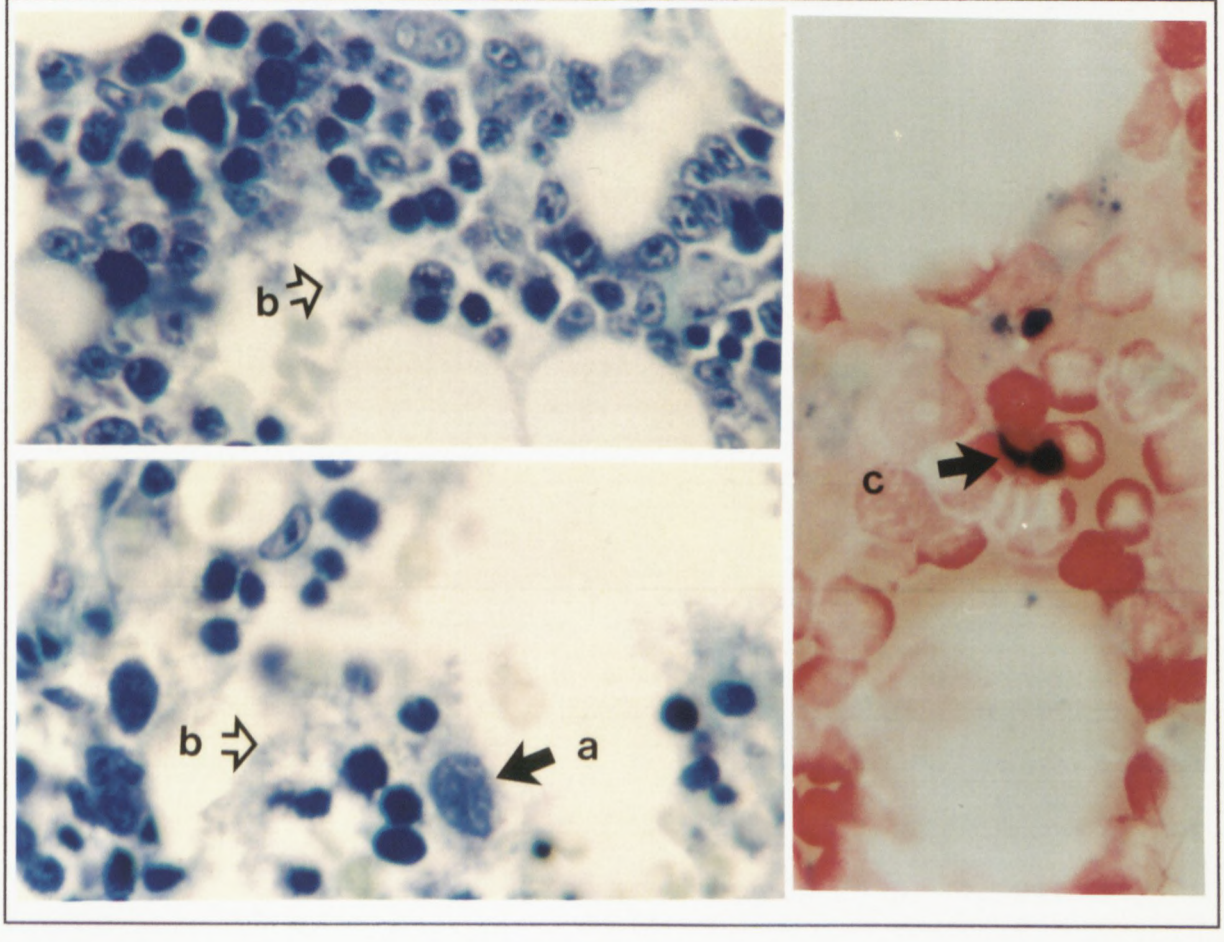

Figure 23

Bone marrow of Rat G-2 and G-3. Arrow A shows a megacaryocyte. Arrow B shows Fibrinous myelitis and cellular debris. Arrow C shows iron in macrophages.

4.4.11 HISTOLOGY

Overall, 30% (G-2) to 50% (G-3) hypocellularity with suppressed megaloblastic erythropoiesis, comprising some 50-60% of marrow cells, was found (Fig. 23). Megakaryocytes were significantly decreased and numerous apoptotic denuded forms were present Granulopoiesis was 8-20% of marrow cells and was left-shifted. Fibrinous myelitis and cellular debris were present in localised areas of the sections. Iron-laden macrophages and pathological sideroblasts were present.

4.4.12 DIAGNOSIS

Hypocellular marrow with fibrinoid myelitis, severe depression of granulopoiesis with evidence of an increased rate of megakaryocyte death in the marrow and altered ferrokinetics. The picture is consistent with hypoplastic injury to the marrow by a cytotoxic agent.

4.4.13 mtDNA LENGTH MEASUREMENTS - RAT G-2, G-3

Table 36

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length Value Rat mtDNA
2.5	1.86	1.53	1.89
2.3	2.04	1.68	
3.1	2.75	2.27	
2.4	2.13	1.76	
2.9	2.58	2.13	
2.0	1.78	1.47	
1.8	1.60	1.32	
2.9	2.58	2.13	
3.0	2.67	2.21	
2.8	2.49	2.06	
3.4	3.03	2.49	
3.1	2.75	2.27	
2.5	2.22	1.83	
2.6	2.31	1.90	
3.2	2.84	2.34	
2.3	2.04	1.68	
2.0	1.78	1.47	
1.7	1.51	1.24	
2.1	1.86	1.53	
3.1	2.75	2.27	
3.0	2.67	2.21	

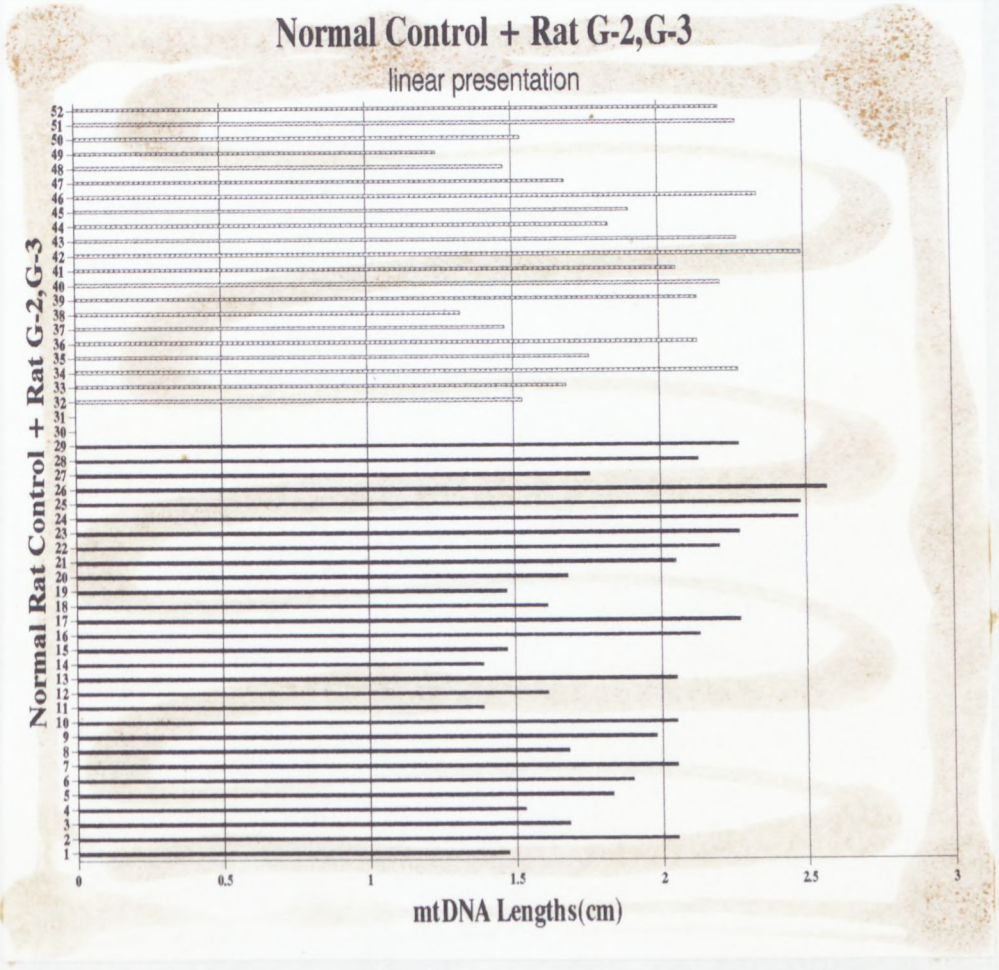

Figure 24

Rat mtDNA mean length values of Rat G-2 and G-3.

Black lines control.

Striped lines Rat G-2, G-3.

4.4.14 RATS G-4; G-5

Procedure	Rat
3 Weeks post-irradiation with #3 400 rad dose X-rays	G-4 G-5

4.4.15 PERIPHERAL COUNTS

RAT	G-4	G-5	UNITS
WBC	1.2	0.9	$x10^3/\mu L$
RBC	4.49	3.21	$x10^6/\mu L$
HGB	11.4	7.9	g/dL
HCT	33.4	23.0	%
MCV	74.3	71.4	fL
MCH	25.4	24.5	pg
MCHC	34.2	34.3	g/dL
PLT	75	33	$x10^3/\mu L$

4.4.16 PERIPHERAL SMEAR

Pancytopenia with anisopoikilocytosis and a sudden increase in polychromatic cells (+++), stomatocytes (+), neutrophil hypersegmentation with hypogranularity, left shift with occasional myelocytes and occasional giant platelets.

4.4.17 BONE MARROW SAMPLE G-4

MYELOGRAM			
Neutrophils	- 7	Monocytes	- 5
Myelocytes	- 0	Mast Cells	- 4
Promyelocytes	- 6	Eosinophils	- 0
Blast Cells	- 5	Lymphocytes	- 10
Iron(Fe)	6/6	Plasma Cells	- 10
		Erythroid Cells	- 55

4.4.18 BONE MARROW SAMPLE G-5

MYELOGRAM			
Neutrophils	- 3	Monocytes	- 5
Myelocytes	- 4	Mast Cells	- 4
Promyelocytes	- 5	Eosinophils	- 0
Blast Cells	- 4	Lymphocytes	- 10
Iron(Fe)	5/6	Plasma Cells	- 10
		Erythroid Cells	- 55

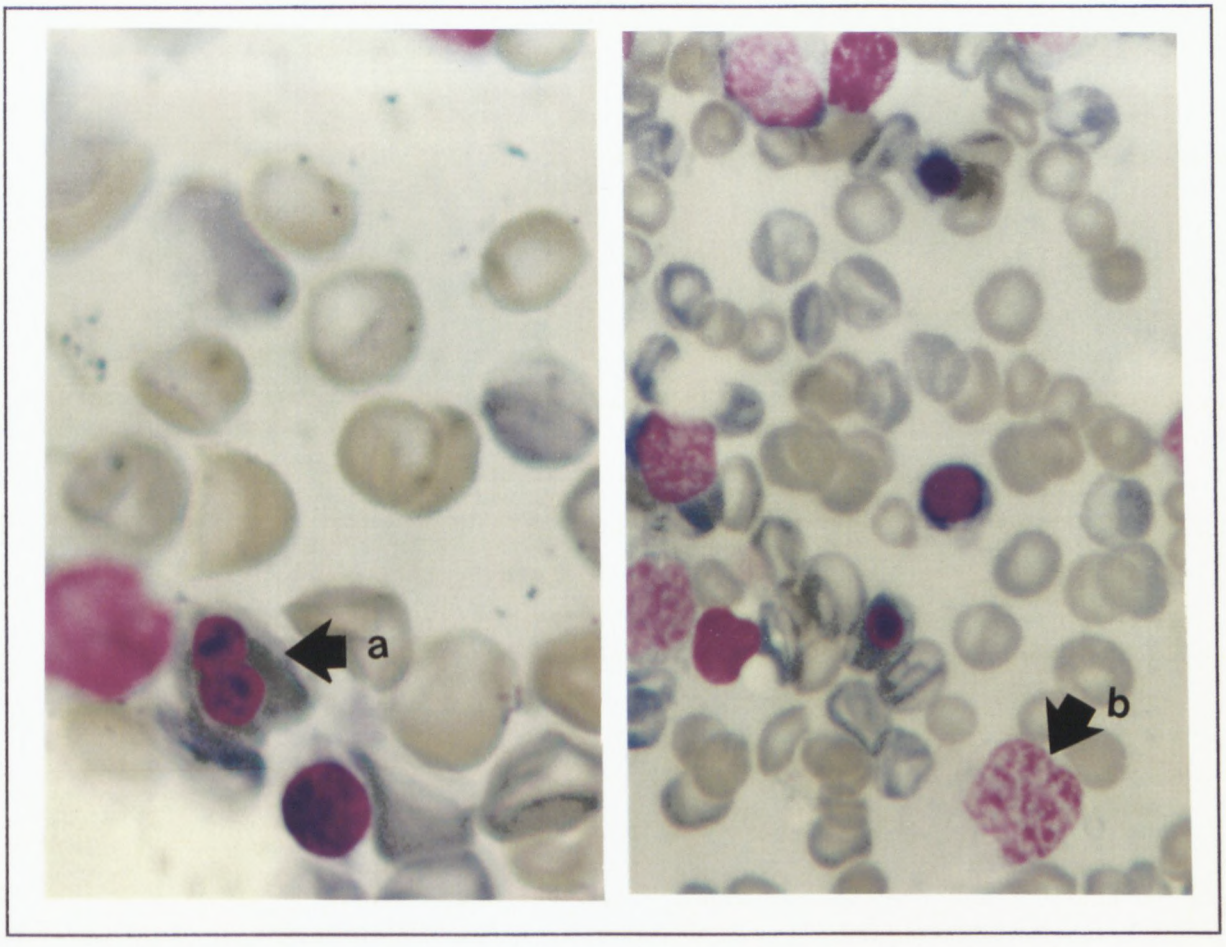

Figure 25

Bone marrow aspirate of Rat G-5

Arrow A shows dyserythropoiesis.

Arrow B show apoptotic forms

4.4.19 BONE MARROW ASPIRATES/IMPRESSIONS

30% hypocellularity with megaloblastic erythropoiesis comprising 35-55% of the marrow cells (Fig. 25). Vacuolated erythroblasts and dyserythropoiesis (Arrow A) is present. Megakaryocytes showed apoptotic denuded forms (Arrow B). Granulopoiesis was severely suppressed with gross maturational delay. Blasts comprised 5% of the marrow cells.

Ferrokinetics were grossly altered with ++++ pathological sideroblasts and occasional ringed sideroblasts.

4.4.20 DIAGNOSIS

Hypocellular marrow with trilineage dysplasia, characterised by hypogranular peripheral neutrophils, myeloid maturational delay, dyserythropoiesis and micromegakaryocytes with grossly altered ferrokinetics. These findings are consistent with an early hypocellular MDS.

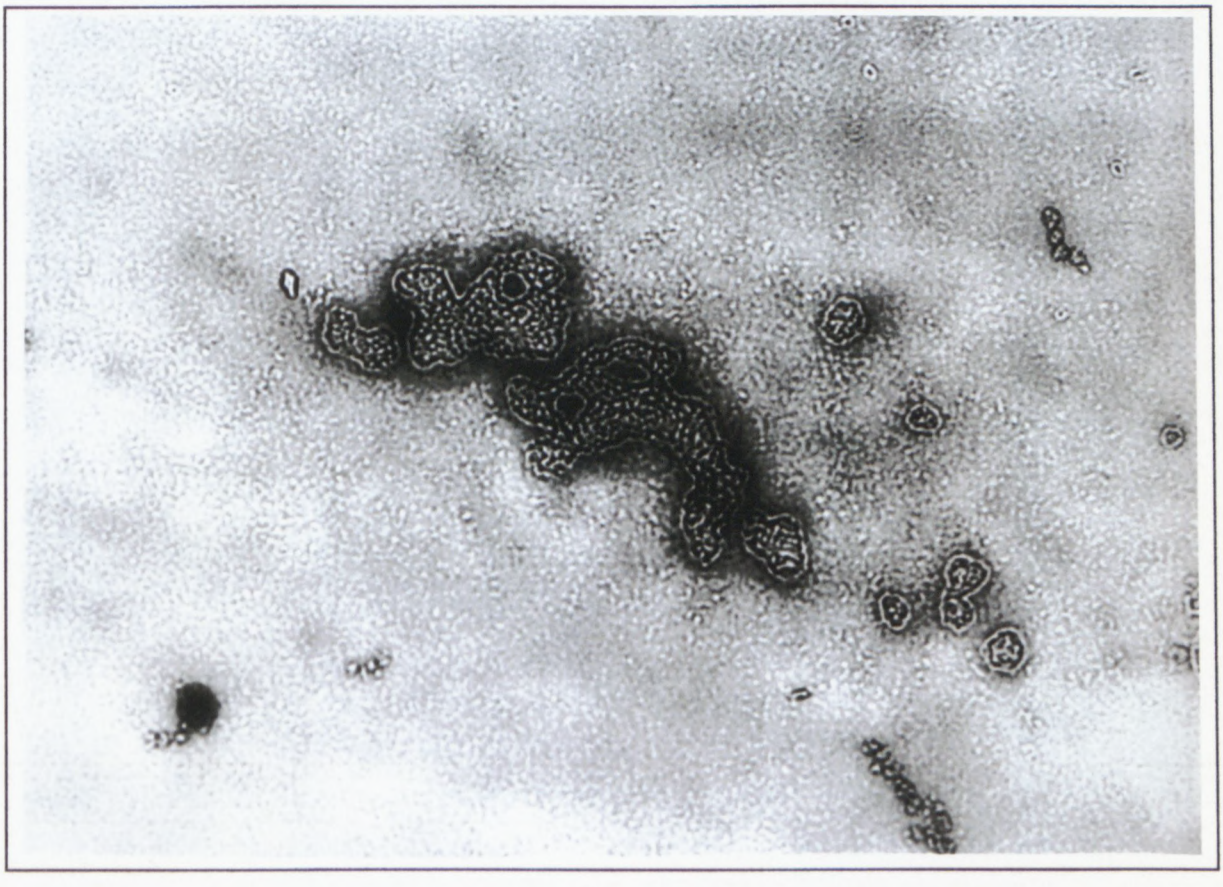

Figure 26

Photomicrograph of Rat G-4 mtDNA

mtDNA strands isolated from mononuclear blood cells

4.4.21 mtDNA LENGTH MEASUREMENTS RAT G-4, RAT G-5

Table 37

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 0.93)	Average Mean Length Value Rat mtDNA
2.0	1.78	1.91	3.37
2.20	1.95	2.09	
1.75	1.55	1.66	
1.50	1.33	1.43	
2.10	1.86	2.0	
1.65	1.46	1.56	
2.20	1.95	2.09	
2.7	2.40	2.58	
2.65	2.35	2.52	
2.35	2.09	2.24	
2.75	2.44	2.62	
10.4	9.25	9.94	
5.10	4.53	4.87	
4.10	3.64	3.91	
4.95	4.40	4.73	
5.4	4.80	5.16	
5.1	4.53	4.87	
4.0	3.56	3.82	
4.25	3.78	4.06	

Table 38

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Tat mtDNA (% 0.93)	Average Mean Length Value Rat mtDNA
2.70	2.4	2.58	3.04
2.6	2.31	2.48	
2.2	1.95	2.09	
2.4	2.13	2.29	
1.75	1.55	1.66	
1.95	1.73	1.86	
2.90	2.58	2.77	
3.70	3.29	3.53	
3.70	3.29	3.53	
4.1	3.64	3.91	
4.0	3.56	3.82	
4.25	3.78	4.06	
3.6	3.20	3.44	
3.55	3.15	3.38	
3.40	3.02	3.24	
3.70	3.29	3.97	

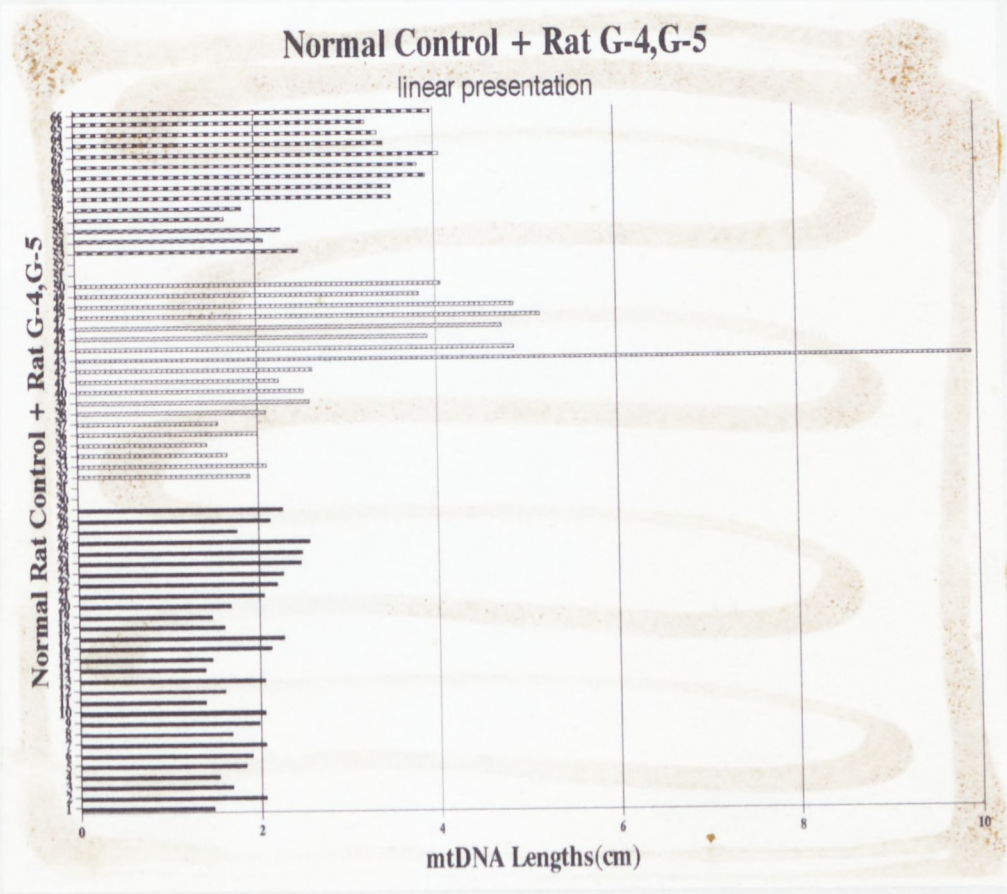

Figure 27

Rat mtDNA mean length values of Rat G-4 and Rat G-5

Linear presentation mtDNA lengths

Black lines are the control

Striped lines are the gamma-irradiated rat mtDNA

4.4.22 RATS G-6; G-7

Procedure	Rat
4 Weeks post-irradiation with #3 400 rad dose X-rays	G-6 G-7

4.4.23 PERIPHERAL COUNTS

RAT	G-6	G-7	UNITS
WBC	1.40	1.34	$x10^3/\mu L$
RBC	2.16	3.07	$x10^6/\mu L$
HGB	5.6	8.0	g/dL
HCT	18.3	25.5	%
MCV	84.6	82.8	fL
MCH	26.7	25.9	pg
MCHC	31.5	31.3	g/dL
PLT	73	230	$x10^3/\mu L$

4.4.24 PERIPHERAL SMEAR

Anisopoikilocytosis with round macrocytes, atypical lymphocytes, neutrophil hypersegmentation with hypogranularity and recovering granulocyte counts were found.

Polychromasia (++) and (++) giant platelets were present.

4.4.25 BONE MARROW RAT G-6

MYELOGRAM			
Neutrophils	- 4	Monocytes	- 8
Myelocytes	- 10	Mast Cells	- 15
Promyelocytes	- 14	Eosinophils	- 2
Blast Cells	- 6	Lymphocytes	- 13
Iron(Fe)	5/6	Plasma Cells	- 5
		Erythroid Cells	- 23

4.4.26 BONE MARROW RAT G-7

MYELOGRAM			
Neutrophils	- 22	Monocytes	- 0
Myelocytes	- 2	Mast Cells	- 8
Promyelocytes	- 8	Eosinophils	- 3
Blast Cells	- 9	Lymphocytes	- 10
Iron(Fe)	5/6	Plasma Cells	- 0
		Erythroid Cells	- 38

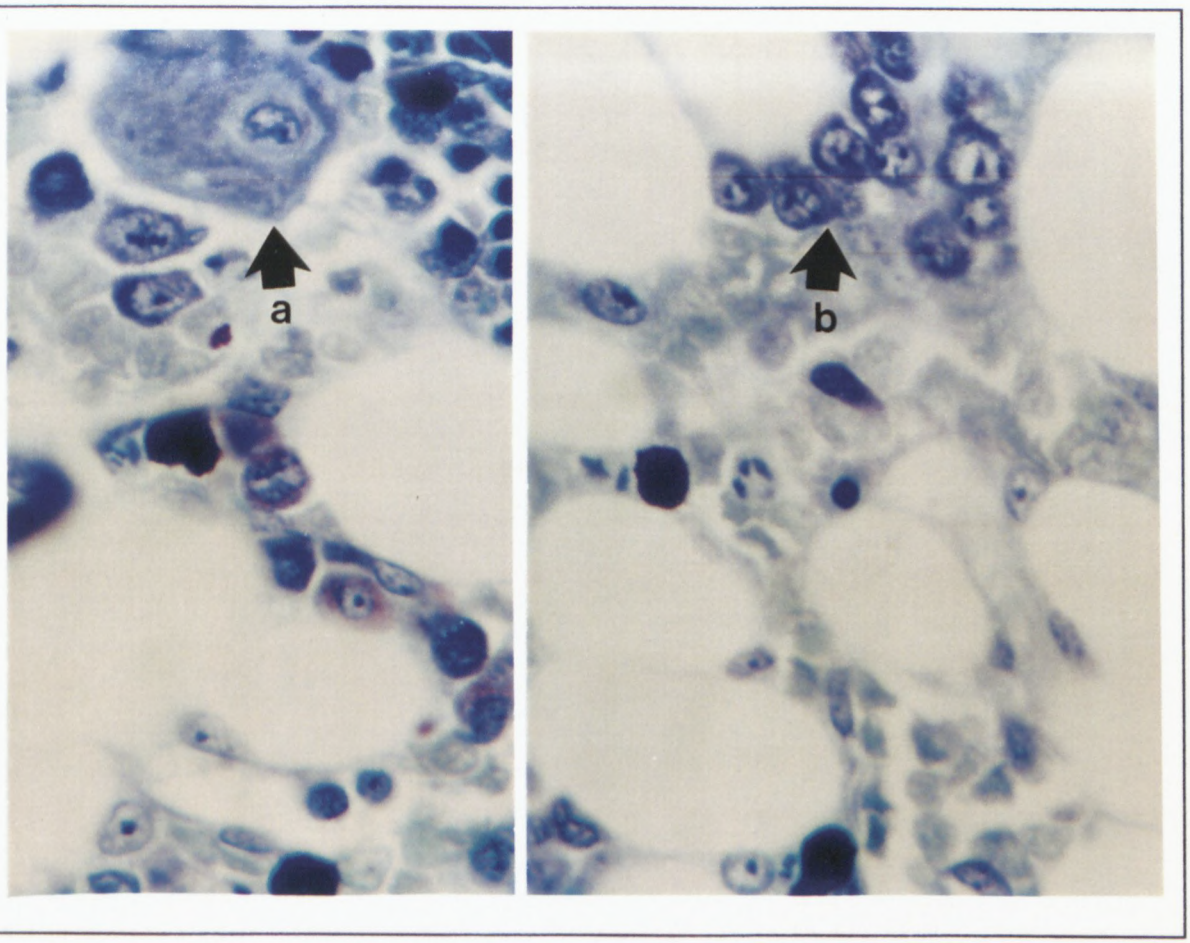

Figure 28

Bone marrow of Rat G-7.

Bone marrow biopsy sections showed 50-60% hypocellularity with megaloblastic erythropoiesis comprising 23-38% of the marrow cells. Megakaryocytes were decreased in number with micromegakaryocytes (Arrow A) and apoptotic denuded forms. Granulopoiesis showed recovery (26%) with significant maturational delay and small areas of ALIP (Arrow B). Mast cells were grossly increased. Pathological sideroblasts (++) and occasional ringed sideroblasts were present.

4.4.27 DIAGNOSIS

Regenerating marrow cellularity with recovering granulopoiesis showing significant maturational delay with ALIP. Dyserythropoiesis was found as well as (++) dysplasia in the megakaryocyte lineage. Ferrokinetics were grossly altered. The marrow picture is consistent with hypocellular trilineage dysplasia, MDS.

4.4.28 mtDNA LENGTH MEASUREMENTS RAT G-6; RAT G-7

Table 39

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 0.93)	Average Length Value Rat mtDNA
2.0	1.78	1.91	3.85
2.20	1.95	2.09	
1.75	1.55	1.66	
1.50	1.33	1.43	
2.20	1.95	2.09	
1.6	1.42	1.53	
2.5	2.22	2.29	
1.8	1.60	1.72	
2.5	2.22	2.39	
3.8	3.38	3.63	
4.2	3.73	4.01	
4.0	3.56	3.83	
4.4	3.91	4.20	
3.0	2.67	2.87	
3.7	3.29	3.54	
6.15	5.47	5.88	
5.3	4.71	5.06	
6.5	5.78	6.21	
5.0	4.45	4.78	
5.1	4.53	4.87	
5.4	4.80	5.16	
1.75	1.55	1.66	
2.85	2.53	2.72	
5.10	4.53	4.87	
4.55	4.04	4.34	
5.05	4.49	4.83	

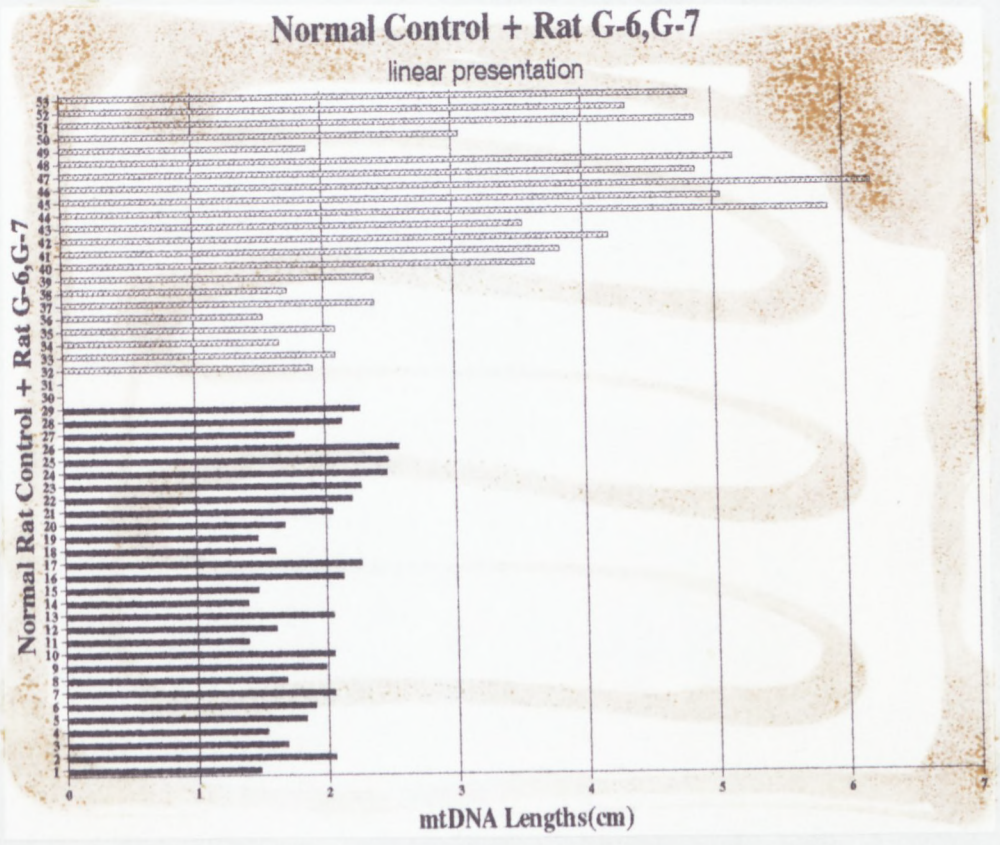

Figure 29

Linear presentation of rat mtDNA length values of Rat G-6 and G-7

Black lines are the control

Striped lines are the gamma-irradiated rat mtDNA

4.4.29 RATS G-8; G-9; G-10

Procedure	Rat
4 Weeks post-irradiation with #3 400 rad dose X-rays	G-8 G-9 G-10

4.4.30 PERIPHERAL COUNTS

RAT	G-8	G-9	G-10	UNITS
WBC	4.06	3.13	2.15	$x10^3/\mu L$
RBC	5.89	5.55	5.35	$x10^6/\mu L$
HGB	13.8	12.6	12.7	g/dL
HCT	44.4	39.3	38.2	%
MCV	75.4	70.7	71.5	fL
MCH	23.4	22.7	23.7	pg
MCHC	31.0	32.1	33.1	g/dL
PLT	736	521	608	$x10^3/\mu L$

4.4.31 PERIPHERAL SMEAR

Normal peripheral cell counts with anisocytosis and round macrocytes, hypersegmented and hypogranular neutrophils with adequate platelets were observed.

4.4.32 BONE MARROW RAT G-8

MYELOGRAM

Neutrophils	- 21	Monocytes	- 6
Myelocytes	- 4	Mast Cells	- 9
Promyelocytes	- 8	Eosinophils	- 4
Blast Cells	- 4	Lymphocytes	- 10
Iron(Fe)	4/6	Plasma Cells	- 3
		Erythroid Cells	- 31

4.4.33 BONE MARROW RAT G-9

MYELOGRAM			
Neutrophils	- 36	Monocytes	- 5
Myelocytes	- 7	Mast Cells	- 7
Promyelocytes	- 10	Eosinophils	- 0
Blast Cells	- 5	Lymphocytes	- 2
Iron(Fe)	4/6	Plasma Cells	- 1
		Erythroid Cells	- 31

4.4.34 BONE MARROW RAT G-10

MYELOGRAM			
Neutrophils	- 36	Monocytes	- 5
Myelocytes	- 7	Mast Cells	- 7
Promyelocytes	- 10	Eosinophils	- 0
Blast cells	- 6	Lymphocytes	- 2
Iron(Fe)	4/6	Plasma Cells	- 1
		Erythroid Cells	- 25

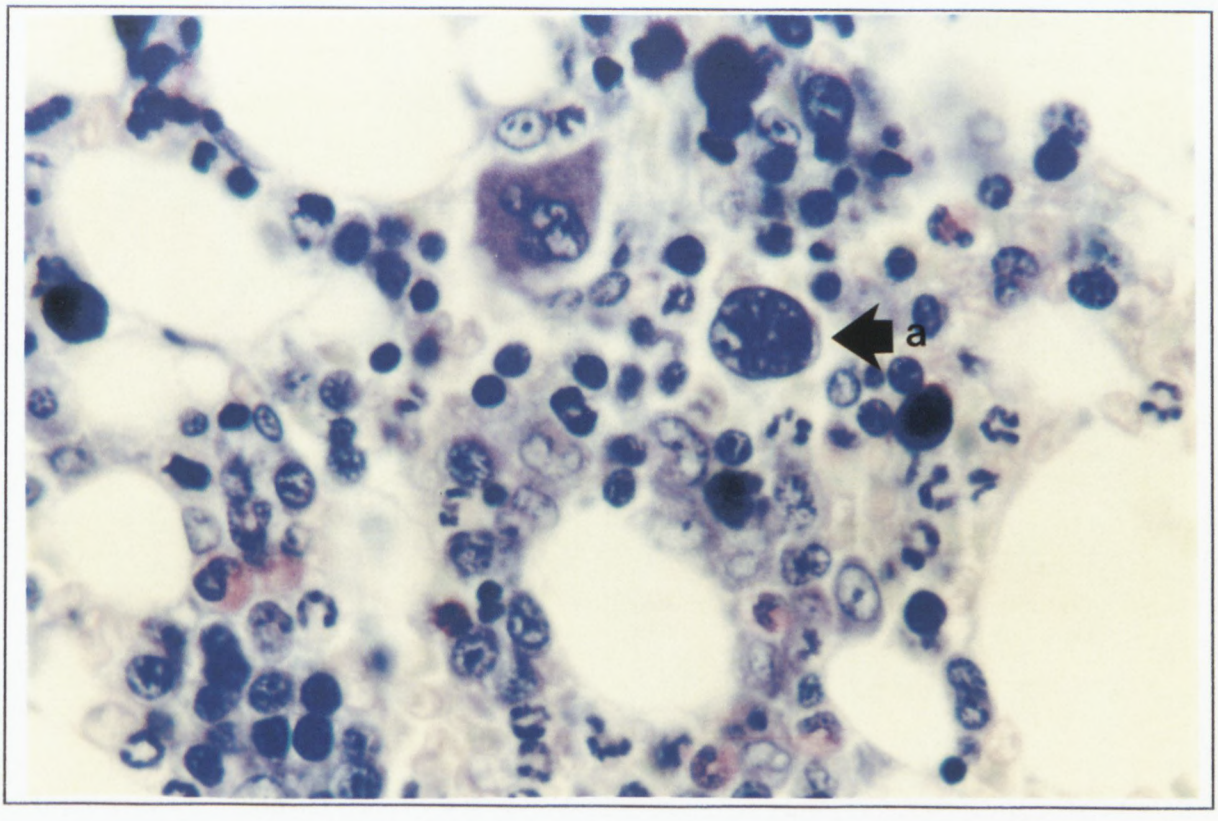

Figure 30

BONE MARROW RAT G8; G9:

Bone marrow (G-8; G-9) with a 60% low normocellular marrow and 30% megaloblastic erythropoiesis. Megakaryocytes were dysplastic with increased apoptotic denuded forms (Arrow A). Granulopoiesis was 37% of marrow cell population. Mast cells were increased in number. There was a significant maturational delay with ALIP

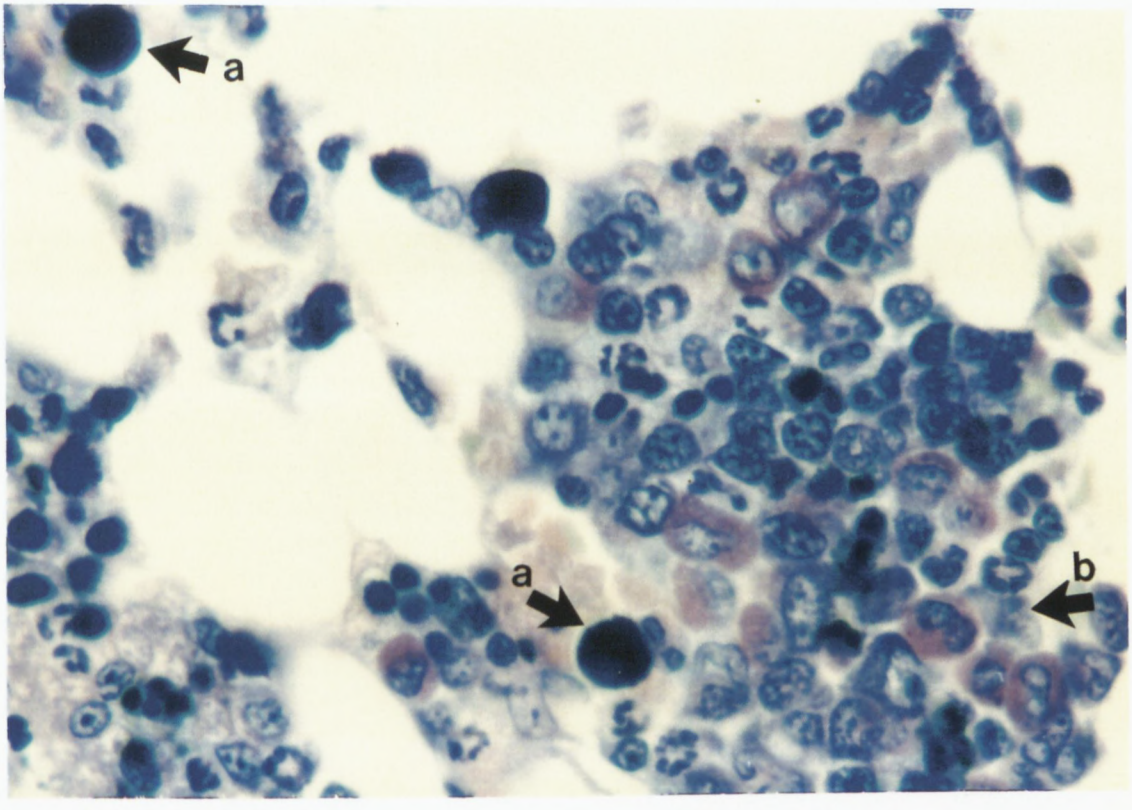

Figure 31

BONE MARROW G8; G9:

Bone marrow (G-8; G-9) with a 60% low normocellular marrow and 30% megaloblastic erythropoiesis. Megakaryocytes were dysplastic with increased apoptotic denuded forms(arrow A). Granulopoiesis was 37% of marrow cell population. Mast cells were increased in number. There was a significant maturational delay with ALIP (arrow B)

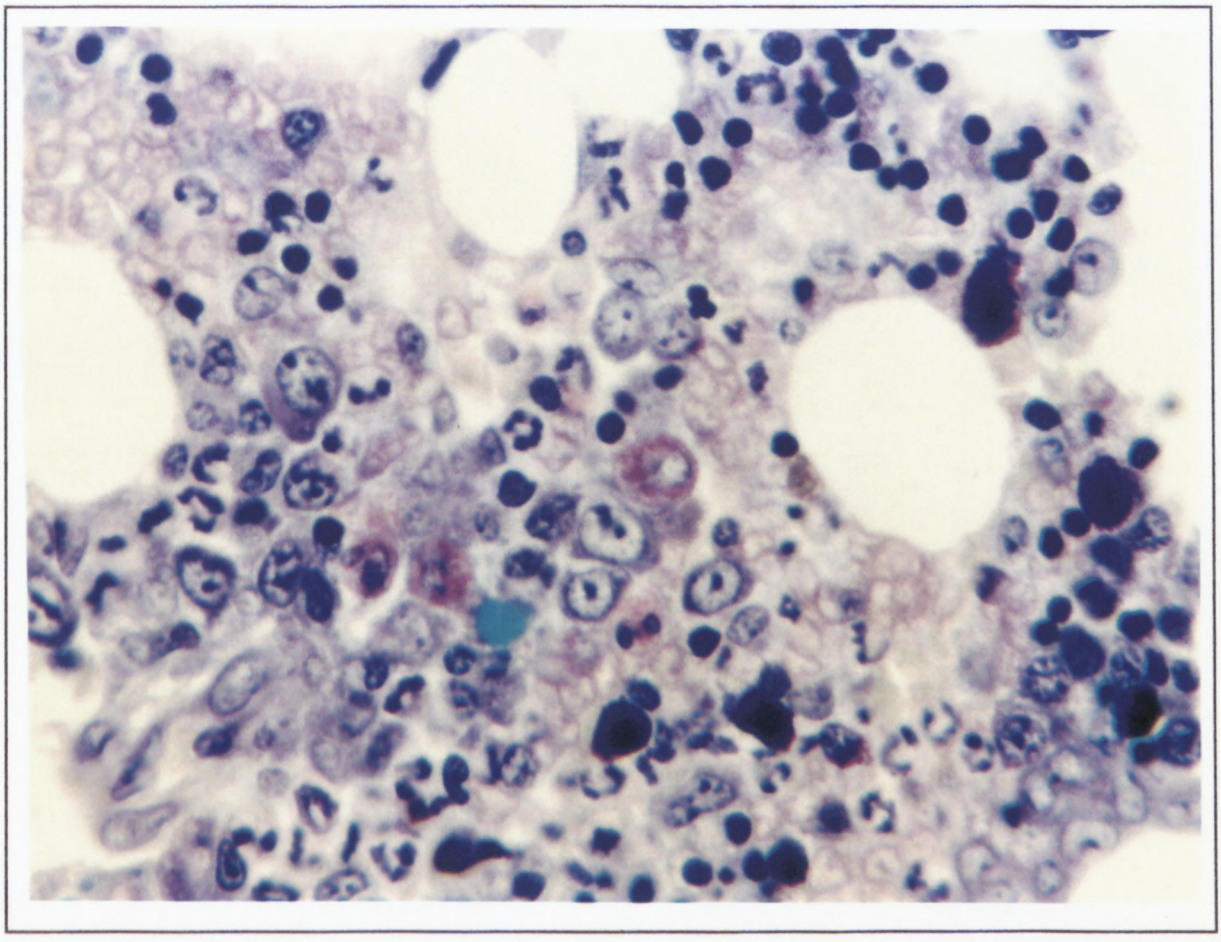

Figure 32

BONE MARROW G-10:

Bone marrow G-10 with 80% normocellular marrow and hyperplastic granulopoiesis with maturational delay and ALIP. Granulopoiesis comprised 67% of the marrow cell population. Blast cells and mast cells were increased in numbers. Erythropoiesis was megaloblastic and suppressed. Megakaryocytes were present with dyserythropoiesis.

4.4.35 DIAGNOSIS

Rat G-8, G-9, G-10 smears and biopsies - the findings were consistent with early MDS (RAEB FAB Subtype).

4.4.36 mtDNA LENGTH MEASUREMENTS RAT G-8, G-9, G-10

Table 40

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 0.93)	Average Length Value Rat mtDNA
2.6	2.31	2.48	2.98
1.8	1.60	1.72	
1.4	1.24	1.33	
2.1	1.86	2.00	
1.5	1.33	1.43	
1.3	1.15	1.23	
1.9	1.69	1.81	
1.5	1.33	1.43	
1.7	1.51	1.62	
1.4	1.24	1.33	
2.3	2.04	2.19	
1.6	1.42	1.52	
2.2	1.95	2.09	
2.7	2.40	2.58	
3.3	2.93	3.15	
3.8	3.39	3.64	
3.4	3.02	3.24	
3.5	3.11	3.34	
2.9	2.58	2.77	
3.9	3.47	3.73	
3.3	3.38	3.15	
3.1	3.47	2.95	
2.8	2.93	2.95	
3.5	2.75	2.67	
4.0	2.49	3.34	
3.9	3.11	3.82	
3.6	3.56	3.73	
3.8	3.47	3.44	
4.12	3.20	3.36	
4.10	3.38	4.10	
4.95	3.82	3.91	
5.4	3.64	4.73	
5.1	4.40	5.16	
4.85	4.80	4.87	
4.73	4.53	5.21	
5.45	4.84	5.08	
5.31	4.72	5.07	

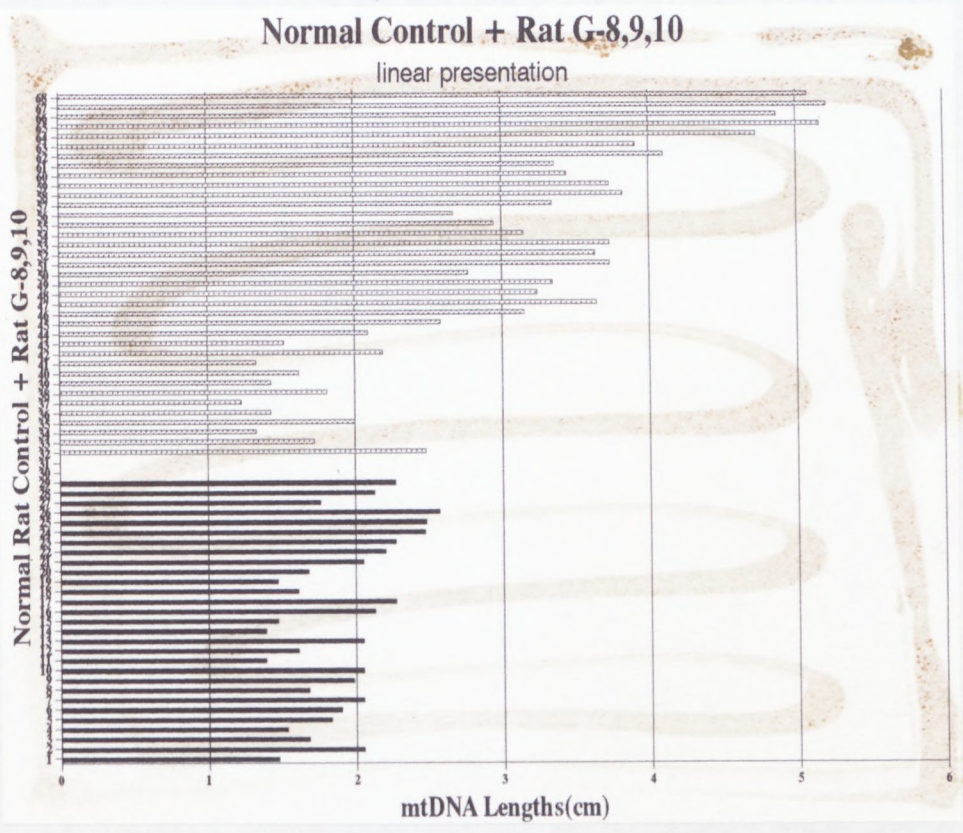

Figure 33

RAT mtDNA MEAN LENGTH VALUES RAT G-8, RAT G-9, RAT G-10

Linear presentation of mtDNA of RAT G-8; G-9; G-10

Black lines are the control

Striped lines are the gamma-irradiated rat mtDNA

4.5 EXPERIMENTAL RESULTS - GAMMA/NEUTRON (GN) IRRADIATED RAT POPULATION

Procedure	Rat
2 Weeks post-irradiation with #1 with 288 rads x/n	GN-1 GN-2

4.5.1 PERIPHERAL BLOOD COUNTS

RAT	GN-1	GN-2	UNITS
WBC	1.7	2.5	$x10^3/\mu L$
RBC	4.70	3.58	$x10^6/\mu L$
HGB	10.3	8.3	g/dL
HCT	30.4	24.0	%
MCV	64.6	66.9	fL
MCH	21.9	23.1	pg
MCHC	33.9	34.4	g/dL
PLT	305	218	$x10^3/\mu L$

4.5.2 PERIPHERAL SMEAR

Anisopoikilocytosis with occasional peripheral normoblasts, giant platelets and left-shifted neutrophils were observed.

4.5.3 BONE MARROW RAT GN-1, GN2

a) RAT GN-1

MYELOGRAM			
Neutrophils	- 14	Monocytes	- 8
Myelocytes	- 4	Mast Cells	- 6
Promyelocytes	- 12	Eosinophils	- 2
Blast Cells	- 3	Lymphocytes	- 12
Iron(Fe)	4/6	Plasma Cells	- 6
		Erythroid Cells	- 33

b) RAT GN-2

MYELOGRAM			
Neutrophils	- 22	Monocytes	- 2
Myelocytes	- 8	Mast Cells	- 4
Promyelocytes	- 14	Eosinophils	- 0
Blast Cells	- 4	Lymphocytes	- 0
Iron(Fe)	3/6	Plasma Cells	- 0
		Erythroid Cells	- 46

4.5.4 BONE MARROW BIOPSIES

Rat GN-1

70-75% low normocellular marrow with megaloblastic erythropoiesis comprising 35-45% of the marrow cell population was found. Megakaryocytes were adequate in number but with dysplastic morphology in the lineage. Granulopoiesis was suppressed and left-shifted with a significant maturational delay evident at the promyelocyte/myelocyte stage and it comprised 14-20% of the total marrow cell population. Mast cells were increased and blast cell numbers were high normal.

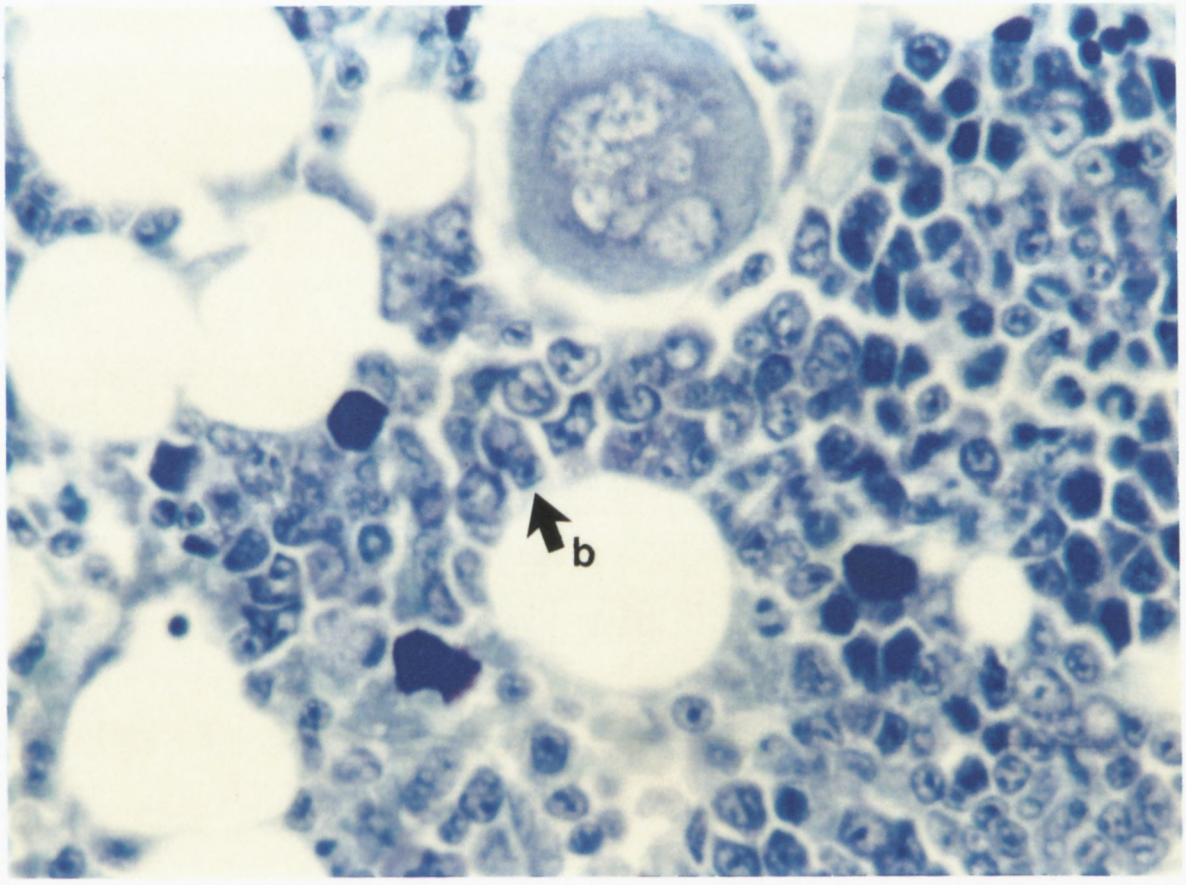

Figure 34

BONE MARROW RAT GN-1:

May-Grünewald Giemsa stain of section from bone marrow Rat GN-1 Arrow B shows maturational delay at the promyelocyte/myelocyte stage.

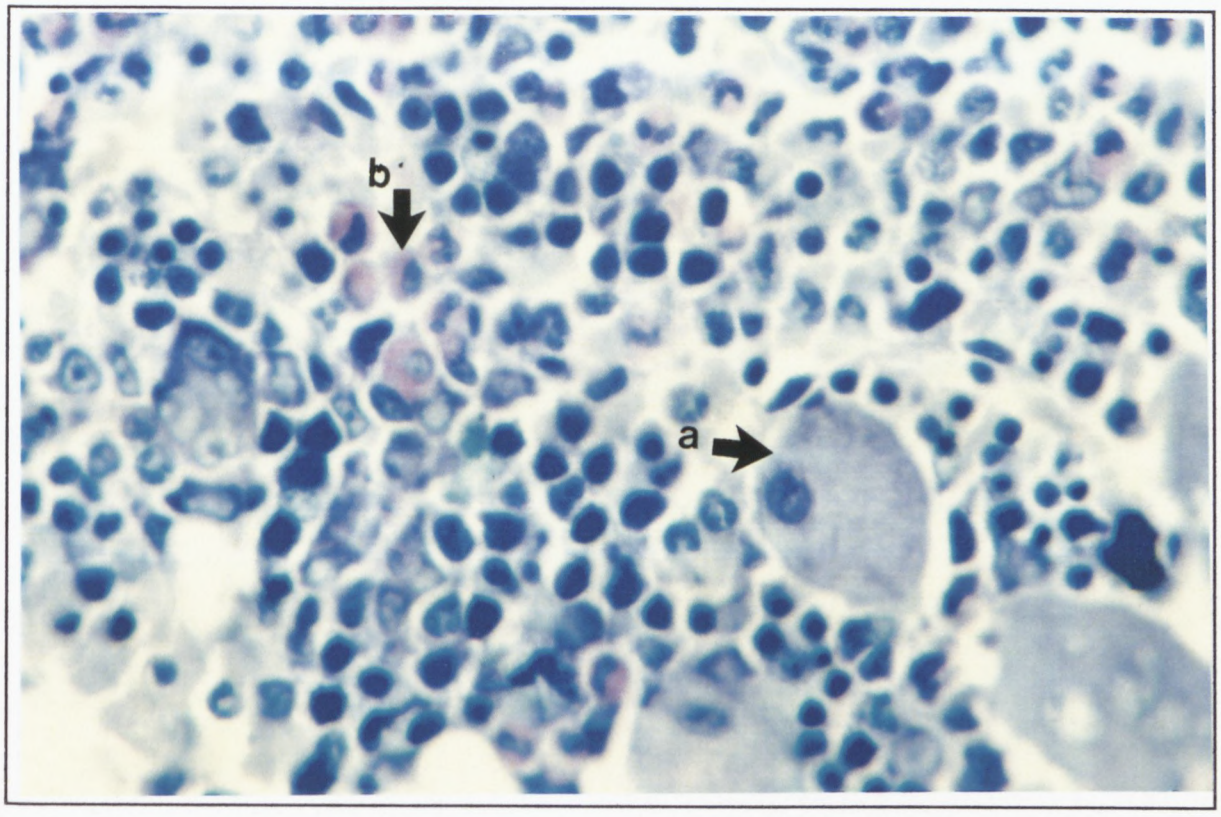

Figure 35

BONE MARROW GN-1:

May-Grünewald Giemsa stain of section of bone marrow of Rat GN-1

Arrow B shows marurational delay at the promyelocyte/myelocyte stage

Arrow A shows a micromegakaryocyte

4.5.5 DIAGNOSIS

Low normocellular marrow with megaloblastic anaemia and maturational delay in the myeloid compartment. The megakaryocytic lineage showed signs of dysplasia. The picture is consistent with possible early MDS (RAEB FAB Subtype).

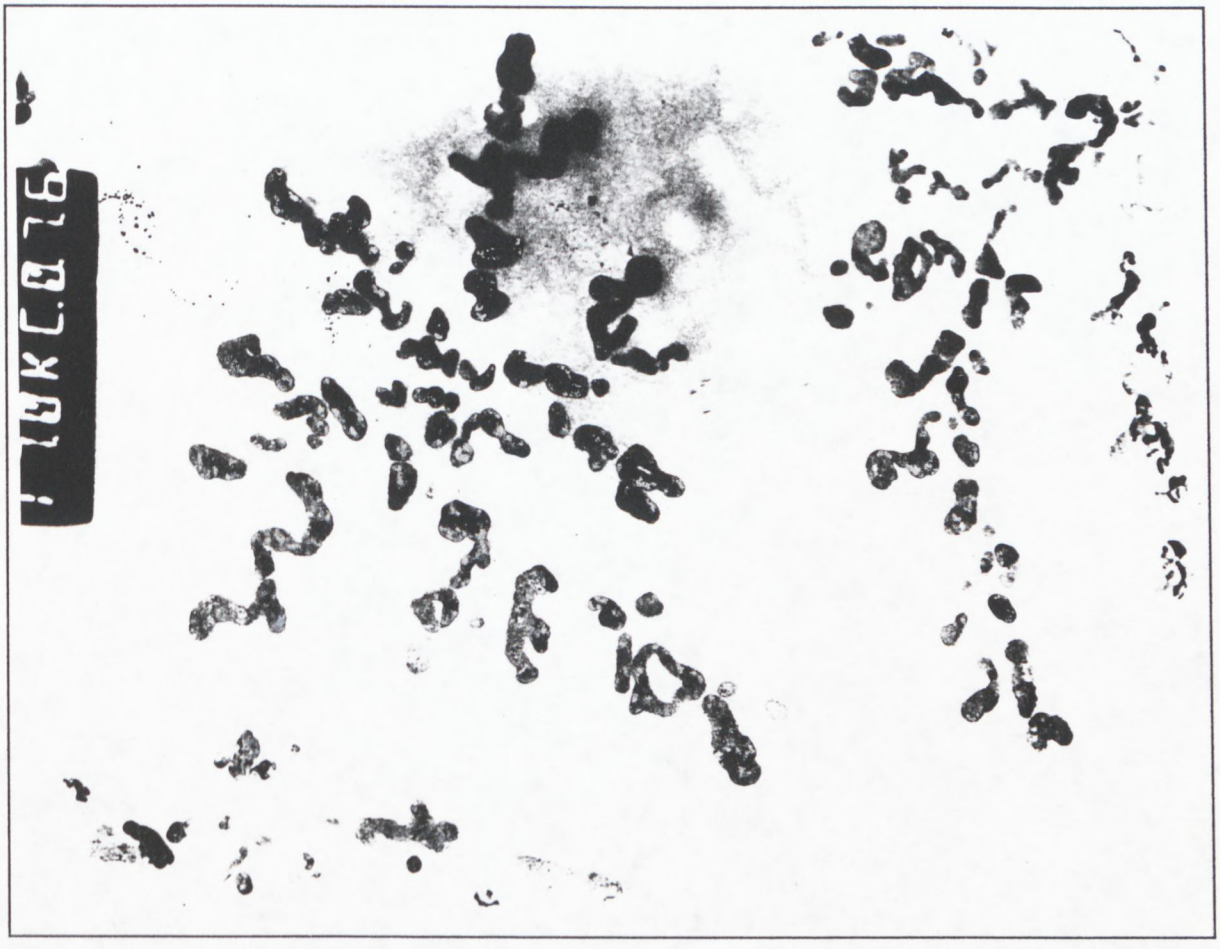

Figure 36

PHOTOMICROGRAPH OF GN-1 mtDNA WITH pBR322 PLASMID AS AN INTERNAL LENGTH STANDARD

Photo shows isolated mtDNA circular bands.

4.5.6 mtDNA LENGTH MEASUREMENTS RAT GN-1, RAT GN-2

Table 41

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length Value Rat mtDNA
1.85	1.64	1.35	3.01
2.0	1.78	1.47	
2.2	1.95	1.61	
1.8	1.60	1.32	
5.1	4.53	3.74	
5.3	4.7	3.88	
7.8	6.94	5.73	
4.8	4.27	3.52	
6.4	5.69	4.70	
7.9	6.03	5.80	
4.4	3.91	3.23	
2.2	1.95	1.61	
2.8	2.49	2.05	
7.0	6.23	5.14	
8.45	7.52	6.21	
5.75	5.42	4.47	
2.0	1.78	1.47	
4.7	4.8	3.96	
6.5	5.78	4.77	
1.8	1.60	1.32	
2.3	2.04	1.68	
2.0	1.78	1.47	
1.8	1.60	1.32	
2.2	1.95	1.61	
2.4	2.13	1.76	

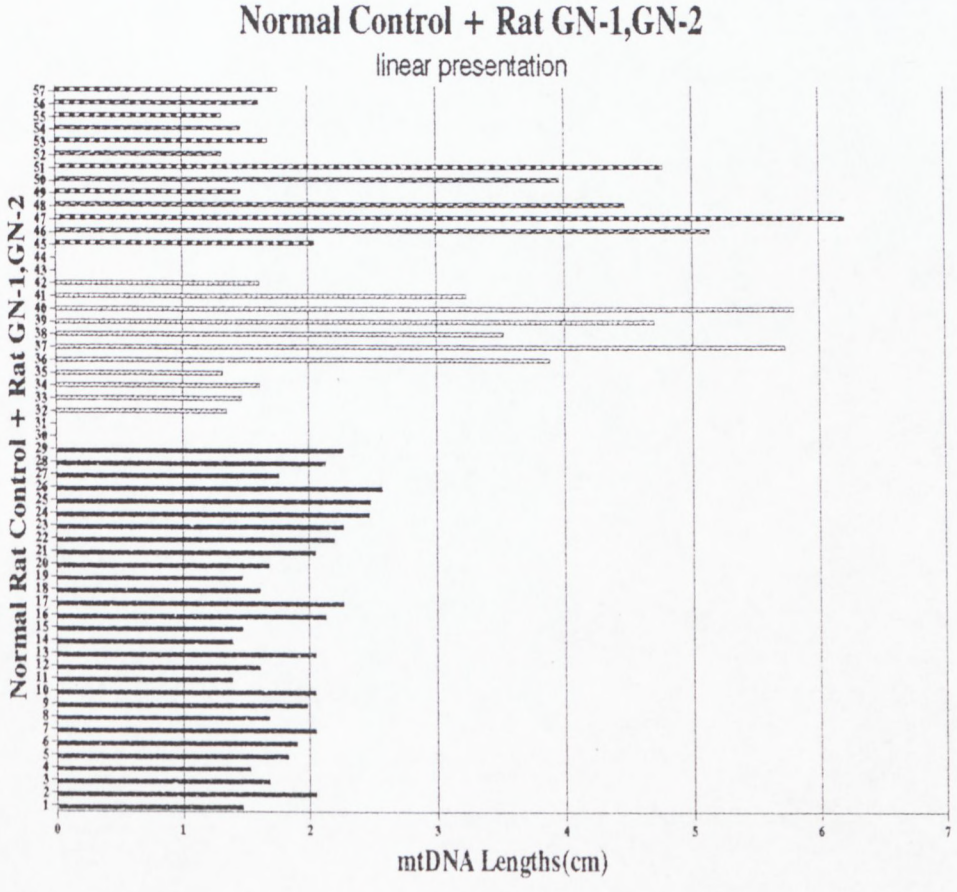

Figure 37

RAT mtDNA MEAN LENGTH VALUES RAT GN-1, RAT GN-2

Linear presentation of mtDNA of Rat GN-1; GN-2

Black lines are the control

Dotted lines are the gamma/neutron irradiated rat samples.

Procedure	Rat
2 Weeks post-irradiation with #2 with 288 rads x/n	GN-3 GN-4

4.5.7 PERIPHERAL BLOOD COUNTS

RAT	GN-3	GN-4	UNITS
WBC	2.6	1.89	$x10^3/\mu L$
RBC	4.56	4.75	$x10^6/\mu L$
HGB	10.5	11.3	g/dL
HCT	30.5	34.8	%
MCV	66.9	73.2	fL
MCH	23.1	23.9	pg
MCHC	34.5	32.6	g/dL
PLT	404	323	$x10^3/\mu L$

4.5.8 PERIPHERAL SMEAR

Anisocytosis with severe neutropenia, round macrocytes and occasional circulating normoblasts were seen. Platelets were mildly decreased in number with occasional large platelets present.

4.5.9 BONE MARROW RAT GN-3; RAT GN-4

a) RAT GN-3

MYELOGRAM			
Neutrophils	- 16	Monocytes	- 5
Myelocytes	- 7	Mast Cells	- 7
Promyelocytes	- 28	Eosinophils	- 2
Blast Cells	- 3	Lymphocytes	- 8
Iron(Fe)	1/6	Plasma Cells	- 4

b) RAT GN-4

MYELOGRAM			
Neutrophils	- 12	Monocytes	- 2
Myelocytes	- 9	Mast Cells	- 5
Promyelocytes	- 23	Eosinophils	- 4
Blast Cells	- 3	Lymphocytes	- 12
Iron(Fe)	1/6	Plasma Cells	- 1
		Erythroid Cells	- 28

4.5.10 BONE MARROW BIOPSIES

45-50% hypocellular marrow with markedly suppressed megaloblastic erythropoiesis comprising 20-30% of the total marrow cell population was observed. Megakaryocytes were adequate in number but with continuing dysplastic morphology and micro-megakaryocytes. Granulopoiesis was hyperplastic comprising 53% of the marrow cells with gross maturational delay and ALIP. Mast Cells were increased and blast cell numbers were high normal.

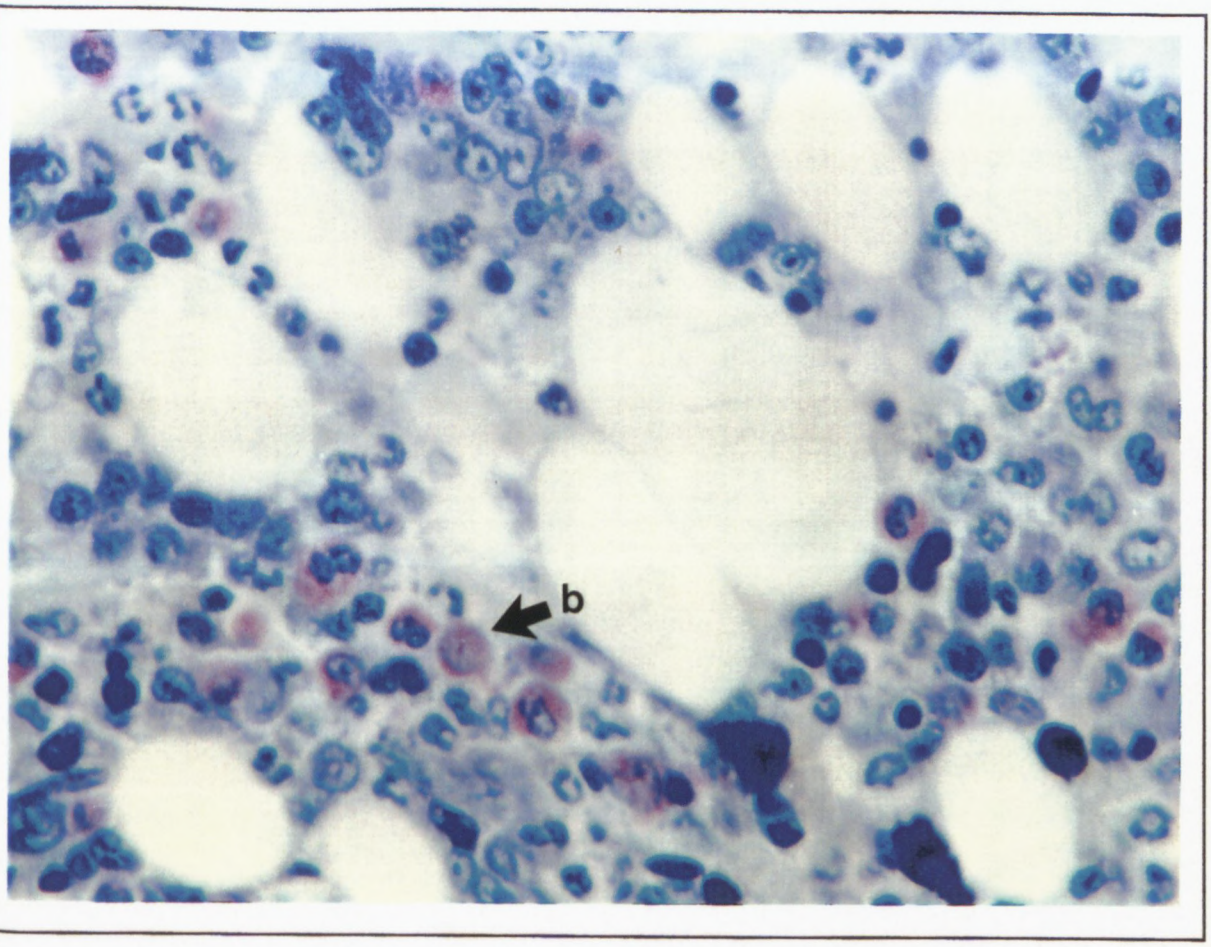

Figure 38

BONE MARROW BIOPSY RAT GN-4

May-Grünewald Giemsa stain of bone marrow section from Rat GN-4

Arrow shows the maturational delay of the granulopoiesis.

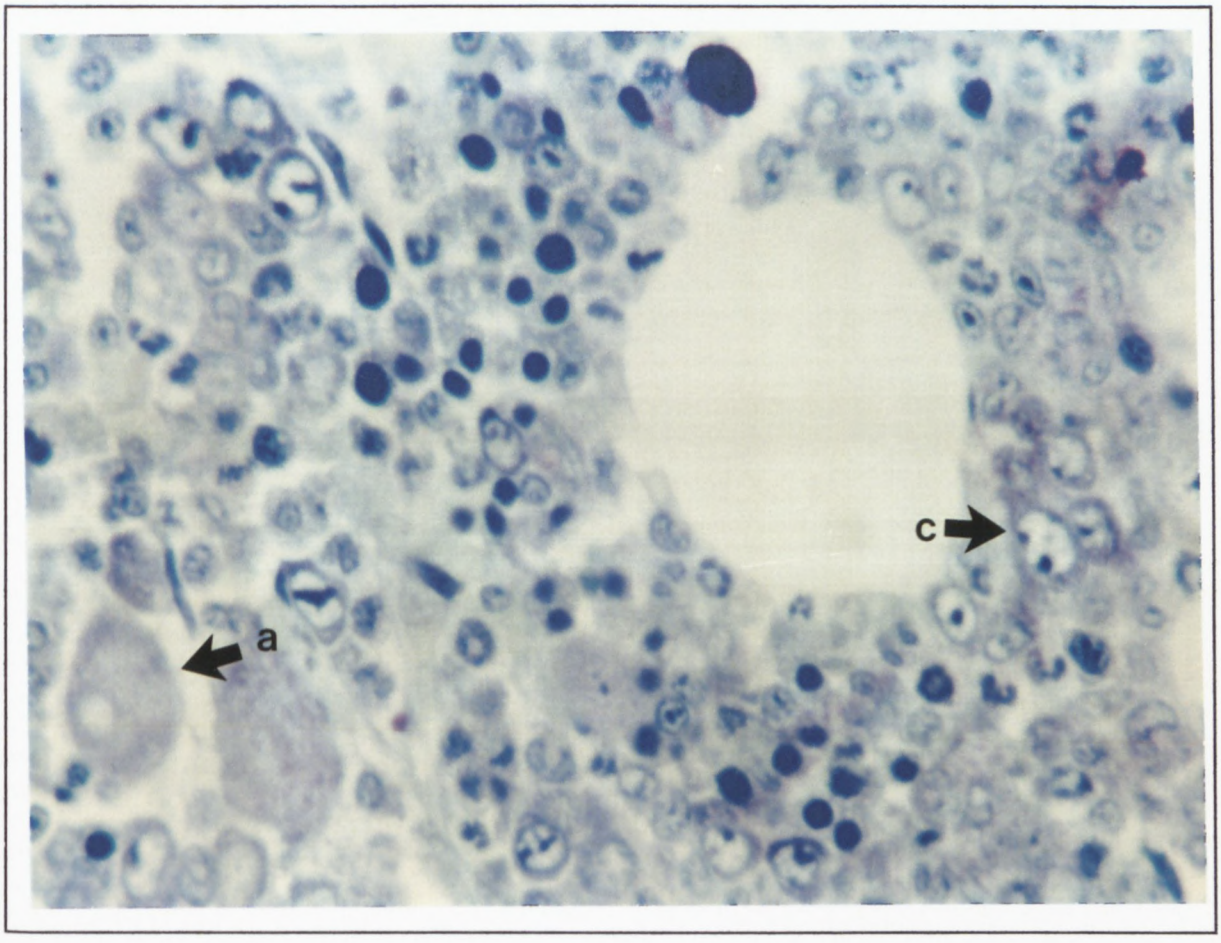

Figure 39

BONE MARROW BIOPSY RAT GN-4

May Grünewald Giemsa stain of bone marrow section of Rat GN-4

Arrow A shows micromegakaryocytes

Arrow C shows abnormal localisation of immature precursors(ALIP.)

4.5.11 DIAGNOSIS

Moderate hypocellular marrow with suppressed megaloblastic erythropoiesis, dysplasia in the megakaryocyte lineage and relatively hyperplastic granulopoiesis with gross maturational delay at the promyelocyte stage and ALIP. The marrow picture is mixed with features typical of MDS (RA with trileanage dysplasia and ALIP) combined with delayed maturation in an over-expanded

myeloid progenitor compartment, as seen in humans with chronic myeloid myeloproliferative disease (MPD).

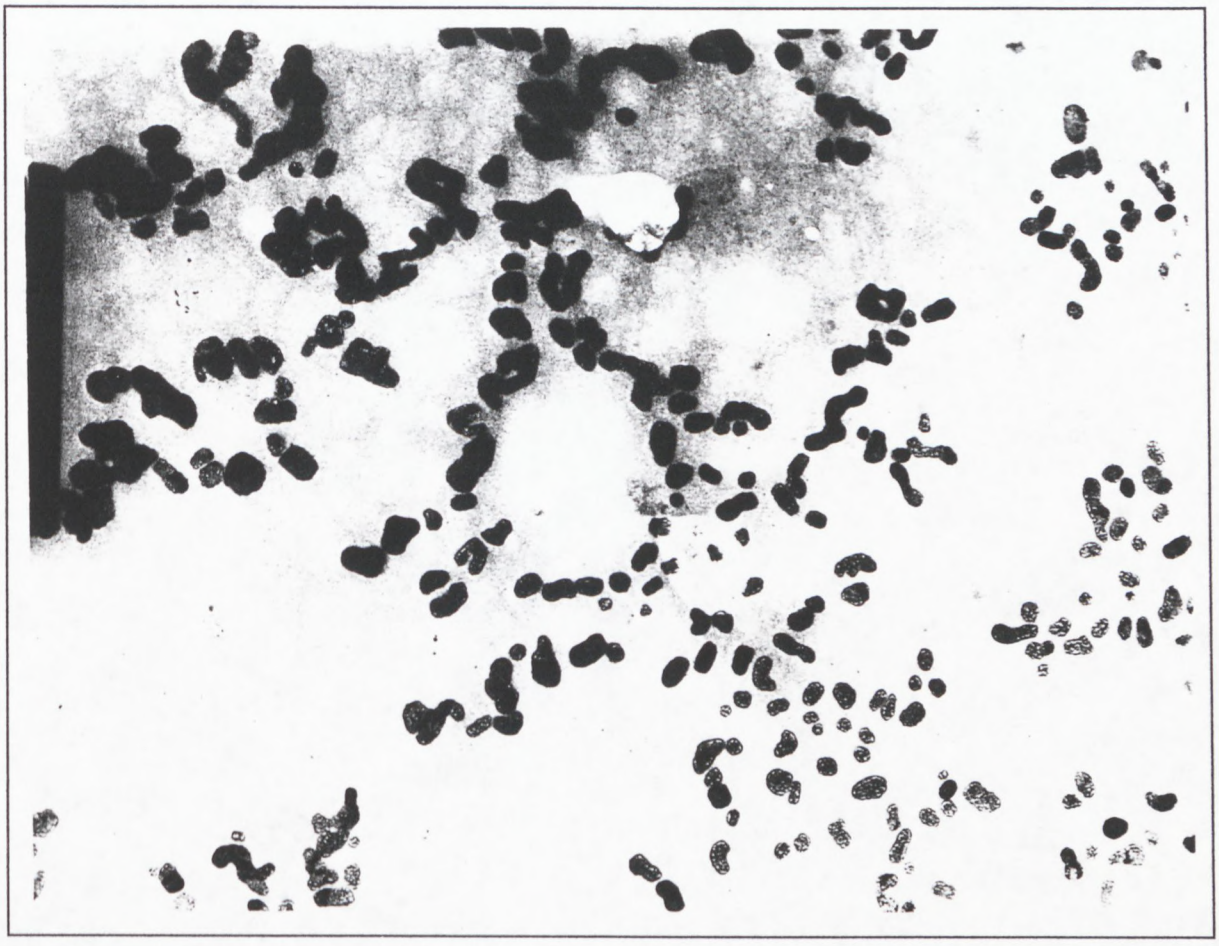

Figure 40

PHOTOMICROGRAPH OF GN-4 RAT mtDNA WITH pBR322 PLASMID AS INTERNAL LENGTH STANDARD

This picture shows isolated mtDNA circular bands.

4.5.12 mtDNA LENGTH MEASUREMENTS RAT GN-3, RAT GN-4

Table 42: GN-3

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length Value Rat mtDNA
3.2	2.84	2.34	2.99
3.8	3.38	2.79	
3.2	2.84	2.34	
2.3	2.04	1.68	
2.8	2.49	2.05	
2.2	1.95	1.61	
6.9	6.14	5.07	
4.8	4.27	3.52	
3.2	2.84	2.34	
6.1	5.42	4.47	
5.8	5.16	4.26	
4.8	4.27	3.52	
3.95	3.51	2.90	

Table 43: GN-4

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Length Value Rat mtDNA
6.0	5.34	4.41	3.77
5.1	4.43	3.66	
6.65	5.91	4.88	
6.5	5.78	4.77	
7.05	6.27	5.18	
5.9	5.25	4.33	
5.8	5.16	4.26	
4.7	4.18	3.45	
3.2	2.84	2.34	
2.9	2.57	2.12	
2.93	2.60	2.14	

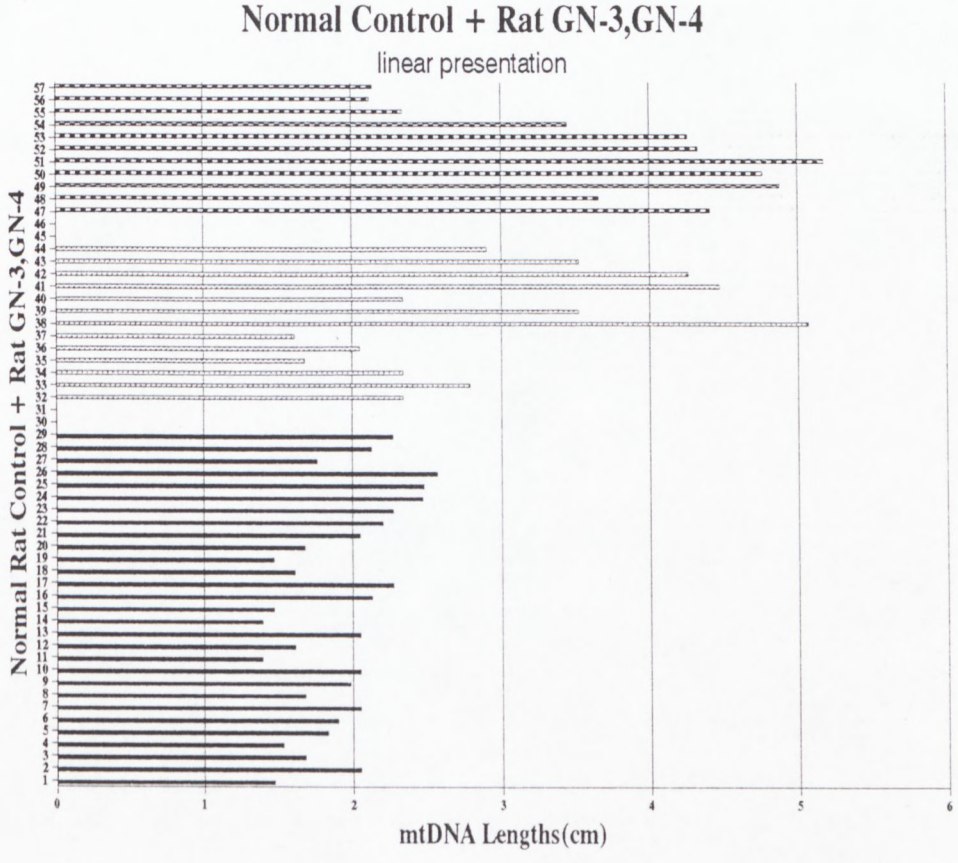

Figure 41

RAT mtDNA MEAN LENGTH VALUES RAT GN-3, RAT GN-4

Linear presentation of rat mtDNA

Black lines are the control

Dotted lines are the mtDNA values of gamma/neutron irradiated rats.

Procedure	Rat
2 Weeks post-irradiation with #3 with 288 rads x/n	GN-5 GN-6

4.5.13 PERIPHERAL BLOOD COUNTS

RAT	GN-5	GN-6	UNITS
WBC	1.52	4.31	$x10^3/\mu L$
RBC	4.97	5.26	$x10^6/\mu L$
HGB	12.1	12.8	g/dL
HCT	36.1	38.0	%
MCV	72.7	72.1	fL
MCH	24.5	24.3	pg
MCHC	33.7	33.7	g/dL
PLT	321	464	$x10^3/\mu L$

4.5.14 PERIPHERAL SMEAR

Anisocytosis with (+) polychromatic cells, stomatocytes (+), atypical lymphocytes and recovering neutrophil counts which showed hypersegmentation/hypo-granulation with severe left-shift to the myeloid stage. Platelet counts were mildly decreased with giant platelets (++).

4.5.15 BONE MARROW SAMPLE RAT GN-5, GN-6

a) RAT GN-5

MYELOGRAM			
Neutrophils	- 10	Monocytes	- 5
Myelocytes	- 8	Mast Cells	- 4
Promyelocytes	- 15	Eosinophils	- 2
Blast Cells	- 5	Lymphocytes	- 8
Iron(Fe)	2/6	Plasma Cells	- 3
		Erythroid Cells	- 34

b) RAT GN-6

MYELOGRAM			
Neutrophils	- 21	Monocytes	- 7
Myelocytes	- 5	Mast Cells	- 6
Promyelocytes	- 12	Eosinophils	- 3
Blast Cells	- 4	Lymphocytes	- 8
Iron(Fe)	2/6	Plasma Cells	- 3
		Erythroid Cells	- 34

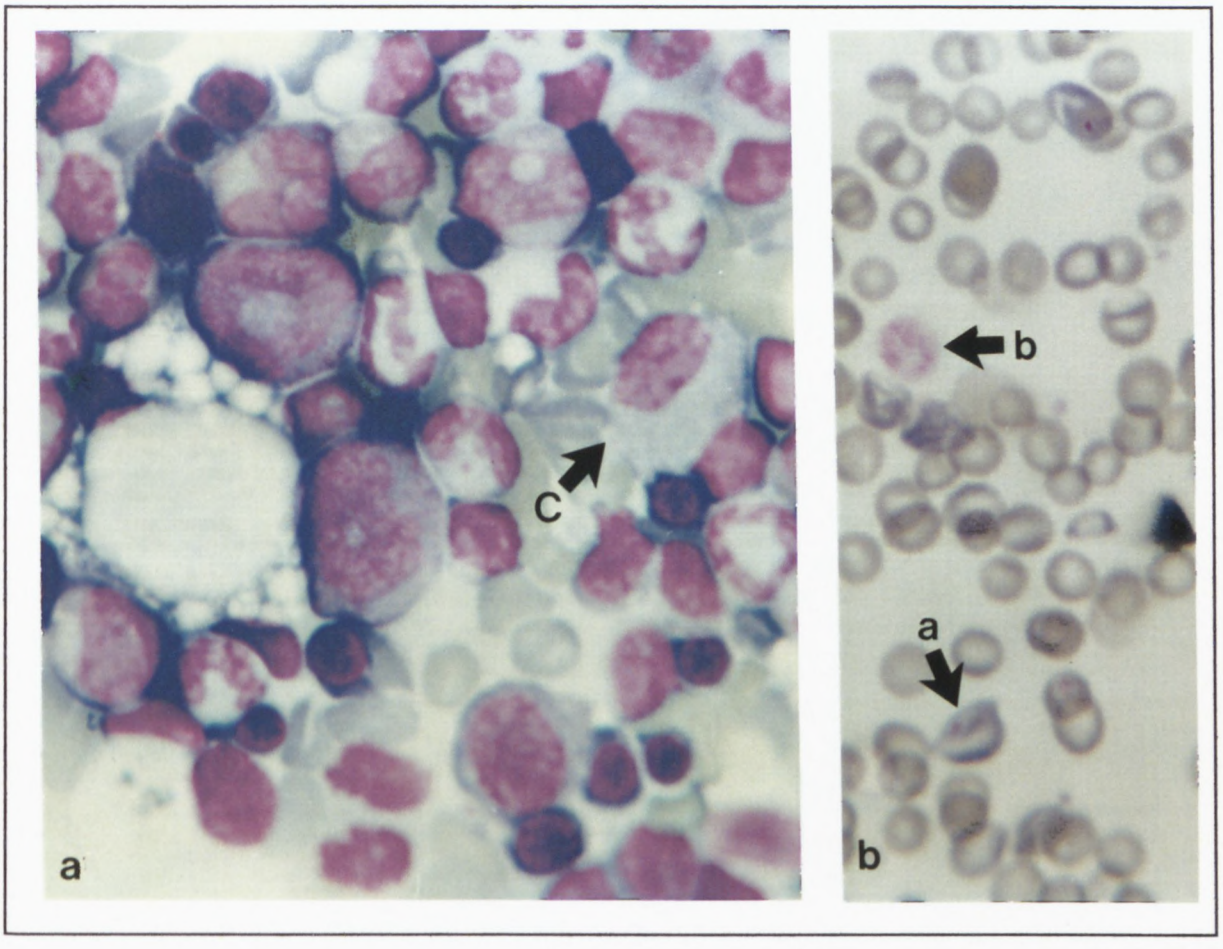

Figure 42

BONE MARROW ASPIRATE/IMPRESSION AND PERIPHERAL BLOOD SMEARS

a) Bone marrow aspirate from Rat GN-5 showing megaloblastic dyserythropoiesis, asynchronous myeloid maturation (nuclear/cytoplasm asynchronous maturation), with increased blast cell numbers (MDS-RAEB). Other areas of the smear demonstrate abnormal nuclear morphology. Micromegakaryocytes are present. (Arrow C x 500).

b) Peripheral blood film from Rat GN-6 demonstrating anisopoikilocytosis with stomatocytes (Arrow A) and giant circulating platelets (Arrow B).

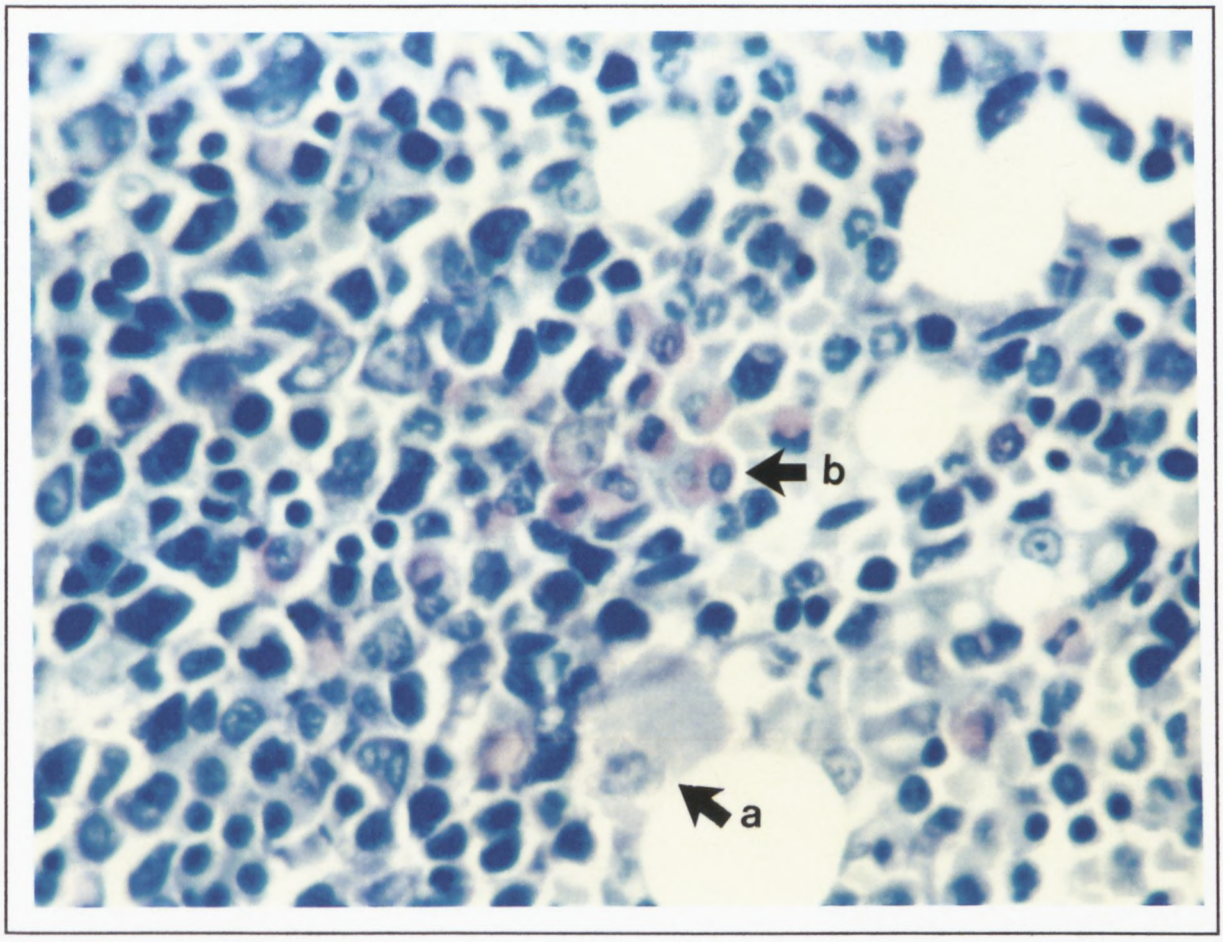

Figure 43

BONE MARROW BIOPSIES

80% normocellular marrow with regenerating megaloblastic dyserythropoiesis were observed. Micromegakaryocytes were present (Arrow A). Granulopoiesis was hyperplastic and left-shifted with gross maturational delay and asynchrony with low numbers of mature neutrophils (Arrow B). Iron stores were normal with occasional pathological sideroblasts present.

4.5.16 DIAGNOSIS

Normocellular marrow recovery and ineffective granulopoiesis with trilineage dysplasia. The picture is consistent with early MDS following hypoplastic marrow insult MDS (RA FAB Subtype).

4.5.17 mtDNA LENGTH MEASUREMENTS RAT GN-5, RAT GN-6

Table 44

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Lemgth Value Rat mtDNA
1.6	1.42	1.17	2.34
1.7	1.51	1.24	
1.8	1.60	1.32	
2.5	2.22	1.83	
5.3	4.71	3.89	
5.0	4.45	3.67	
4.4	3.91	3.23	

Table 45

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.21)	Average Mean Lenth Value Rat mtDNA
2.5	2.22	1.83	2.79
3.8	3.38	2.79	
4.2	3.73	3.08	
4.0	3.56	2.94	
3.4	3.02	2.49	
5.0	4.45	3.67	
3.4	3.02	2.49	
4.4	3.91	3.23	
3.7	3.29	2.71	
3.4	3.02	2.49	
3.0	2.67	2.20	
3.5	3.11	2.57	
5.3	4.71	3.89	
3.6	3.20	2.64	

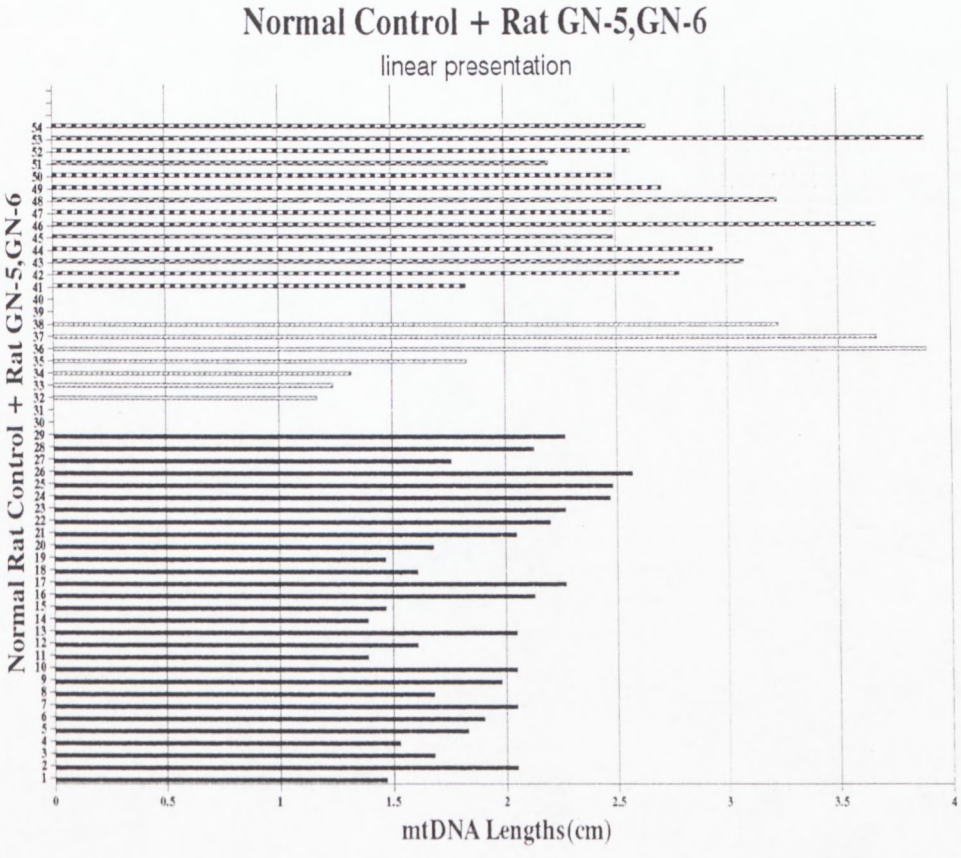

Figure 44

RAT mtDNA MEAN LENGTH VALUES RAT GN-5, RAT GN-6

Linear presentation of mtDNA of Rat GN-5; GN-6

Black lines show the control

Dotted lines show mtDNA values of gamma/neutron irradiated rats

4.5.18 RATS GN-7; GN-8; GN-9; GN-10

Procedure	Rat
4 Weeks post-irradiation with #3 288 rads x/n	GN-7 GN-8 GN-9 GN-10

4.5.19 PERIPHERAL COUNTS

RAT	GN-7	GN-8	GN-9	GN-10	UNITS
WBC	3.54	3.17	3.29	3.27	$x10^3/\mu L$
RBC	5.66	5.45	5.67	5.85	$x10^6/\mu L$
HGB	13.8	12.8	13.8	13.6	g/dL
HCT	42.1	38.9	42.3	41.6	%
MCV	74.4	71.4	74.6	71.1	fL
MCH	24.4	23.5	24.3	23.3	pg
MCHC	32.8	33.0	32.6	32.8	g/dL
PLT	657	602	685	807	$x10^3/\mu L$

4.5.20 PERIPHERAL SMEAR

Anisocytosis with (+) polychromatic cells,macrocytes, small platelets and hypersegmented hypogranular neutrophils, some with nuclear "drumsticks" were observed.

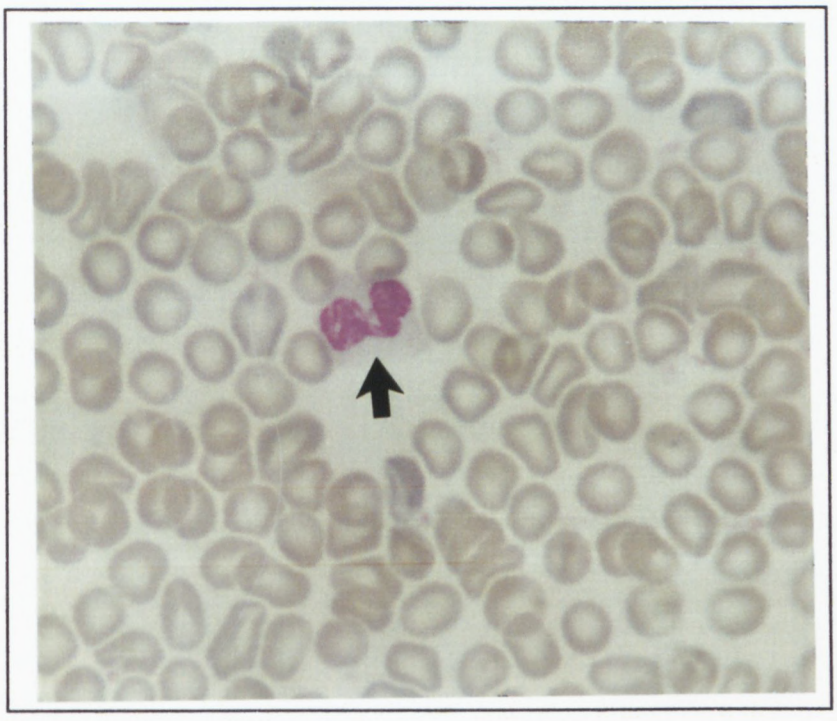

Figure 45

A peripheral smear of Rat GN-10 showing a Pseudo-Pelger-Huët cell (arrow) typical of myeloidysplasia.

4.5.21 BONE MARROW SAMPLE RAT GN-7, GN-8, GN-9, GN-10

a) RAT GN-7

MYELOGRAM			
Neutrophils	- 26	Monocytes	- 8
Myelocytes	- 7	Mast Cells	- 6
Promyelocytes	- 10	Eosinophils	- 3
Blast Cells	- 6	Lymphocytes	- 10
Iron(Fe)	3/6	Plasma Cells	- 3
		Erythroid Cells	- 19

b) RAT GN-8

MYELOGRAM			
Neutrophils	- 24	Monocytes	- 10
Myelocytes	- 12	Mast Cells	- 10
Promyelocytes	- 8	Eosinophils	- 4
Blast Cells	- 5	Lymphocytes	- 9
Iron(Fe)	4/6	Plasma Cells	- 6
		Erythroid Cells	- 17

c) RAT GN-9

MYELOGRAM			
Neutrophils	- 15	Monocytes	- 10
Myelocytes	- 11	Mast Cells	- 10
Promyelocytes	- 9	Eosinophils	- 4
Blast Cells	- 5	Lymphocytes	- 12
Iron(Fe)	4/6	Plasma Cells	- 5
		Erythroid Cells	- 19

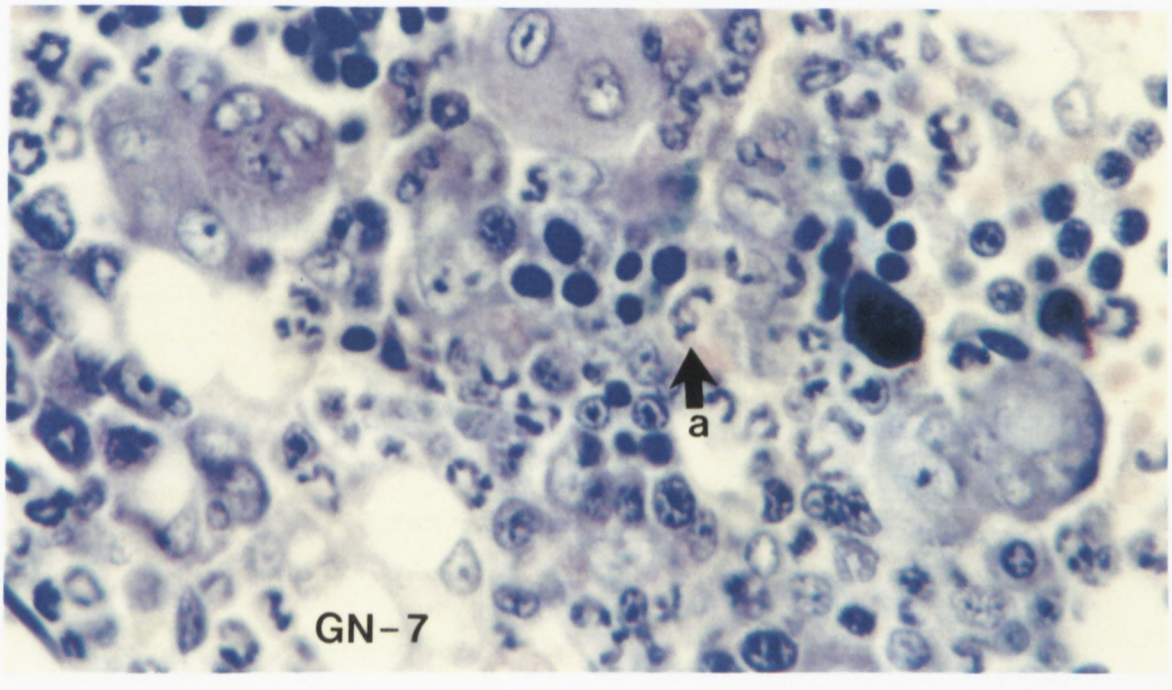

Figure 46

Photomicrograph of bone marrow section of Rat GN-7.

Arrow A shows suppressed megaloblastic erythropoiesis.

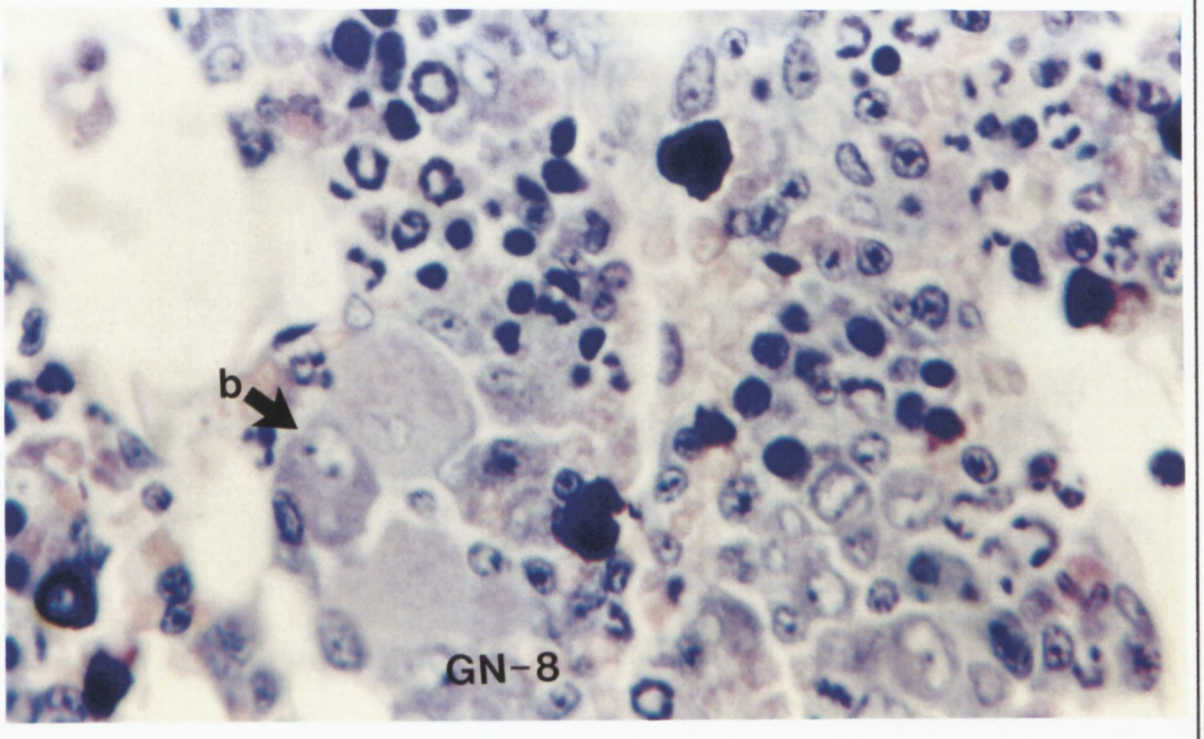

Figure 47

Photomicrograph of bone marrow section of Rat GN-8.

Arrow B shows a micromegakaryocyte.

80-100% hypercellular marrow with suppressed megaloblastic erythropoiesis (Arrow A p.135) comprising 19-21% of the marrow cells was observed. Megakaryocytes were increased in number with (++) dysplasia and micromegakaryocytes (Arrow B). Granulopoiesis was left-shifted with maturational delay and ALIP (Arrow C p.137). The marrow stroma was disorganised and blast cells were increased above normal numbers.

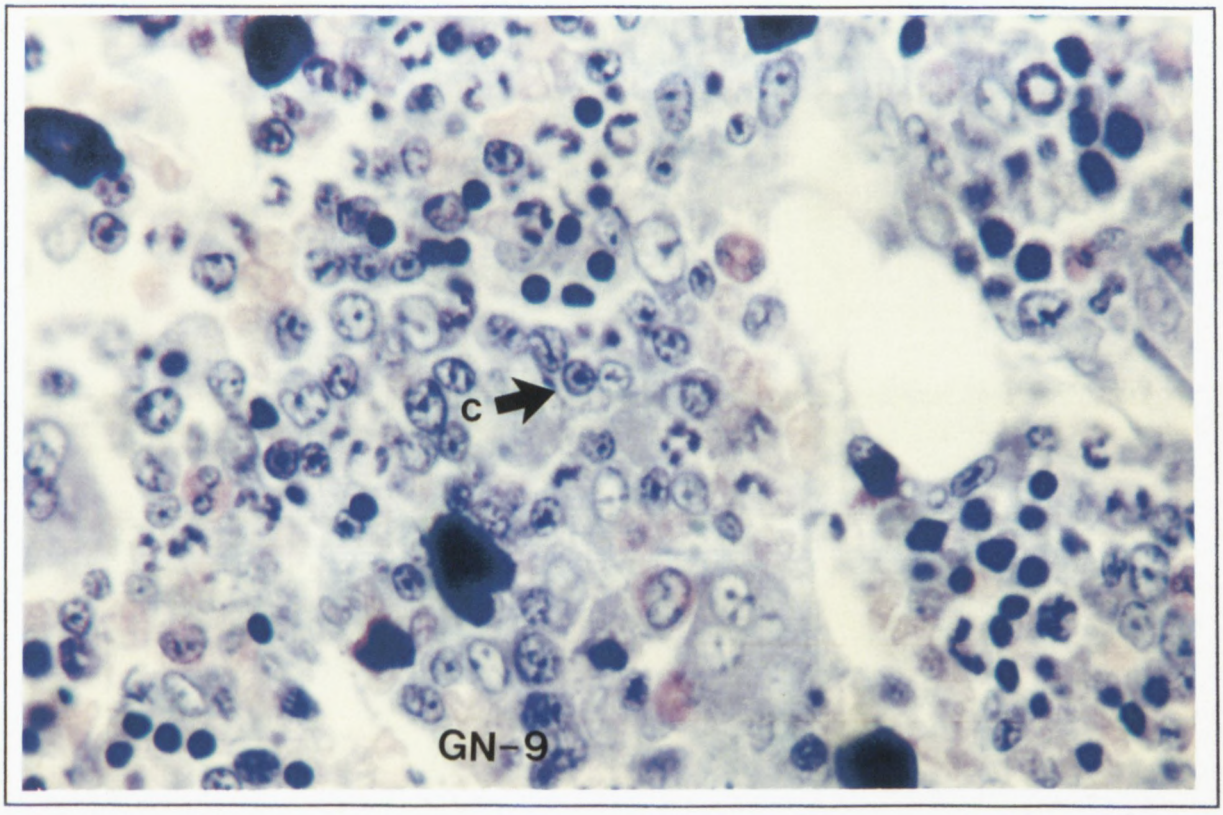

Figure 48

Photomicrograph of bone marrow section of Rat GN-9

Arrow C shows abnormal localisation of immature precursors(ALIP)

d) RAT GN-10

MYELOGRAM			
Neutrophils	- 21	Monocytes	- 8
Myelocytes	- 10	Mast Cells	- 4
Promyelocytes	- 11	Eosinophils	- 3
Blast Cells	- 6	Lymphocytes	- 10
Iron(Fe)	3/6	Plasma Cells	- 6
		Erythroid Cells	- 21

e) BONE MARROW BIOPSY RAT GN-10

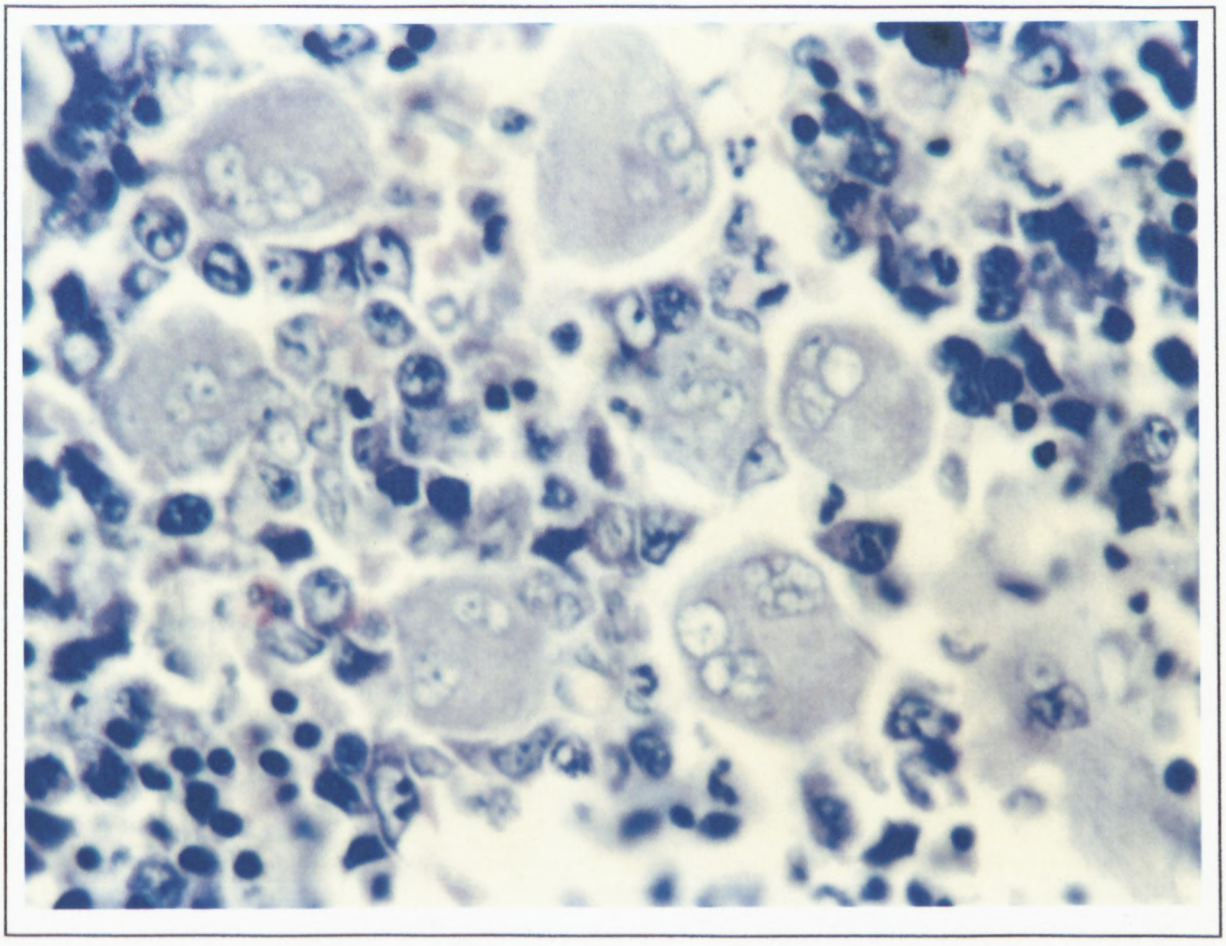

Figure 49

Photomicrograph of bone marrow section of Rat GN-10 showing hyperplastic megakaryocytes.

100% hypercellular marrow with suppressed scattered megaloblastic erythropoiesis were present. Megakaryocytes were grossly hyperplastic but showed little dysplasia. Granulopoiesis was hyperplastic, with maturational delay. There was ineffective granulopoiesis and abnormal numbers of blast cells with small areas showing ALIP. Mast cells were not increased and the marrow stroma was disorganised. Occasional pathological sideroblasts were present.

4.5.22 DIAGNOSIS RAT GN-7; RAT GN-8; RAT GN-9

The findings were consistent with MDS in Rats GN-7, GN-8 and GN-9. This appears to be slightly progressing towards RAEB.

4.5.23 DIAGNOSIS FOR MARROW BIOPSY GN-10

Rat GN-10 was more difficult to diagnose. Rat GN-10 demonstrated a peripheral thrombocytosis of 807,000/mm. The haemoglobin value was 13g/dl and there was stainable iron in the marrow. The platelets appeared morphologically normal. Occasional circulating immature granulocytes were present including a small number of Pseudo-Pelger-Huët cells. The granulocytic lineage was the only marrow element to show definite ineffective cytopoiesis, with an increase in the number of precursor cells not matched by a comparative increase in the number of mature granulocytes in the peripheral circulation. This picture most closely resembles an early phase of a chronic myeloproliferative disorder such as Essential Thrombocytopenia (ET), or chronic myeloid leukaemia (CML). The FAB criteria for ET are listed in Table 46,p.140.

Table 46

Essential Thrombocythemia - Diagnostic Criteria

1. Platelet count >600,000/μL

2. Haemoglobin ≥ 13 gm/dl or normal red cell mass (males >36 mL/kg ; females <32 mL/kg)

3. Stainable iron in marrow or failure of iron trial (<1 gm/dl rise in haemoglobin after 1 month of iron therapy)

4. No Philadelphia chromosome

5. Increased bone marrow reticulin

   A) Absent or
   B) <½ biopsy area without both splenomegaly and leuco-erythroblastic reaction

6. No known cause for reactive thrombocytosis

Marrow reticulin was not stained for, but the lack of circulating teardrop red cells in the peripheral blood suggested marrow reticulin, if present, was minimal.

4.5.24 mtDNA LENGTH MEASUREMENTS RAT GN-7, GN-8, GN-9

Table 47

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.19)	Average Mean Length Value Rat mtDNA
3.23	2.84	2.38	4.82
3.51	3.08	2.59	
4.19	3.68	3.09	
4.22	3.71	3.11	
7.92	6.96	5.84	
7.68	6.75	5.76	
8.52	7.49	6.29	
8.21	7.22	6.06	
11.63	10.23	8.59	
7.93	6.97	5.85	
7.76	6.82	5.73	
8.31	7.31	6.14	
6.54	5.75	4.83	
6.92	6.08	5.10	
6.89	6.27	5.09	
7.13	4.26	5.26	
4.85	3.74	3.57	
4.25	4.20	3.14	
4.78	3.96	3.52	
4.50	7.14	3.32	
8.12		6.00	

4.5.25 mtDNA LENGTH MEASUREMENT RAT GN-10

Table 48

Rat mtDNA Length (cm)	Conversion (0.888)	Mean Length Value Rat mtDNA (% 1.19)	Average Mean Length Value Rat mtDNA
8.26	7.26	6.10	5.20
9.86	8.67	7.28	
10.32	9.08	7.63	
8.73	7.68	6.45	
8.12	7.14	6.00	
7.78	6.84	5.74	
9.21	8.10	6.80	
4.30	3.78	3.17	
4.20	3.69	3.10	
4.50	3.96	3.32	
4.85	4.26	3.57	
4.40	3.87	3.25	

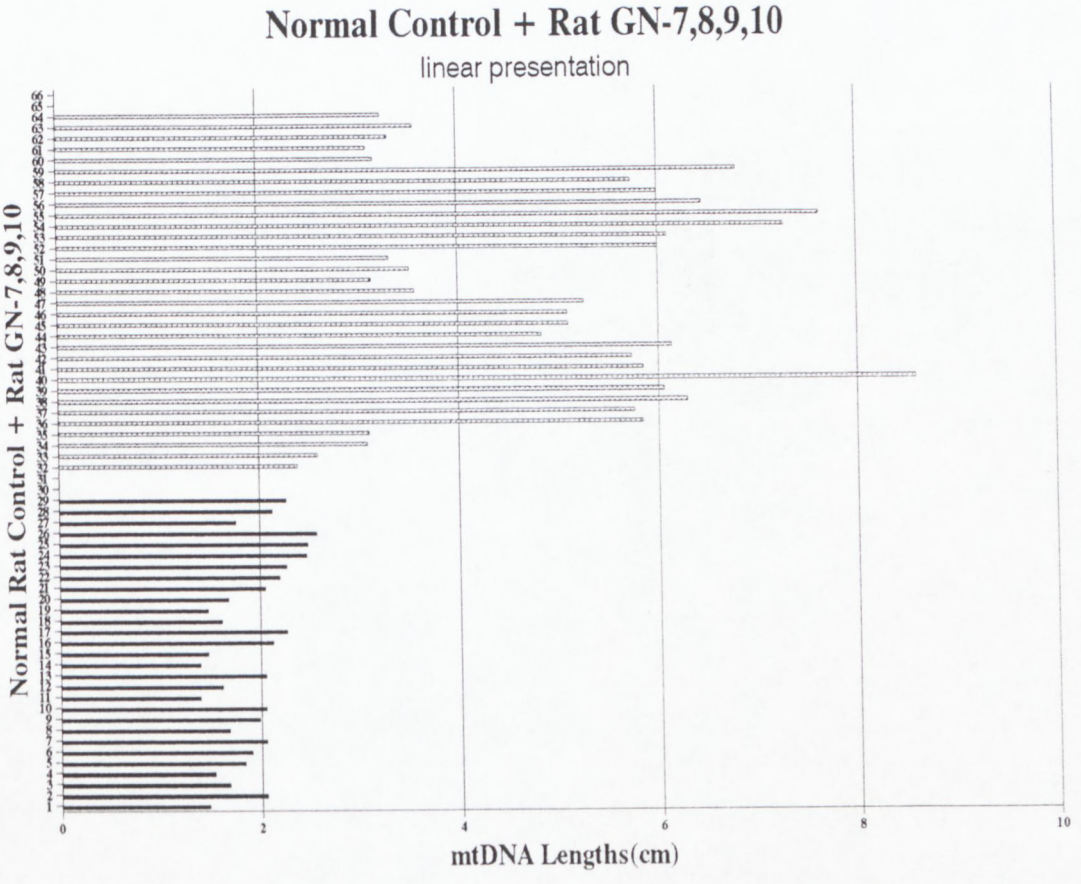

Figure 50

RAT mtDNA MEAN LENGTH VALUES RAT GN-7, GN-8, GN-9, GN-10

Linear presentation of mtDNA of gamma/neutron irradiated rats.

Table 49

Summary of bone marrow biopsy data for gamma-irradiated rat population

G-1	75% Cellularity
Week 3	Mild myeloid maturation delay
	Suppressed erythropoiesis
G-2	30% Hypocellularity
G-3	Fibrinoid myelitis
Week 7	Trilineage dysplasia
G-4	30% Hypocellularity
G-5	Trilineage suppression
Week 11	Trilineage dysplasia
	Micromegakayocytes
G-6	50-60% Recovering cellularity
G-7	++ Mast cells
Week 12	Trilineage dysplasia
	Recovering granulopoiesis with small areas ALIP
	Decreased erythropoiesis
	9% Blasts
G-8	60% Recovering cellularity
G-9	Apoptic megakaryocytes ++
G-10	Maturation delay-ALIP
Week 20	Decreased erythropoiesis
	6% Blasts

Table 50

Summary of bone marrow biopsy data for gamma-neutron irradiated rat population

GN-1	75% Cellularity
GN-2	Suppressed granulopoiesis
Week 3	Myeloid maturation delay
	Dysplastic megakaryocytes
	Suppressed erythropoiesis
	Hypoplastic trilineage dysplasia
GN-3	50% Hypocellularity
GN-4	Suppressed erythropoiesis
Week 7	Dysplastic megakaryocytes
	Hypoplastic granulopoiesis with maturational delay and ALIP
GN-5	Normocellular
GN-6	Recovering erythropoiesis
Week 12	Hyperplastic granulopoiesis with asynchronous delayed maturation
	Micromegakaryocytes - giant platelets
GN-7	100% Hypercellularity
GN-8	Suppressed erythropoiesis
GN-9	Increased megakaryocytes ++ with dysplasia and micromegakaryocytes
Week 14	Hyperplastic granulopoiesis with ALIP
GN-10	Hyperplasctic granulopoiesis
Week 14	Megakaryocyte hyperplasia
	Chronic myeloproliferative disease (Essential thrombocythemia)

CHAPTER 5

DISCUSSION

mtDNA was successfully isolated from 4 normal individuals and from 6 patients with MDS. The average measured length of plasmid pBR322 was 1.56cm on the photographic negative, and the average measured length of normal mtDNA was 3.85cm. The ratio of the molecular size of pBR322(4.3Kb) to normal mtDNA (16.5Kb) was 3.83Kb. This is in close approximation to the average mean length values for mtDNA calculated by these experiments,(3.85Kb).

The molecular weight of pBR322 is $2.83x10^7$ daltons, so that the molecular weight of normal mtDNA is calculated to be;($2.83x10^7$ daltons) x(3.85Kb) = 1.08^9x108 daltons. This is equivocable to the weight of mtDNA calculated from the number of base pairs in the molecule; 1Kb double strand DNA = $6.6x10^6$ daltons.

(16.5Kb) x ($6.6x10^6$ daltons)=$1.089x10^8$ daltons.

Thus, the experimental techniques and the resulting data derived from these mtDNA length measurements was quantitatively significant.

Six patients (4 male,2 female) with MDS were examined for possible mtDNA length mutations in their hematopoietic cell compartment. The clinical data of these patients is summarized in Table 31. All MDS patients demonstrated a significant mtDNA length heteroplasmy which was not observed in the normal control. Two patients with firmly established late phase MDS (Patients A and C) exhibited mtDNA length measurements consistent with gross abnormal dimer formation. These dimers comprised roughly 50% of the total mtDNA measured. As can be observed, the number of these observed mtDNA dimers could not be correlated with the number of blast cells in the individual's marrow; (50% blasts Patient A, 15% blasts Patient C). However, what does appear to correlate

well, is the percentage of myeloid cells (neutrophils, myelocytes, promyelocytes and blasts) in relation to the percentage of erythroid cells in the marrow.

This suggests that the observed mtDNA dimers are formed mainly in the myeloid compartment. The finding that abnormal mtDNA dimers are present in both neoplastic leukaemia cells (the dominant cell type in Patient A) and in the myeloid progenitor cells (the dominant cell type in Patient C), suggests this feature may represent a link between the pathogenesis of MDS and its transformation into acute leukaemia. Also significant, is that the mtDNA dimers found in Patients C and B, were found in patients diagnosed as having secondary MDS as a result of prior radiation/alkylating drug exposure (Patient B) or chronic benzene exposure (Patient C). The myelodyplastic syndromes comprises a heterogeneous group of disorders (RA, RARS, RAEB, RAEB-T and CMML). Although the clinical picture and prognosis vary from case to case, it is thought that MDS patients share a common abnormality in the multipotential stem cell population. This leads to the later formation of abnormal progenitor cells in the 3 primary lineages of the bone marrow. The small number of mtDNA dimers found in the patients with early primary MDS (Patient E and F) suggests abnormal dimer formation may be a critical feature in establishing the severity of MDS at the time of presentation. Dimer formation may also be useful as a marker for the impending development of secondary MDS, following ionizing radiation or chemotherpy (alkylating agent) exposure. In this respect, it was decided to conduct a controlled study of radiation-induced myelodysplasia in an animal model, in an

attempt to determine precisely what phase of MDS development can be associated with abnormal mtDNA dimer formation.

Bone marrow and peripheral blood changes were observed in the gamma-irradiated rat population. The gamma-irradiated rats showed a progressive peripheral pancytopenia following the first and subsequent two additional radiation exposures. Granulopoiesis was markedly suppressed in the marrow following initial radiation exposure, and the overall marrow cellularity remained low until 2 weeks after the last irradiation, when cellularity began a gradual return to normal (marrow regeneration). Erythropoiesis appeared to be less affected than granulopoiesis by the fractionated doses. This was in contrast to the rat population exposed to mixed gamma-neutron radiation doses. Rats in this population demonstrated a severe bilineage decrease in both erythropoiesis and granulopoiesis. Both the gamma and gamma-neutron irradiated populations seemed to develop some degree of radio-resistance after the second fractionated dose of both gamma and gamma-neutron radiation. This was evidenced by the continuing recovery of marrow cellularity even during, and through the third fractionated dose to both rat populations.

There was a distinct lack of the rapid compensatory rise in erythropoiesis in the gamma-neutron (GN) irradiated rat population, which was observed in the gamma-irradiated rats.

This was felt to be indicative of the greater RBE cell killing in the GN rats provided by the neutron component.

The period of hypoplastic marrow recovery began at Week 11 and continued to the end of the experiments for the gamma irradiated rats. Erythropoiesis recovered first, followed by granulopoiesis This was unlike the period of recovery seen in the GN rats, which started at Week 7 with both erythropoiesis and granulopoiesis regenerating and lasting until about Week 12, when there was a sudden decrease in erythropoiesis. Bone marrow samples were taken from the gamma-irradiated and GN rat populations.

From the biopsy summaries (page 143 and 144), it can be seen that the bone marrow response was fundamentally different in the gamma and gamma/neutron rat populations.

The gamma-irradiated rats showed an initial mild bone marrow suppression two weeks after the first fractional dose of radiation. While the GN rats also showed only a low normocellularity two weeks after, the first fractionated dose of gamma-neutrons, trilineage dysplasia similar to MDS and a high normal blast count were prominent features of the marrow.

Two weeks after the second fractionted dose, the gamma-irradiated rats demonstrated hypocellular marrow injury, while the GN rat population showed mixed signs of both MDS and early myeloproliferative disease.

Two weeks after the third fractionated dose, the gamma-irradiated rats demonstrated trileanage dysplasia and a marrow picture consistent with hypocellular MDS.

The GN rats showed definite signs of established MDS with ALIP and micromegakaryocytes. By Week 8 and Week 10 respectively, the gamma and GN rat population had established MDS; however, only the GN rats demonstrated an increase in the number of megakaryocytes in the marrow and 10% of the rats in this population appeared to develop a chronic myeloproliferative disorder (Essential Thrombocythemia), at this time.

Rat mtDNA was successfully isolated from all 20 rats used in these experiments. The average measured length of plasmid pBR322 was 1.51cm on the photographic negatives, and the average mean length value of normal rat mtDNA (16.5Kb) was 3.83cm. This was in close approximation to average mean length of normal rat mtDNA measured in these experiments, and was considered to be quantitatively significant.

Bone marrow samples were taken from the gamma-irradiated rats and analysed for mtDNA dimer formation. Abnormal dimers of mtDNA were first detected in proportionally low numbers to normal in rats G-4 and G-5 during Week 11 of the experiment. This co-incided with increased marrow cellularity granulopoiesis starting to increase over the rate of erythropoiesis. An increased percentage number of mtDNA dimers were present at Week 20 of the experiment when the last rats were sacrificed.

Samples of mtDNA from the gamma-neutron irradiated rat population, also demonstrated abnormal mtDNA dimer molecules not found in the control (non-irradiated) rat population.

These mtDNA dimers were first detected during Week 12 during the period of hypoplastic recovery. The dimers were still present two weeks later (Week 19), when the last specimens were taken. There appeared to be a greater proportional number of these dimers present in the marrow than was demonstratable at Week 20 for the gamma-irradiated population.

It is possible that mtDNA dimers could have appeared anytime between Weeks 7 and 12, but this is uncertain because of the lack of samples taken for this period.

The results of the animal study confirmed an association between radiation-induced MDS and the presence of abnormal mtDNA dimers in the bone marrow compartment. These dimers appear to develop during the period of marrow regeneration following hypoplastic injury. The lack of any hypoplastic effect caused by the third fractionated dose in both the gamma and GN rat populations, may indicate the de-novo development to some degree of radio-resistence by the marrow progenitor cells.

Both the gamma and the GN irradiated rat populations showed an increase in the number of apoptotic megakaryocytes in the marrow. The apoptotic cells were first visible in the gamma-irradiated population at the time of hypoplastic injury, and were later observed in increased numbers in association with the establishment of MDS.

It is as yet uncertain if the increase in apoptotic

megakaryocytes was due to the direct effect of radiation, or if this was a feature of the resulting induced myelodysplasia.

CHAPTER 6

CONCLUSIONS

This study included several objectives which were considered striving for. First, it was necessary to focus attention on the present uncertainties concerning the leukaemogenic risks of low dose/dose-rate radiation exposure. Secondly, it was to find a relative simple yet reliable technique to serve as a platform for individual life scientists to express viewpoints, and to consider the recent radiobiological data on low-dose mutagenesis and its importance for secondary MDS induction.
Lastly, it outlines a possible marker for further study, which may be central to the pathogenesis of MDS and may provide important spin-offs for leukaemia complications associated with conventional chemotherapy and radiotherapy treatment regimes.

In the present study patients with MDS were examined for the presence of mtDNA length mutations(dimers and cocantamers), however, such topological forms have already been reported in the literature in association with human leukaemia where steric considerations suggest that mtDNA dimers are probably non-functional due to supercoiling (Firkin,F.C. *et al*,1979). Thus, it was felt that a progressive accumulation of non-functional dimers in the hematopoietic compartment could account for many of the clinical features associated with MDS. Abnormal mtDNA dimer formation was found in all instances. The proportional number of these dimers were found to roughly correlate with the myeloid/erythroid cell ratio in the bone marrow and it appears likely that the dimers were generated in the myeloid compartment during MDS.

Controlled radiation studies were performed on 20 Wistar rats in an attempt to elucidate the approximate time when abnormal mtDNA dimer formation occured, following fractionated gamma or gamma/neutron irradiation.

Gamma-irradiated rats demonstrated abnormal mtDNA dimer formation at the time hypoplastic marrow recovery was first observed. The lack of any discernable further hypocellular damage to approximately 60% of the marrow, exposed to 400 rad gamma rays, suggests this period of recovery was associated with an acquired radio-resistance in the marrow progenitor cells. Erythropoiesis was least affected in this study.

In contrast, rats exposed to fractionated doses of mixed gamma/neutron radiation, showed a severe decrease in both erythropoiesis and granulopoiesis. This was probably due to the higher RBE for cell killing, associated with the neutron component of the radiation. This population also appeared to develop an acquired radio-resistance after a second fractionated dose. This phenomenon was evidenced by the continued recovery of marrow cellularity during the time the third fractionated dose was administered.

Unlike the gamma-irradiated population, the gamma-neutron irradiated rats showed simultaneous recovery of both erythropoiesis and granulopoiesis. This bilineage recovery continued until Week 12 of the experiment when erythropoiesis abruptly decreased, and the myeloid compartment began hyperplastic over-expansion. The hyperplasia was accompanied by asynchronous myeloid maturation which culminated in the

appearance of myelodysplasia and what appeared to be a case of myeloproliferative disease.

Abnormal mtDNA dimers and concatamers, were initially present in low numbers in the gamma/neutron population at 2 weeks after the first radiation insult. The dimers initially increased in number and were accompanied by the development of a population of what appeared to be deleted dimers during the period of marrow recovery.

At the time of myeloid hyperplasia, the hematopoietic compartment contained a mixture of normal mtDNA dimers, deleted dimers, and a small number of mtDNA cocantamers which had now reappeared. Overall, mtDNA dimers were less in number than normal mtDNA molecules.

This is in contrast with the gamma-irradiated rat population which did not exhibit mtDNA dimers until well into period of marrow regeneration. Overall, the number of dimers tended to increase with time. This correlated with the slow recovery of marrow cellularity with ineffective hematopoiesis as evidenced by the continuing peripheral cytopenia with histological adequate marrow reserve.

Histology studies of the irradiated rat populations, revealed the presence of increased numbers of apoptotic megakaryocytes in the gamma-irradiated rats. A subsequent retrospective study performed on MDS patients confirmed that megakaryocyte apoptosis is a distintive feature of human MDS. A significant correlation was found between the number of apoptotic megakaryocytes in the marrow and increased reticulin. The results support the theory

of Castro-Malaspena (1984) and will most probably be shown to have a prognostic significance should more patients have been studied.

The major findings of this thesis can thus be listed as follows:
1) This study implicates mtDNA in the pathogenesis of secondary MDS and leukaemia.
Hatfill, S.J.; La Cock,C.J.R.; Laubscher,R.; Downing,T.G.; Kirby,R. A role for mtDNA in the pathogenesis of radiation-induced myelodysplasia and secondary leukaemia. Leukemia Research.(1993) Vol 17,11,907-913.
2) mtDNA length heteroplasmy appears to be an early marker of MDS induced by fractionated gamma, or gamma-neutron irradiation. It is possible that this may serve to identify astronauts with an enhanced leukaemia risk during LDSM. An automated in flight assay could probably be developed to detect mtDNA heteroplasmy based on a rotating pulse-field electrophoresis system.
Hatfill,S.J.; La Cock,C.J.R.; Kirby,R. Mitochondrial DNA heteroplasmy in an animal model of secondary human myelodysplasia. Radiation Research,(1994).

3) The apparent relationship between acquired radio-resistance and the appearance of abnormal mtDNA dimers in bone marrow progenitor cells, requires further exploration.

The mechanisms responsible for the generation of abnormal topological forms of mtDNA are unknown, but similar monomeric dimers of plasmid DNA are known to develop only in bacteria,

which express a functional Rec A error-prone DNA repair system.(Clayton D.A.,1969). The presence of abnormal error-prone DNA polymerase activity in acute leukaemia was first demonstrated by Springgate and Loeb in 1973. They demonstrated a 10 fold increased rate of base-pair mismatch when leukaemia cell lysates were subjected to synthetic oligonucleotide replication assay.

Support for the involvement of error-prone DNA repair in radiation-induced leukaemia is provided by studies on Co irradiated Beagle dogs, in which hypoplastic recovery of the bone marrow during chronic irradiation was associated with the appearance of myeloid progenitors with enhanced DNA repair capability, increased radio-resistance, and abnormal leukaemogenic potential (Seed,T.M. *et al*,1987).

It is thus reasonable to assume that a similar error-prone repair system may be involved in the formation of mtDNA dimers in leukaemia, and this could also be in operation during the establishment of secondary MDS.

The hematopoietic mitochondria represent a large cross-sectional target area. It is therefor reasonable to assume that the repair of hypoplastic marrow damage, induced by radiation or alkylating agents, is associated with the replication of stem cells which contain a dose-related number of damaged mitochondria.

Free radical moderation, or the activation of endogenous phospholipase A2 activity, could facilitate the dissociation of these mitochondria from the cytoskeleton in a time-related manner. Several ultrastructural studies have already shown the ability of mtDNA to translocate to the nucleus. Mitochondria have

been observed to undergo fusion with the nuclear membrane of erythroblasts during dyserythropoeisis (Frish,B. *et al*, 1975), and leukaemia blast cells may exhibit direct contact of the inner mitochondrial matrix with the nuclear chromatin over a significant distance (Schumacher,H.R. *et al*,1973). Such fusion events could expose the genomic DNA to a sudden flux of mitochondrial superoxides, hydroxyl radicals and reactive iron atoms which could act to promote error-prone DNA repair activity. It is conceivable that the hypoplastic regeneration of the marrow is accompanied by the repeated fusion of oxidatively damaged "free-floating" mitochondria with the stem-cell or progenitor cell nuclear membrane. These fusion events could induce repeated error-prone DNA repair activity and create cell sub-populations which exhibit progressive DNA sequence randomization, loss of growth control, abnormal maturation and eventual neoplastic transformation. This proposed mechanism is diagrammed in Figure 51.

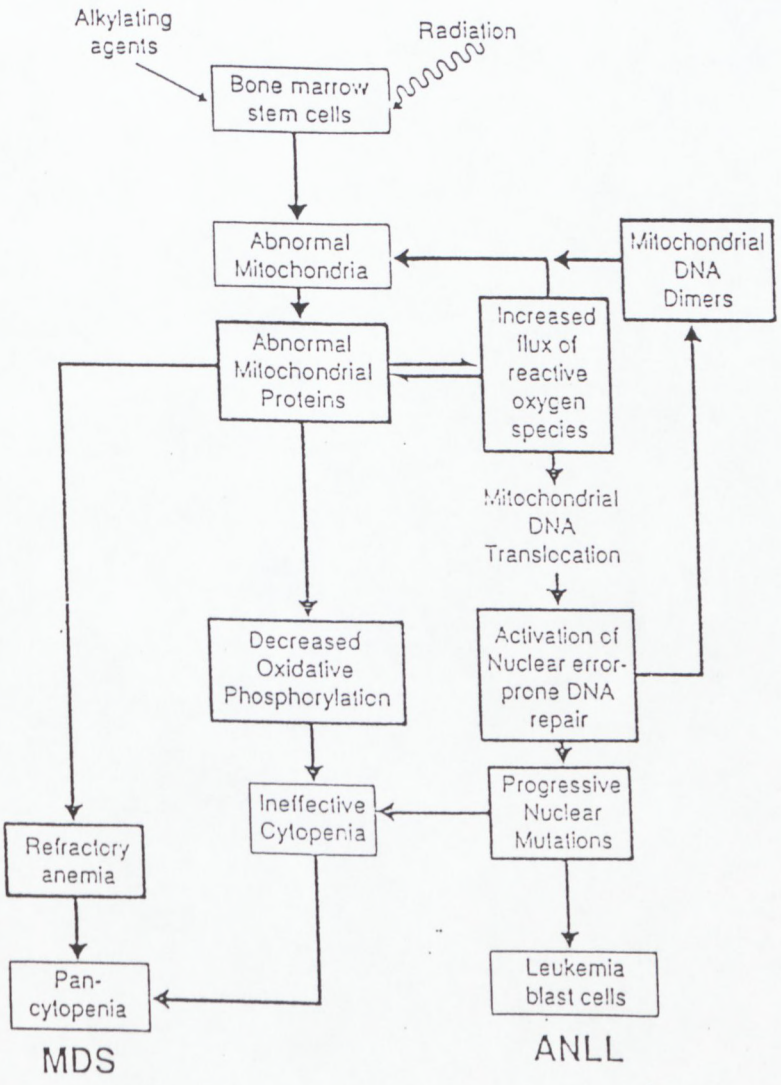

Figure 51

Proposed pathogenesis of secondary myelodysplastic syndrome. A suggested model for the pathogenesis of secondary myelodysplasia and its transformation into acute leukaemia.

##########

REFERENCES

Adel A. Yanis; Seminars in Haematology Vol.10 No.3, 1973

Aksoy M., Erdem S. & Dincol G. (1976) Types of leukemia in chronic benzene poisoning. A study of thirty-four patients. Acta Haematologica 55, 65-72

Bader-Meunier,B.; Rötig,A.(1994) Refractory anaemia and mitochondrial cytopathy in childhood. British Journal of Haematology, 87, 381-385.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Gralnick, H.R., Sultan, C. (1985): Proposed revised classification of acute myeloid leukemia. Am Intern Med 103:626

Castro-Malaspena, H.: Pathogenesis of myelofibrosis: Role of inefective megakaryopoiesis and megakaryocyte components. In Myelofibrosis and the Biology of Connective Tissue. Edited by P.D. Berk, H. Castro-Malaspena, L.R. Wasserman. Alan R. Liss, Inc., New York, 1984

Clayton D.A. & Vinoquad J. (1969) Complex mitochondrial DNA in leukemia and normal human myeloid cells. Proc. Natn. Acad. Sci USA 62, 1077.

Colowick, S.P., Kaplan, N.O. in: Methods in Enzymology Gene Visualization Techniques (edited by Grossman L.) (1969) Volume XXI pp. 417-425

Countenay V.D. (1969) Radioresistant Mutants of L5178y cells. Radiat. Res 38, 186

Cronkite, EP "Kinetics of Cell proliferation" in; Perspectives in Leukemia (Dameshek, Dutcher, eds.) 1968, Grune and Stratton Hon N.Y., 169.

D'Ambrosio S.M., Setlow, R.B., (1978) Postreplication repair in xeroderma pigmentosum cells. Cancer. Res. 38, 1147

Firkin F.C. & Clark-Walker G.D. (1979) Abnormal mitochondrial DNA in acute leukemia and lymphoma. Br. J. Haemat. 43, 201-206

Frisch, B., Lewis, S.M., & Sherman D. (1975). The ultrastructure of dyserythropoiesis in aplastic anemia. Br. J. Haemat, 29, 545.

Fukushi O.F., Akiko, M., et al. (1988) Mutation induction by very low dose y-rays in L5178y cells. Radiat. Res 1145, 273

Galton DAG: The myelodysplastic syndromes. Clin Lab Haematol 6:99, 1984

Greenberg PL, Mara B: The preleykemic syndrome. Correlation of in vitro parameters of granulopoiesis with clinical features. Am J Med 66:951, 1979

Greenberg, P.L., Mara B: The preleukemic correlation of in-vitro parameters of granulopoiesis with clinical features. Am J Med 66:951, 1979.

Groopman, J.E: The pathogenesis of myelofibrosis in myeloproliferative disorders. Ann Intern Med 92:857 1990.

Hall E.J., Miller, R.C., Brenner, D.J. (1991) Neoplastic transformation and the inverse-dose-rate effect for neutrons. Radiat Res 128, 575

Hamblin TJ, Oscier DG: The myelodysplastic syndrome - a practical guide. Hematol Oncol 5:19, 1987

Hatfill S.J.; La Cock,C.J.R.; Laubscher,R: Downing.T.G.; Kirby,R. A role for mtDNA in the pathogenesis of radiation induced myelodysplasia and secondary leukaemia. Leukemia Research.(1993) Vol 17,11,907-913.

Hatfill,S.J.; La Cock,C.J.R.; Kirby,R. Mitochondrial DNA heteroplasmy in an animal model of secondary human myelodysplasia. Radiation Research (1994).

Ihle, J.N., (1978) Conceptual Approaches to Studies of Leukemia; in: Sem. in Hematol., XV(2), 109

Kumana C.R. (1988) Chloramphenicol use and childhood leukemia. Lancet, Feb. 27 (1), 476

Kalf G.F. Rushmore T. & Snyder R. (1982) Benzene inhibits RNA synthesis in mitochondria from liver and bone marrow. Cham.-Biol. Interactions 42, 353-370

Lichter,A.S.; Lawrence,T.S.(1995) Review article: Recent advances in radiation oncolgy. New England Journal of Medicine Vol. 332 No. 6

Lownsend, L.W., Cucinotta, F.A., Wilson, J.W. (1992) Interplanetary Crew Exposure Estimates for GCR. Radiat. Res. 129, 48-52

Letaw J.R., Silberg R., TS20, C.H., (1987) Radiation hazards on Space Missions. Nature, 330, p709

Manyan DR., Arimura G.K. & Yunis A.A. (1972) Chloramphenicol induced erythroid suppression and bone marrow ferrochelase activity in dogs. J. Lab. Clin. Med 79, 137

Michels S.D. et al,1985. Therapy related acute myeloid leukaemia and myelodysplasia: A clinical and morphological study of 65 cases. Blood 65 :1364

Mufti G.J.; Galton D.A.G. The Myelodysplastic Syndrome (1992)

Nachtwey, D.S. (1984) NASA technical memorandum 58255. p31

Olivieri, G., Bodycote, J., Wolff, S., (1984) Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. Science, 223, 594-597

Pearson H.A.; Lobel J.S. ect. The journal of pediatrics Vol 95 No 6 1979 pp 976-984.

Pierre RV: Preleukemic syndrome. Virchows Arch (Cell Pathol) 29:29, 1978

Pitcock JA, Reinhard EH, Justus BW, et al: (1962) A clinical and pathologic study of seventy cases of myelofibrosis. Ann Intern Med 57:73

Rotblat, J. (1988) A Tale of Two Cities. New Scientist. Jan 7, 46-50

Roesch, W.C., et al. (1987) Final Report: US Japan Joint Reassessment of A-Bomb Radiation in Hiroshima and Nagasaki., Rad Effects Res. Foundation, Hiroshima.

Rowley J.D. Golomb H.M. et al. (1981) Nonrandom Chromosome abnormalities in secondary acute nonlymphocytic leukemia Blood, 58 : 759-67

Ruutu, T., The myelodysplastic syndromes (1986) Scand. J. of Haem. Supplement 45, Vol 36

Roth DG, Richman CM, Rowley JD: (1980) Chronic myelodysplastic syndrome (preleukemia) with the Philadelphia chromosome. Blood 56:262

Rotig A., Colanna M., Munnich A., et al. (1988) Deletion of blood mitochondrial DNA in pancytopenia. Lancet, Sept. 3, 567

Saarni M.I., Linman J,W: Preleukaemia:(1973) The haematologic syndrome preceding acute leukaemia. Am J Med 55:38

Sankaranarayanan, K. (1991) Nature of mutations induced by ionizing radiation Mut Res. 258, 3-122

Sankaranarayanan, K. (1993) Ionizing Radiation, Genetic Risk Estimation and Molecular Biology: Impact and Inferences. Trends in Genetics, 9. No. 3. 79-83

Seed, T.M., Kasper, L.V., Tolle, D.V. & Fritz, T.E. (1987) Chronic radiation leukemogenesis: postnatal hematopathologic effects. Leukemia Res. 11, 2171.

Seed, T.M., Kaspar L.V. et al. (1986) Hematopoietic repair modification during preclinical phases of radiation induced myeloproliferative disease. Expl. Hematol. 14 (6) 443

Seed T.M., Kaspar, L.V. et al. (1987) Chronic radiation leukemogenesis Radiat. Res 11(2)171
Saarni MI, Linman JW: Preleukemia: (1973) The hematologic syndrome preceding acute leukemia. Am J Med 55:38

Seed T.M., Chubb G.T. et al (1981) Sequential changes in the bone marrow during continuous low dose gamma irradiation. Scan. Elect. Microsc. 1981 (I) 61

Scoazec J,; Imbert M.; Crists M. etal. (1985) Myelodysplastic syndrome or acute myeloid leukemia? Cancer 55:2390

Springgate, C.F., & Loeb, L.A. (1973) On mutagenic DNA polymerases in human leukemia cells. Proc, natn. Acad. Sci, U.S.A. 70, 245.

Schumacher H.R., Szekely, I.E., & Fisher D.R. (1973) Mitochondria in oncogenesis revisted. Lancet. Nov. 24, 2 (839), 1207.

Silverstein MN: (1974) Postpolycythemia myeloid metaplasia. Arch Intern Med 134:113

Storniolo AM, Moloney WC, Rosenthal D, et al:(1990) The chronic myelomonocytic syndromes. Leukemia,Nov. 4(11) p766-70

Streuli RA, Testa JR, Vardiman JW, et al: (1980) Dysmyelopoietic syndrome: Sequential clinical and cyto-genetic studies. Blood 55:636

vallespi T.; Torrabadella M.; Julia A. et al: Myelodysplastic syndromes: A study of 101 cases according to the F.A.B. classification. Br J Haematology 61:83, 1985

Wallace D.C.(1989) Reviews Human Genetic Disease, Vol 5 no.1 Mitochondrial DNA mutations and neuromuscular disease.

Wetmur J.G., N. Davidson, and J.V. Scaletti, (1966) Biochem, Biophys, Res. Commun. 25, 684

Yau, T.M., Gregg E.C. et al. (1979) Production of radio-resistant mutants in murine L5178y Lymphoma cells. Radiat. Res. 80, 502