INTERACTIONS BETWEEN THE HAEMATOPOIETIC STEM CELL AND THE MYELOID MICROENVIRONMENT IN APLASTIC ANAEMIA.

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DEDICATIONS

TO JULIA, IGOR AND ALEXANDER FOR THEIR UNSTINTING SUPPORT, UNDERSTANDING AND PATIENCE. TO MY PARENTS WHO TAUGHT ME TO SEEK THE TRUTH THROUGH REASON AND WORK.

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"In aplastic anaemia following antilymphocyte globulin therapy the marrow stroma functions normally, while the haematopoietic progenitors have a reduced

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INTERACTIONS BETWEEN THE HAEMATOPOIETIC STEM CELL AND THE MYELOID MICROENVIRONMENT IN APLASTIC ANAEMIA.

SUMMARY

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SUMMARY

In patients with aplastic anaemia that respond to immunosuppressive therapy, quantitative, morphological and functional haematologic derangement have been reported. To explain these findings, abnormalities in the marrow stroma or the stem cell have been postulated. To define the relative contribution of each of the latter, the integrity of the bone marrow from sixteen patients that responded to anti-lymphocyte globulin and high dose methyl prednisolone was compared to normal individuals.

Bone marrow mononuclear cells were divided into two fractions. From the first, stroma was cultured in aMEM containing 12.5% of both horse and foetal calf serum and 10^{-5} M hydrocortisone at 37° C in 5% CO_2 in 90% humidity. The medium was changed weekly. Upon confluence, these stromal layers were studied morphologically and with cytospin preparations stained with Sudan black, O red oil, alkaline and acid phosphatases. The remainder was monocyte and lymphocyte depleted, CD 34+ progenitors were selected with paramagnetic beads and the population morphologically and immunophenotypically defined. To determine the functional status, control or patient CD 34+ progenitors, were suspended for two hours on normal or aplastic stroma for adherence to take place. The non-adhesive fraction was decanted by standardised washing and cultured for fourteen days in the presence of PHA-conditioned medium in the CFU-gm assay. Stroma-adherent progenitors were covered with 0.3% agar and cultured for five days. Aggregates with more than twenty cells were scored (CFU-bl). The remaining CD 34+ cells were cultured in the mixed colony assay with combinations of recombinant cytokines belonging to the G protein super-family and the tyrosine kinase group in dose response studies.

Light density cells from patients with treated aplasia contained significantly fewer CD 34+ cells than those present in the control suspensions (mean 0.65%, SD 0.35% vs 1.62%, SD 1.4%; $p= 0.002$). Normal and aplastic stroma became confluent at three and four weeks. There was no difference on the morphology or the cytochemical stains between the two groups. Functionally, aplastic bone marrow stroma supported CFU-bl formation no differently from normal layers. However, CD 34+ precursors from the patients cultured on

control stroma resulted in significantly fewer CFU-bl (p= 0.0002,) and CFU-gm (p= 0.0009). This work provides original evidence supporting the reduced clonogenicity of the corresponding populations of CFU-bl from patients with aplasia is unrelated to attachment to the stroma, but intrinsic to the CD 34+ cells. Moreover, this study shows for the first time that exposure of these progenitors to growth factors belonging to the G protein and tyrosine kinase receptor families have defective responses, correctable only at supra physiological concentrations, while effects on combinations containing c-kit ligand, appear preserved.

Following immunosuppressive therapy, the bone marrow is repopulated by a hypoproliferative progenitor cell population which responds suboptimally to physiological cytokine stimulation. This suggests that abnormal interactions between receptors and their ligands or alterations in the signal transduction for cell division by the cytokines belonging to the G superfamily lead to suboptimal growth.

25 August, 1993.

TABLE OF CONTENTS

PAGE

Preface i
Dedications ii
Acknowledgments. iii
Congress Presentations and Publications iii
Summary v
List of Illustrations xii
List of Abbreviations xiv
Abstract xv

1. APLASTIC ANAEMIA. BONE MARROW FAILURE OF MULTIPLE ETIOLOGIES

1.1 INTRODUCTION 1

1.2 HISTORICAL BACKGROUND 1

1.2.1 Historical Notes 1

1.3 CLINICAL VARIANTS IN APLASTIC ANAEMIA 2

1.3.1 Congenital Aplastic Anaemia 2
1.3.2 Acquired Aplastic Anaemia 5
1.3.2.a Drug Induced Aplastic Anaemia 5
1.3.2.b Radiation Damage to the Bone Marrow 8
1.3.2.c Aplasia Induced by Viral Infections 8
1.3.2.d Paroxysmal Nocturnal Haemoglobinuria (PNH) 9
1.3.2.e Aplastic Anaemia Associated with Pregnancy 10
1.3.3 Epidemiological Studies in Aplastic Anaemia 11
1.3.4 Bone Marrow Failure. Clinical Features 12
1.3.5 Bone Marrow Failure. Laboratory Findings 12
1.3.6 Aplastic Anaemia. Therapy 14

1.4 PATHOPHYSIOLOGY: INTERACTIONS BETWEEN THE HAEMATOPOIETIC STEM CELL AND THE BONE MARROW MICROENVIRONMENT IN APLASTIC ANAEMIA. SEED OR SOIL? 16

1.4.1 Normal Haematopoiesis 16
1.4.1.a Introduction. Normal Bone Marrow Function 16
1.4.1.b Haematopoietic Growth Factors 17
1.4.1.c Organization of the Haematopoietic System 20
1.4.2 Haematopoiesis in Aplastic Anaemia 23
1.4.3 Rationale for the Study and Concluding Remarks 27

2. QUALITATIVE ABNORMALITIES CHARACTERISE HAEMATOPOIESIS THAT RESTORES MARROW FUNCTION FOLLOWING THERAPY WITH ANTILYMPHOCYTE GLOBULIN (ALG) AND HIGH DOSE METHYL PREDNISOLONE (HDMP) IN APLASTIC ANAEMIA

2.1 INTRODUCTION 30

2.2 STUDY PATIENT POPULATION 31

2.2.1 Aplastic Anaemia. Therapy 32
2.2.2 Laboratory Investigations 32
2.2.2 a Morphological Assessment of the Haematopoiesis in Aplastic Anaemia 32
2.2.2 b Cytogenetic Studies of the Bone Marrow in Patients with Aplastic Anaemia 33
2.2.2 c Granulocyte Functions in Aplastic Anaemia 33
2.2.2 d Lymphocyte Functions and Serum Immunoglobulin Levels 35
2.2.2 e Determination of the Platelet Functions 36
2.2.3 Statistical Analysis 37

2.3 RESULTS OF THE CLINICAL, MORPHOLOGICAL AND FUNCTIONAL STUDIES ON THE BLOOD ELEMENTS IN APLASTIC ANAEMIA 37

2.3.1 Response to Therapy and Long Term Follow up 37
2.3.2 Study Patient Population 39
2.3.2.a Morphological Assessment of the Haematopoiesis in Aplastic Anaemia 39
2.3.2.b Cytogenetic Studies 40
2.3.2.c Results of the Granulocyte Function Studies 40
2.3.2.d Results of Lymphocyte Functions and Serum Immunoglobulin Levels 41
2.3.2.e Functional Studies of the Platelets. Results 41

2.4 DISCUSSION OF THE FINDINGS 43

3 BONE MARROW PURIFICATION AND SELECTION FOR THE PROGENITOR CELL POPULATION. RESPONSE TO RECOMBINANT GROWTH FACTORS

3.1 INTRODUCTION 48

3.2 METHODS FOR THE PURIFICATION AND SELECTION OF THE HAEMATOPOIETIC STEM CELL POPULATION 49

3.2.1 Progenitor Cell Selection 50
3.2.1.a Progenitor Cell Antibody-Mediated Plate Binding (Greenberg 1986) 50
3.2.1.b Antibody-Coated Magnetic Bead progenitor Cell Selection 51
3.2.2 Immunofluorescence Studies of the Selected Cell Populations 52
3.2.3 Definition of the Selected Cell Population. Clonogenic Assays 52
3.2.3.a Haematopoietic Growth Factors 52
3.2.3.b Clonogenic Assays of the Selected Population 53
3.2.3.c Dose Response to Growth Factor Combinations 53

3.3 RESULTS OF THE SELECTION FOR THE HAEMATOPOIETIC PROGENITOR CELLS 53

3.3.1 Progenitor Cell Selection: Morphology and Immunofluorescence Studies 53
3.3.1.a Selection by Antibody-Mediated Plate Binding 54
3.3.1.b Selection by Antibody-Coated Magnetic Beads 54
3.3.2 Clonogenic Assays. Dose Response 54

3.4 DISCUSSION OF THE RESULTS 60

4 BONE MARROW STROMA AND STEM CELL FUNCTION IN UNTREATED PATIENTS AND PATIENTS WITH APLASTIC ANAEMIA RESPONDING TO ANTI-LYMPHOCYTE GLOBULIN. PILOT STUDY

4.1 INTRODUCTION 63

4.2 PATIENTS WITH APLASTIC ANAEMIA AND METHODS 64
4.2.1 Description of the Patient Population 64
4.2.2 Bone Marrow Cells 65
4.2.2.a Cultures of the Bone Marrow Stroma 65
4.2.2.b Blastic Colony Assay (CFU-bl) 65
4.2.2.c Immunomagnetic Selection of the Haematopoietic Progenitor Cells 66
4.2.2.d Clonogenic Tests. Mixed Colony Assay 66

4.3 RESULTS 66
4.3.1 Cultures of the Bone Marrow Stroma 66
4.3.2 Blastic Colony Assay 67
4.3.3 Immunomagnetic Progenitor Cell Selection and Mixed Colony Assay 67

4.4 DISCUSSION OF THE RESULTS 67

5 IN APLASTIC ANAEMIA THE BONE MARROW STROMA DOES NOT INHIBIT NORMAL CLONOGENIC GROWTH

5.1 INTRODUCTION 71

5.2 STUDY POPULATION 73
5.2.1 Patients with Aplastic Anaemia 73
5.2.2 Normal Control Population 73

5.3 METHODOLOGY FOR THE CULTURE OF THE BONE MARROW STROMA AND THE BLASTIC COLONY ASSAY 74

5.3.1 Bone Marrow Cells 74
5.3.2 Cultures of the Bone Marrow Stroma 74
5.3.3 Selection of the Haematopoietic Progenitor Cell 75
5.3.4 Blastic Colony Assay 75
5.3.5 Morphological and Cytochemical Assessment 76
5.3.6 Statistical Analysis 77

5.4 THE BONE MARROW STROMA IN APLASTIC ANAEMIA. RESULTS OF THE BLASTIC COLONY ASSAY 77
5.4.1 Morphology and Cytochemical Staining of the Stroma 77
5.4.2 Selection of the Haematopoietic Progenitor Cells 78
5.4.3 Blastic Colony Assay 78
5.4.4 Stroma Non-Adherent Clonogenic Growth 80

5.5 DISCUSSION OF THE RESULTS 81

6 THE BONE MARROW PROGENITOR CELLS IN APLASTIC ANAEMIA HAVE A SUB OPTIMAL RESPONSE TO GROWTH FACTORS

6.1 INTRODUCTION 88

6.2 PATIENT POPULATION AND METHODOLOGY 89
6.2.1 Selection of the Haematopoietic Progenitor Cells 90
6.2.2 Clonogenic Assays of the Selected Progenitor Cell Population of the Bone Marrow 90
6.2.2.a Haematopoietic Growth Factors 90
6.2.2.b Clonogenic Assays. Dose-Response 91
6.2.3 Statistical Analysis 91

6.3 RESULTS OF DOSE RESPONSE AND GROWTH FACTOR COMBINATION STUDIES 92

6.3.1 Selection of the Bone Marrow Progenitor Cells 92
6.3.1.a Clonogenic Assays. Dose Response 92
6.3.1.a (i) Erythropoietin (Epo) 92
6.3.1.a (ii) Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) 93
6.3.1.a (iii) Interleukin 3 (IL-3) 93
6.3.1.a (iv) Stem Cell Factor (c-kit ligand) 95
6.3.1.b Growth Factors in Combinations 96
6.3.2 Multivariant Analysis 96

6.4 DISCUSSION OF THE RESULTS 97

7 SUMMARY OF THE OBSERVATIONS AND FINAL CONCLUSIONS

7.1 INTRODUCTION 104

7.2 MAIN OBSERVATIONS OF THE THESIS 106

7.3 SIGNIFICANT CONCLUSIONS OF THE THESIS 108

7.4 FINAL COMMENTS 110

8 REFERENCES 113

LIST OF ILLUSTRATIONS

PAGE

TABLE

1.1 Severe acute aplastic anaemia (Diagnostic Criteria) 13
2.1 Post-acute aplastic anaemia 40
2.2 Quantitative evaluation 41
2.3 Morphological evaluation 42
2.4 Granulocyte functions 44
2.5 Platelet functions 45
3.1 Progenitor cell selection. Comparative study 56
3.2 Dose response 57
4.1 Study population. Clinical and laboratory data 64
4.2 Selected populations (Colony formation) 69
5.1 Cytochemical definition of the bone marrow stromal layers 78
5.2 Progenitor cell selection 82
5.3 Stroma adherent and non-adherent colonies 83
6.1 Clonogenic assays 94
6.2 Growth factors in combinations 97

FIGURES

2.1 Survival of patients with acute aplastic anaemia treated with ALG + HDMP 38
3.1 Immunomagnetic selection 55
3.2 Morphological details of the positively selected population 55
3.3 Morphology of a BFU-e colony at a plateau concentrations of growth factors 58
3.4 Details of an immature BFU-e colony 58
3.5 Morphological details of a mixed erythroid and myeloid colony 59
3.6 Eosinophilic colony 59

3.7 Granulocyte-Macrophage colony cultured in the presence of GM-CSF 61
3.8 Morphological details of a granulocytic colony 61
4.1 Stromal blast cell colony assay on normal and aplastic stroma 68
4.2 GEMM: CFU assays results 69
5.1 Normal confluent bone marrow stroma 79
5.2 Normal confluent bone marrow stroma. Contrast microscopy 80
5.3 Blastic colony assay. Contrast Microscopy 81
5.4 Blastic colony assay. May Grunewald Giemsa 84
5.5 Details of a day 5 stromal colony. May Grunewald Giemsa 84
5.6 Contrast microscopy of a day 7 stromal colony derived from Aplastic CD 34+ progenitors 85
5.7 Clonogenic growth. Stroma adherent versus non-adherent CD 34+ progenitors 86
6.1 a) and b) Dose response of aplastic and control CD 34+ cells 99
6.2 Per cent change in clonogenic growth by the addition of growth factor combinations. 101

LIST OF ABBREVIATIONS

ALG:	Anti-lymphocyte Globulin
ALG+HDMP:	Anti-lymphocyte Globulin plus High Dose Methyl Prednisolone.
aMEM:	Alpha Minimal Essential Medium.
BFU-e:	Burst Forming Unit-erythroid.
CD:	Cluster Designation.
CFU-bl:	Blastic Colonies.
CFU-e:	Colony Forming Unit-erythroid.
CFU-gm:	Colony Forming Units-granulocyte, macrophage.
CFU-S:	Colony Forming Units-Spleen.
CSA:	Cyclosporin A.
CSF:	Colony Stimulating Factors.
Epo:	Erythropoietin.
G-CSF:	Granulocyte-Colony Stimulating Factor.
GF:	Growth Factor.
GM-CSF:	Granulocyte-Macrophage Colony Stimulating Factor
GPI:	Glycosylphosphatidyl-Inositol-Linked Proteins
HSC:	Haematopoietic Stem Cell
IFN-g:	Interferon gamma
IL-3:	Interleukin 3.
IMDM:	Iscove's Modified Dulbecco's Medium.
MDS:	Myelodysplastic syndromes
PNH:	Paroxysmal Nocturnal Haemoglobinuria.
PBS:	Phosphate Buffered Saline.
ppm:	Parts Per Million.
***Sl*:**	Steel Locus.
SAA:	Severe Aplastic Anaemia.
SCF:	Stem Cell Factor; c-kit ligand.
SL:	Stromal Layers.
TNF:	Tumor Necrosis Factor
W/W:	White Spotting Locus.

ABSTRACT

Introduction: In patients with aplastic anaemia lacking a bone marrow transplant option, immunosuppressive therapy can restore haematopoiesis. However, recovery is often incomplete and a proportion of the responders relapse. Extended follow up has indicated that in some, morphological and functional abnormalities of the blood elements occur, while in the bone marrow a marked reduction in the *in vitro* clonogenic pool has been described. In this group, particularly, clonal and malignant disorders may arise.

Based on *in vitro* bone marrow culture and animal models, alterations in the myeloid microenvironment and quantitative or qualitative abnormalities of the haematopoietic stem cell have been proposed. The objective of the current thesis was to define the contributions of each pathophysiological defect in a well-defined cohort of patients.

Patients: Thirty-six subjects that presented with features of severe aplasia received intravenously an infusion of horse anti-human lymphocyte globulin (ALG) 50 mg/kg, following a bolus of 500 mg/kg methylprednisolone. Seventy one percent responded and at a median follow-up of four years, 65% are alive in response. Of this group, 16 were available and consented to participate in this study.

Methods: This analysis consisted of morphological and quantitative determinations of the blood elements At the same time, peripheral blood functional tests were undertaken and these included generation of hydrogen peroxide, migration, phagocytosis and granulocytes killing of Candida Albicans, mitogenic lymphocyte responses and immunoglobulin production, bleeding time with platelet aggregometry and complement sensitivity of the red cell (Ham's test) by standard techniques. At the same time, bone marrow trephine biopsy and aspirates were obtained for morphological appraisal, cytogenetics studies with *in vitro* cultures of selected clonogenic progenitors and their marrow stroma and compared with 7 normal volunteers.

For the evaluation of the haematopoiesis, bone marrow mononuclear cells were recovered by density gradient sedimentation and divided into two fractions. From the first, confluent stromal layers were prepared, while from with the second a lymphocyte and monocyte depleted population was obtained and enhanced for CD 34+ cells by immunomagnetic selection.

In cross-culture experiments, 1 $x10^4$ CD 34+ progenitors from patients or controls were seeded on to preformed, confluent normal and aplastic bone marrow stromal layers. Adhesive precursors were scored for the formation of blastic colonies (CFU-bl) after 5 days incubation. Non adherent positively selected cells were defined in the CFU-gm assay. In a second step a corresponding aliquot of the CD 34+ fraction was subjected to dose response studies in the mixed colony assay using combinations of erythropoietin, G, GM-CSF, IL-3 and c-kit ligand.

Results: At a median of 4 years (range 1-8) following therapy, the median haemoglobin was 134.5 g/L (SD 27.2), MCV 98 fL (SD 7.17), granulocytes 2.9 $x10^9$/L (SD 1.89), lymphocytes of 1.65 $x10^9$/L (SD 0.71), monocytes of 0.27 $x10^9$/L (SD 0.2) and platelets 127 $x10^9$/L (SD 80.44). Two patients developed a positive Ham's test with clinical features of paroxysmal nocturnal haemoglobinuria (PNH). Peripheral blood and bone marrow morphology was normal in three patients only. Variable degrees of dyserythropoiesis and megaloblastosis were seen in the rest. Although excessive numbers of blasts were not found, features of dysplasia were documented in one (2 patients), two (6 patients) and three cell lineages (5 patients) with abnormal localization of premature precursors (3 patients).

Suitable metaphases in 15 patients showed a constitutionally abnormal karyotype in one subject (47,XX +C), while in 5, random chromosomal breaks and gaps ranging from 13 to 40% of cells were demonstrated.

Of 13 subjects tested, granulocyte functions were within the normal range in only one patient. For the group, these were significantly abnormal for the generation of peroxide at 2.79 ηmol/min/10^6 (SD 0.6) ($p= 0.007$) and phagocytosis at 53% (SD 12.8) ($p= 0.002$).

Results of mixed lymphocyte cultures and following stimulation of immunocytes with mitogens were normal in 12 patients but were subnormal in both subjects receiving cyclosporine therapy. Serum immunoglobulins were found to be within the normal range in all. Assessment of the platelet function demonstrated that the median bleeding time was 8.5 minutes (SD 3.4), was normal in five subjects of the fifteen tested, while in 3 it was more than 15 minutes. Aggregation studies were deranged in all the patients, with significantly sub optimal values for ADP, adrenaline, collagen and Ristocetin.

Studies on the bone marrow showed that when mononuclear bone marrow cells were corrected to equal numbers, there were significantly fewer light density cells displaying CD 34 antigen in the aplastic group (mean 0.65%, SD 0.35%) compared to normal control (1.62%, SD 1.4%; $p= 0.002$).

Both normal and aplastic stroma became confluent at a median of four weeks. They were morphologically and cytochemically indistinguishable, supporting equally the growth of CFU-bl from normal marrow selected cells (mean 117, SD 20.4 vs 103.1 SD 30.4 respectively); However, for the CD 34+ aplastic progenitors cultured on control layers this value was significantly reduced (mean 41.06; SD 42.9; $p= 0.0002$, exact two tailed test). In a similar manner, the stroma non-adherent positively selected population had a reduction in the number of CFU-gm (mean 142.6; SD 104.8) when compared to normal precursors (mean 361.7; SD 91.3) CFU-gm with significantly lower total (CFU-bl+CFU-gm) clonogenic output ($p= 0.0009$).

In the mixed colony assay, results were conflicting. At low concentrations of Erythropoietin, IL-3 and GM-CSF the positively selected aplastic progenitor cells had uniformly inferior colony formation compared to control cultures ($p < 0.05$). However, they improved significantly, matching normal clonogenic growth with escalating doses. In contrast, cultures supplemented with growth factors

containing c-kit ligand, were significantly stimulated over the baseline values, proportionally more intensely than control progenitors. At effective concentrations, colony scores for BFU-e and CFU-gm were highest than any other combination, with values not significantly different from the control studies. Multivariate analysis segregated combinations containing appropriate concentrations of this ligand, suggesting that interactions originated by this cytokine were independent from all other combinations.

From these simultaneous studies, it is concluded that even after an extended period following effective immunosuppressive therapy there are widespread morphological, functional and cytogenetic abnormalities in the haematopoitic cells. *In vitro* studies of the marrow progenitors demonstrated that the stroma derived from patients with aplasia is not morphologically different from control adherent layers, and supports the growth of control blastic colonies normally. Therefore, the reduction in the clonogenic growth of the corresponding populations of CFU-bl in the study group was unrelated to defective attachment to the marrow stroma, but intrinsic to the CD 34+ cells.

Furthermore, exposure of these selected progenitors to growth factors belonging to the GTPase and tyrosine kinase receptor families, demonstrated defective responses to the former group that were correctable only at supraphysiological concentrations of cytokines. However, growth in culture supplemented with combinations containing c-kit ligand, which belongs to the later group, appeared preserved.

It is reasoned that following immunosuppressive therapy the bone marrow is repopulated by a hypoproliferative progenitor cell population which responds suboptimally to physiological cytokine stimulation. This is consistent with the clinical experience. Although it remains unexplained, it suggests that in these bone marrow precursors either abnormal interactions between receptors and their ligands or alterations in the signal transduction for cell division by the cytokines belonging to the G superfamily lead to suboptimal growth. This explains the persistent subnormal blood values in patients with aplastic anaemia and may relate to the morphological and functional derangements described.

CHAPTER 1

APLASTIC ANAEMIA. BONE MARROW FAILURE OF MULTIPLE AETIOLOGIES

1. 1 INTRODUCTION

Normal haematopoiesis requires .the daily production of 1-2 x 10^{11} erythrocytes, granulocytes and platelets (Gale *et al,* 1981). These high generation rates and the relatively short life span of the differentiated cells mean that any compromise in the normal bone marrow function will produce serious clinical consequences.

Aplastic anaemia is a disorder characterized by a partial or total arrest in the haematopoiesis due to a loss of this marrow regenerative capacity, leading to symptomatic pancytopenia that without appropriate therapy, will result in death from irreversible bone marrow failure.

1. 2 HISTORICAL BACKGROUND

1. 2. 1 Historical Notes

Paul Erlich in 1888 gave the first description of this disease, reporting peripheral blood pancytopenia with "yellow" hypo cellular marrow in a 21 year-old pregnant female (Erlich, 1888). He vividly described the triad of presenting symptoms: anaemia, infection and haemorrhage. Ehrlich deduced that the primary lesion was located in the haematopoietic tissues, expressing itself as failure of regeneration. However it was Chauffard who first gave the term aplasia to this syndrome (Chauffard, 1904). Since then several thousand patients have been described with similar clinical presentations.

Regrettably, in the early days marrow examination was not a routine diagnostic procedure, so that the terminology was employed interchangeably with other terms for bone marrow failure used at the time, including hypoplasia of the marrow, aleukaemia haemorrhagica, toxic paralytic anaemia, Fanconi's anaemia, Diamond-Blackfan anaemia, etc.

With the development of radiation biology during the 1940s, the bone marrow was recognized as a particularly susceptible organ to this type of radiation-induced damage, directed particularly at the stem cell pool. This observation led to the development of reproducible models for aplasia. Animal systems were designed to quantify marrow injury and study the kinetics of cell recovery. These methods suggested that in humans, bone marrow stem cell infusions might have a potential curative role in aplasia.

However, reports of similar presentations, where pancytopenia was associated with the exposure to certain chemicals such as benzene derivatives, insecticides, drugs, and viral infections led to a more systematic search for the pathogenetic mechanisms initiating this syndrome. Recently, the epidemiology and the clinical course of the disease were more precisely defined, this led to practices where the supportive use of haematopoietic growth factors were combined with definitive therapies such as bone marrow transplantation or immune modulation, resulting in significant improvement in the outcome.

1. 3 CLINICAL VARIANTS IN APLASTIC ANAEMIA

1. 3. 1 Congenital Aplastic Anaemia

The etiology of aplastic anaemia is unknown. Certain associations have been proposed, however. The occurrence of congenital somatic abnormalities in siblings with this disorder was first described by Fanconi (Fanconi, 1927). Following this report, children without the visceral and bony deformities or a clear-cut family history have also been cited (O'Gorman *et al,* 1974), bringing some confusion into the characterization of this group.

It was further proposed that the term of "constitutional anaemia" be used to describe this heterogeneous group of inherited diseases. This group included congenital hypoplastic anemia (Diamond-Blackfan) (Viskochil *et al,* 1990), familial aplastic anaemia with or without physical abnormalities and congenital amegakaryocytic thrombocytopenia. It was then suggested that the term Fanconi's anemia be applied to the familial marrow hypoplasia with somatic malformations, generally manifested during the first decade of life (Williams *et al,* 1985).

However, the current working definition of Fanconi's Anaemia requires the demonstration in lymphocyte cultures of chromosomal breakage, with cross linking agents such as mitomycin C or diepoxibutane. Employing these criteria, a recent survey by the International Fanconi Anaemia Registry (IFAR) noted that 37% had no physical abnormalities, 31% had no aplasia while 7% had neither (Auerbach *et al,* 1989, b).

Inheritance in this type of genetic anaemia, follows an autosomal recessive pattern. Nevertheless, familial occurrence has also been described, with common HLA-DR2 in another two reported cases. Since this phenotype is frequently found in patients with various autoimmune disorders, this could suggest a genetically inherited susceptibility in the development of the disease (Shimoda *et al,* 1990).

Although the precise pathogenetic mechanisms still remain unknown, reduction in the clearance of oxygen radicals (Porfirio *et al.* 1989) and increased cellular sensitivity to DNA cross linking agents that appeared related to defective endonuclease activities, have been reported (Lambert *et al,* 1992). Furthermore, employing functional complementation methods, a specific mutant genetic product has been cloned representing a particular response to the DNA damage (Strathdee *et al,* 1992).

On the cellular level, it is postulated that these abnormalities interact with the multiple signals that regulate the development of the haematopoietic and other cells. This is consistent with the recent finding of abnormal *in vitro* production of interleukin 6 (IL-6) by bone marrow cells. Since this cytokine is intimately involved

in the differentiation of early haematopoietic progenitors, these results may explain some of the described clinical and laboratory observations (Roselli *et al,* 1992).

Clinical presentation occurs at a median age of 8 years (0-48) and is typically characterized by manifestations of progressive pancytopenia with marrow hypocellularity. Chromosomal analysis demonstrate cytogenetic abnormalities in high frequency (Athale *et al,* 1991). About 10% will develop acute leukaemia.

Although the only cure for Fanconi's anemia is bone marrow transplantation, initially patients are responsive to a variety of treatments. These typically include corticosteroids and androgenic steroids, individually or in various combinations (Rogers *et al,* 1989). The projected median survival is 27 years for those reported to IFAR (Auerbach *et al,* 1989 b). Male fertility is apparently reduced, with abnormal spermatogenesis and hypogonadism. Interestingly, as many female patients are now surviving beyond puberty despite an increased risk for toxemia, more successful pregnancies are being reported. During this period significant reductions in the blood counts are often observed. These may require supportive measures, which include blood product transfusions (Alter *et al,* 1992).

The congenital anaemia described by Diamond and Blackfan is characterised by intense reticulocytopenia with a normocellular bone marrow, occurring during early childhood. Inheritance is heterogeneous, with examples of autosomal recessive, autosomal dominant, or more often of sporadic occurrences. Typically fetal haemoglobin is elevated with the fetal γ / α pattern and over-expression of the *i* antigen on red cell membranes (Halperin & Freedman, 1989).

Although in this disease the emphasis remains on the erythroid precursors, abnormalities in the lymphoid differentiation have also been described. *In vitro* studies indicate a diversity of defects, with resistance to erythropoietin (Tsai *et al,* 1989) or IL-3, and suggest an intrinsic progenitor cell disorder. Typically, patients respond rapidly to corticosteroid therapy, nevertheless relapses have been reported. Those failing to improve or needing large maintenance doses of hormonal treatment may require long term blood transfusions. In these

circumstances provision should be made for iron chelation therapy.

1. 3. 2 Acquired Aplastic Anaemia

Acquired aplasia is by far the most frequent presentation and is the subject of this thesis.

1. 3. 2 (a) Drug Induced Aplastic Anaemia

Drugs are a well known cause of marrow failure and in some instances such as the myelosuppression associated with cancer therapy, this is predictable. However, many other chemicals have also been implicated in bone marrow toxicity as a side effect, but because this occurrence is infrequent, cause and effect have been difficult to demonstrate (Roberts, 1990). In others as exemplified by the antibiotic chloramphenicol (Yunis *et al,* 1980), or certain anti-thyroid preparations (thyouracil, methymazole) (Biswas *et al,* 1991) it is an undesirable toxic effect. This is frequently unforeseen and unrelated to dose. It can occur weeks to months after the drug treatment has been initiated, or even after the agent has been withdrawn, reflecting a genetic predisposition (Roberts *et al,* 1990; Kelly *et al,* 1991).

Spurred on by the potential toxicity of pharmaceutical preparations and particularly following the description of chloramphenicol-induced marrow damage, the American Medical Association Council on Drugs established a Sub-Committee on Drug Induced Blood Dyscrasias in 1955. This group had the task of giving the medical profession early warnings on the haematological side effects of new pharmaceutical products. Many such medications have been included in a list, although most of those described have a low occurrence for the aplastic complication. Nevertheless, to facilitate rapid reporting of such events, recently in Paris, standard designations of the various forms of drug induced cytopenias and criteria for causality assessment have been more clearly defined (Standardisation of definition and criteria of causality assessment of adverse drug reactions, 1991).

In the case of chloramphenicol, two syndromes have been described. One follows a dose-dependent inhibition of the bone marrow, while the other is a dose-unrelated irreversible aplasia. The postulated mechanisms in the former are alterations in mitochondrial protein synthesis, causing suppression of haem production and damage to cytochromes, leading to arrest of respiration and proliferation. These manifestations, however, are reversible upon withdrawal of the agent.

The second clinical problem occurs in genetically predisposed individuals where the NO_2 radical undergoes metabolic reduction. This reaction leads to the formation of highly reactive nitroso groups, which are the effectors for the irreversible DNA damage and lethal marrow aplasia (Yunis *et al,* 1980, 1984). The related compound Tiamfenicol, which differs chemically in the NO_2 radical, also induces a dose-dependent marrow inhibition. However, with this agent, the irreversible aplasia has not been reported.

In a recent population-based case control study of patients receiving cardiovascular drugs conducted in Europe and Israel which surveyed a population of 23 million inhabitants, the risk for agranulocytosis or aplastic anaemia was found to be significant for propanolol (2.5), dipyridamole (3.8), digoxin (2.5) and acetyldigoxin (9.9). In addition, excess toxicities were also found with cinepazide, procainamide and furosemide (Kelly *et al,* 1991).

Irreversible idiosyncratic reactions to carbamazepine, although rare, have been increasingly reported. This has resulted in specific warnings and recommendations from the manufacturer for regular monitoring of the haematological parameters in those receiving this therapy. Leukopenia has been described in 7% of adults and 12% of children, but has often reversed despite continuation of the drug (Wyllie & Wyllie, 1991, Sobotka *et al,* 1990). Those with initial low leukocyte counts appear to carry the highest risk (Sobotka *et al,* 1990).

Among the anti-inflamatory medications, similar reactions were reported following phenylbutazone intake, leading to restriction in its use. Nevertheless, despite attempts to produce safer preparations, blood discrasias were described in

association with nearly every antirheumatic agents, including diclofenac (Eustace *et al,* 1989), ticlopidine (Pioda *et al,* 1989), naproxene (Sanal & Gur-Lavi, 1992), D-penicillamine (Fishel *et al,* 1989). However, when scientific epidemiological principles were applied in the reporting of some of these agents, a significant rise in the incidence of blood discrasias could not be confirmed (Imperiale & Horwitz, 1989).

Benzene, used as a solvent in industry for more than 50 years, has been inconsistently implicated in cases of marrow aplasia (Paci *et al,* 1989; Aksoy, 1989). Although this chemical has been consistently classified as non-mutagenic on the Ames test, it has also been shown to cause pancytopenia and significant cytogenetic abnormalities in laboratory animals (Kissling & Speck, 1972).

The metabolism is complex, yielding glucuronide and sulphated conjugates of phenol, quinol and catechol, L-phenylmercaptopuric acid and muconaldehide. Quinol is oxidized to p-benzoquinone. This compound binds to vital cellular components or undergoes redox cycling to generate oxygen radicals. Muconaldehides are also toxic through depletion of intracellular glutathione. The formation of oxygen radicals by the described steps, appears to be the major mechanism of benzene toxicity. However, other pathways including synergism between arylation and glutathione depletion that lead to the creation of more reactive oxygen radicals, may also become operative. These reactions lead to suppression of DNA synthesis or chromosomal breaks (Yardley-Jones *et al,* 1991).

In the industry, safety regulations by the US Government initially limited the atmospheric exposure of benzene to 10 parts per million (ppm) (Elkins & Pagnata, 1956), but recently this was lowered to 5 ppm and to 1 ppm in the United Kingdom.

However a number of related chemicals used as household cleaners or in the general industry have also been associated with this toxicity including toluene, the insecticides DDT (Sanchez-Medal *et al,* 1963, Rugman & Cosstick, 1990), lindane (Rauch *et al,* 1990) and others. Despite extended experience, the long term-safety

of organochlorines remains doubtful, as they were introduced before adequate toxicological screening tests had been developed.

Nevertheless, despite careful history taking, in more than 50% of patients, none of the drugs or chemicals commonly associated with aplasia can be elicited (Haak, 1978, Novitzky *et al,* 1991 b). This may follow the general perception that certain medications purchased over the counter, cosmetics and hair or other dyes are not considered as drugs and often not remembered.

This and other susceptibilities have been linked to specific HLA types. In this regard the association of aplastic anaemia and HLA phenotype has been investigated by several researchers. Although pilot studies suggested such an interaction, in larger series, no excess frequencies for any antigen or haplotypes were found (Albert *et al*, 1976, Haak, 1978).

1. 3. 2 (b) Radiation Damage to the Bone Marrow

Ionizing X-rays, gamma rays or neutron radiation in sufficient doses cause molecular changes with formation of ions, peroxides and free radicals, which in turn may transfer the absorbed energy to critical macromolecules, leading to cell death (Borel *et al,* 1965).

In the marrow, all haematopoietic elements are highly sensitive to this form of energy. Long-term continuous exposure to small amounts of external or internally deposited radiation have been followed by aplastic anaemia, which may develop even months or years after exposure (Court-Brown & Dooll 1957).

1. 3. 2 (c) Aplasia Induced by Viral Infections

Viruses have also been extensively implicated in bone marrow failure (Rosenfeld & Young 1991). Post hepatitis aplasia is typically caused by the C virus, an organism similar to the flaviridiae, a family of microbes of known cytopathic effects on the myeloid organ. Onset of aplasia occurs while jaundice and other manifestations are resolving, with an incidence of 0.1-0.2% of those infected, representing 2.5 - 8.7% of cases of aplastic anaemia. With a lower frequency, both hepatitis A and B have

similarly been implicated, although with the latter, coexistence with the C virus further complicates the etiologic interpretations.

However, as some patients with active hepatitis and marrow aplasia may lack the described serological or hepatic tissue viral markers, a distinct non-A, non-B, non-C organism which induces a CD 8+ lymphocytosis is increasingly being imputed (Hibbs *et al*, 1992).

In a similar fashion, Dengue virus, Cytomegalovirus, Epstein Bar, B 19 parvovirus and HIV (Human Immunodeficiency) are now part of a growing list of other probable infectious agents with similar damaging potential on the marrow cells (Rosenfeld & Young, 1991).

Duncan's Syndrome is an X-linked recessive lymphoproliferation where subjects are unable to mount an efficient immune response to the Epstein-Barr virus, leading to a spectrum of disorders which include aplastic anaemia, hypogammaglobulinemia and malignancies (Schuster *et al*, 1991). The specific pathogenetic mechanisms resulting in marrow failure are unknown, although direct cytopathic effects have been proposed. These may include, as for CMV, loss of relevant genetic sequences for crucial haemopoietic growth factors (Simmons *et al*, 1990). Alternatively, as suggested by the response to immunosuppressives (Frickhofen *et al*, 1991), or the presence of extensive lymphoid infiltrates in the bone marrow (Te Velde & Haak, 1977) a distortion of the immunological response directed against the viral proteins may mediate the destruction of the myeloid elements (Bierman & Nelson, 1965).

1. 3. 2 (d) Paroxysmal Nocturnal Haemoglobinuria (PNH)

Paroxysmal nocturnal haemoglobinuria is a clonal disorder characterized by abnormalities in the glycosylphosphatidyl-inositol-linked proteins (GPI) (Rosse 1990, Rotoli & Luzzatto, 1989). This results in enhanced sensitivity to complement and haemolysis. Investigations into the haematopoiesis in this disease have also revealed variable degrees of pancytopenia including typical aplasia and leukaemia.

In the bone marrow cells, several biochemical and kinetic alterations have been reported. GPI linked molecules include multiple proteins that control the cell interactions with complement such as decay accelerating factor (DAF or CD 55), membrane inhibitor of reactive lysis (MIRL or CD 59), and C8 binding protein. These sequences regulate on the cell membrane, the dynamics of proteins belonging to the complement system. Abnormalities at this level result in unrestrained cell lysis (Hilmen *et al,* 1992). Similarly, GPI anchors the lymphocyte function antigen-3 (LFA-3 or CD 58) to cellular membranes. This recognition molecule for cytotoxic lymphocytes is also deficient in PNH. During the initial immunological aggression that results in bone marrow failure, loss of these receptors may give a survival advantage to these clones. On the other hand, loss of these molecules may also lead to decreased recognition by natural killer (NK) cells of abnormally transformed clones and to leukaemic transformation.

There is agreement that PNH derives from the expansion of an abnormal population which develops through a somatic mutation. In most patients it persists in equilibrium with the normal haematopoiesis, while in others it virtually replaces the normal myeloid tissue (Young, 1992). *In vitro* bone marrow cultures have demonstrated a reduction in the number of clonogenic progenitors, giving further support for this close relation with aplasia. Clinically these patients suffer from chronic haemolytic anaemia often associated with recurrent episodes of nocturnal abdominal pain and exacerbation of the haemolytic state with haemoglobinuria. A thrombophilic state has also been described, with a significantly higher incidence in arterial and venous thrombotic episodes (Rotoli & Luzzatto, 1989).

Bone marrow aplasia can be the initiating event or, following immunosuppressive therapy, appear with recovery of the haematopoiesis. This clonal proliferation replaces the normal myeloid tissue, resulting in clinical haemolysis and a positive Ham's test. Alternatively, this cell membrane disorder may develop as a primary process (Young, 1992).

1. 3. 2 (e) Aplastic Anaemia Associated to Pregnancy

The first description of aplastic anaemia was in a pregnant female (Erlich, 1888). Since then, many case reports and some series have been published recently

(Aitchson *et al,* 1989, van Besien *et al,* 1991) exemplifying the problem.

Aplasia increases the risk for both the mother and the foetus due to anaemia, related toxemia and bleeding. Furthermore, pregnancy often worsens the haematological picture of aplasia while delivery may lead to recovery. If the decision is to carry the pregnancy to term, close observation of the mother and the foetus is recommended. Although the mechanisms of the bone marrow damage remain unclear, hormonal imbalances or unidentified foetal proteins appear to interact with the haematopoietic stem cell or the immune system of the mother.

1. 3. 3 Epidemiological Studies in Aplastic Anaemia

Aplastic anaemia is a relatively infrequent disorder. Although the specific incidence in Africa is unknown, series in Europe, the Far East and the Americas suggest a yearly occurrence ranging from 1.5 to 13 new cases per million inhabitants (Hine *et al,* 1990, Mary *et al,* 1991, Bottiger & Westerholm, 1972, Bottiger & Bottinger, 1981, Szklo *et al,* 1985). Frequency appears to be higher and severity greater among young adults.

Third World countries seem particularly affected, as evidenced by data from the Orient (Young *et al,* 1986). However figures in Thailand and China appear to resemble more the European data (Yang & Zhang, 1991, Issaragrisil *et al,* 1991).

The reasons for these marked differences are unclear, although environmental factors such as the more liberal use of chloramphenicol-containing pharmaceuticals, or poor control in the manipulation of toxic insecticides or pesticides that have been implicated in the disease, are postulated. Nevertheless, the generally low frequency of this disorder means that large series can only be compiled over a long time and that controlled clinical trials can only be performed by large centres or cooperative groups.

1. 3. 4 Bone Marrow Failure. Clinical Features

The clinical presentation in aplasia is heterogeneous, and depends on the severity of the disease. There appear to be at least two categories, a larger group with a very high initial mortality, where patients die at a rate of 20% per month following diagnosis and those patients where long-term survival is possible with a lowered risk of death to approximately 2% monthly (Nathan, 1984). This wide clinical variability can therefore lead to conflicting conclusions during the analysis of small therapeutic trials.

Presentation, however, is often insidious. Patients are usually referred at late stages of the disease, when the complications of the pancytopenia have become clinically obvious with weakness and fatigue from the anaemia, neutropenic fever or fulminant septicaemia and a bleeding tendency as a consequence of thrombocytopenia. Occasionally intermittent menorrhagia or epistaxis persuade patients to seek professional advice earlier.

Physical examination is frequently unremarkable except for pallor, purpura and petechial bleeding, with local or systemic manifestations of infection such as tachycardia and bounding pulses. The presence of splenomegaly or significant lymphadenopathy on presentation is unusual and more often suggestive of a lymphoproliferative disorder.

1. 3. 5 Bone Marrow Failure. Laboratory Findings

By definition, in aplasia, pancytopenia is a universal finding. It may, however, be of variable intensity. Anaemia is typically normocytic or macrocytic (Marsh *et al,* 1987, Novitzky *et al,* 1991), with a low corrected reticulocyte count. The leukocyte count is also reduced, with a granulocytopenia often below 1 x 10^9/L, absolute lymphopenia with inverted CD4/CD8 ratios and sometimes a relative monocytosis. Monocytopenia which is seen in two thirds of patients carries a more severe prognosis. In a similar manner the platelet count is decreased, in direct relation to clinical bleeding.

Based on the severity of the hematological picture, it has become customary to divide this disease according to the presentation peripheral blood values into hypoplasia, severe aplastic anaemia (SAA) (Camitta & Thomas 1978) (Table N° 1.1) and more recently very severe aplasia (VSAA) (granulocytes <0.2 x 10^9/L, and platelets <10 x 10^9/L) (Marsh *et al,* 1987).

TABLE N° 1. 1

SEVERE ACUTE APLASTIC ANEMIA (DIAGNOSTIC CRITERIA)

1.-Reticulocytes	<1% (corrected for Hct.)
Granulocytes	<0.5 x10^9/L
Platelets	< 20 x10^9/L
2.-Bone marrow cellularity	< 25% of normal or 25-50% with > 70% non-haematopoietic tissue.

(Camitta et al 1978)

The value of this division resides in allowing an improved selection of patients for therapy, as those with the more severe forms are unlikely to respond to supportive management or androgenic steroids (Camitta & Thomas, 1978). Here, if a phenotypically HLA identical sibling is available, bone marrow transplantation remains the treatment of choice.

Typically, peripheral blood morphology shows a normocytic picture, but there is often evidence of macrocytosis of the red cells (Marsh *et al,* 1987) with some megaloblastic features being frequently described. Granulocytes are scanty, and may show features of dysplasia. Morphology of the bone marrow aspirates may range from absent particles and scanty cells in the trails, to variable fat replacement and reduction in all series, while iron stores are typically preserved.

Trephine biopsy confirms hypoplasia, often showing frank absence of any haematopoietic elements. In a proportion of patients, inflammatory changes that include lymphocytes and macrophages have been described. Although it was initially suggested that patients with this lymphohistiocytic infiltrate may represent a more favorable prognostic group (Te Velde & Haak, 1977), this observation has not been subsequently confirmed (de Planque *et al,* 1989).

Ferrokinetic studies show a significant delay in the clearance and negligible utilization of the isotope ^{59}Fe (Williams *et al,* 1985). Moreover, for the same concentration of haemoglobin, erythropoietin blood levels and 24 hours urinary excretion rates often exceed those found in most other types of anaemia that are due to production defects (Bray *et al,* 1992, Takeichei *et al,* 1988). These findings are explained by a reduction in the utilization of the hormone by the remaining bone marrow elements.

1. 3. 6 Aplastic Anaemia. Therapy

Initial retrospective clinical studies suggested that treatment with androgenic and adreno-corticoid steroids could lead to an appropriate stimulation of the haematopoiesis (Sanchez Medal *et al,* 1969). However, controlled trials demonstrated that when aplasia was severe, they were ineffective (Camitta & Thomas, 1978). From these studies, based on the presentation blood values and bone marrow picture selection criteria were proposed, where more intensive modalities such as bone marrow transplantation should be explored (Table 1, page 13) (Camitta & Thomas, 1978).

On admission, management requires a balance in the risks and benefits between the initial supportive approach and the hazards of curative therapies. In the severe subgroup, if a suitable donor is available, bone marrow transplantation has become the therapy of choice (Storb *et al,* 1991). However the outcome may become compromised by initial sensitization with transfusions of blood products which are aimed at a symptomatic relief only. On the other hand profound pancytopenia may cause the patient to be at an excessive risk of death from bleeding or severe anaemia. Nevertheless, aggressive initial therapy of infections with specific or

broad spectrum antibiotic combinations together with avoidance of medications that interfere with platelet function are beyond debate. However, since only about one quarter of individuals may have a suitable donor, alternative therapeutic options are needed. Based on the observation that immune mechanisms may suppress haematopoiesis (Cline & Golde, 1978) and in some patients despite intensive preparative regimens, autologous reconstitution may follow unsuccessful bone marrow allografting (Territo, 1977), a number of clinical trials have examined the role of immunomodulating agents. Various schedules of high-dose corticosteroids (Marmont *et al,* 1983, Kitamura & Urabe, 1989, Lopez-Karpovich *et al,* 1991), cyclosporine A (Stryckmans *et al,* 1984) anti lymphocyte globulin (Marsh *et al,* 1987, Novitzky *et al,* 1991) or monoclonal antibodies (Doney *et al,* 1985) have been reported with variable outcomes.

Prednisone alone may result in transfusion independence in 20 to 32% of patients (Marmont *et al,* 1983, Kitamura *et al,* 1989), leaving for more intensive modalities to those primarily unresponsive to corticosteroids. This approach, however, leads to a delay in the delivery of an effective treatment for the majority.

Anti-lymphocyte globulin has been widely used as an immunosuppressive in the prevention and treatment of rejection of solid organ transplantation. It also appears effective in reversing the pancytopenia of marrow aplasia in the majority of the patients treated (Speck *et al,* 1986, Novitzky *et al,* 1991, Novitzky & Jacobs, 1992 [c]).

The mechanism of action remains to be elucidated, as monoclonal antibodies raised against T lymphocytes were ineffective in inducing a similar clinical response (Doney *et al,* 1985). The combined actions of both immunosuppressive and other modulating properties on the accessory cell populations of the bone marrow are therefore postulated (Gascon & Scala, 1988, Nimer *et al,* 1991). Despite clinical responses and improvement in the haematology, blood counts normalise in only one third of the patients, while recurrence of the disease occurs in another 20% (Novitzky *et al,* 1991 [b]). Nevertheless, despite subnormal blood values, responding subjects are able to lead normal lives and enjoy a good performance status (Novitzky *et al,* 1992 [b]).

Following the discovery of cyclosporine, a fungal undecapeptide with selective action on lymphocytes derived from the thymus (Borel *et al,* 77), several groups studied the effects of this immunosuppressive in severe aplasia. After initial encouraging results (Totterman *et al,* 1989), recent controlled studies have confirmed the effectiveness of this agent in untreated patients (Esperou *et al,* 1989), even in those initially unresponsive to ALG (Hinterberger-Fischer *et al,* 1989) or after clinical relapse (Novitzky, unpublished observations). Furthermore, when employed in combination with ALG and corticosteroids, it seemed particularly effective in the very severe variety of the disease (Frickhofen *et al,* 1991).

Nevertheless, in responding patients, after extended follow up, morphological and functional derangements of the mature blood elements have been described, with features similar to those reported in patients with myelodysplastic syndromes and pre-leukaemia. Other patients may display excessive sensitivity to complement as demonstrated by a positive Ham's test, and develop clinical evidence of PNH (de Planque *et al,* 1988, Novitzky *et al,* 1992 b). Furthermore, a proportion seems to transform to acute leukaemia, and this risk of developing clonal disorders appears to increase with time (Tichelli *et al,* 1988). This indicates that, either the marrow was repopulated by unstable progenitor cells with a high potential for clonal progression, or that the originating insult persisted chronically, leading to the described morphological and functional derangements, thus also explaining the prevailing risk of relapse.

1. 4 PATHOPHYSIOLOGY: INTERACTIONS BETWEEN THE HAEMATOPOIETIC STEM CELL AND THE BONE MARROW MICROENVIRONMENT IN APLASTIC ANAEMIA. SEED OR SOIL?

1. 4. 1 Normal Haematopoiesis

1. 4. 1 (a).- Introduction. Normal Bone Marrow Function

It has been postulated since 1906 (Carnot & Deflandre) and proven in 1950

(Reissman, 1950, Erslev, 1953) that erythropoiesis is under the control of a humoral factor, later named Erythropoietin. The hunt for the corresponding "granulopoietins" took another three decades. In the 1960's Pluznik and Bradley (Pluznik *et al,* 1965, Bradley & Metcalf, 1966) developed techniques for the growth of bone marrow cells *in vitro* as colonies. These clonogenic assays have become useful tools and allowed further research into the cell interactions and their growth requirements of the bone marrow progenitors. Initial studies suggested that the organization of the haematopoiesis was hierarchical, where mature cells arise from multipotent precursors or stem cells, via intermediate precursors (Metcalf & Moore 1971).

These concepts were confirmed by radio biological experiments in the 1940s and provided evidence that these myeloid progenitors could also reconstitute a lethally irradiated animal, forming early multipotent colonies first in the spleen (CFU-S) and later repopulating the bone marrow (Till & McCullock, 1964).

Initially, these *in vitro* clonogenic assays employed agar, methyl-cellulose or fibrin clots (Metcalf 1977) as cell supportive structures and required supernatants of epithelial, leukocyte (Iscove *et al,* 1971) or fibroblastic cultures (Burguess *et al,* 1977). These conditioned media contained survival factors that stimulated growth and differentiation of bone marrow cells. Chemical purification of the extracts (Burgess *et al* 1987, Miyake *et al,* 1977, Stanley & Guilbert, 1981, Nicola *et al,* 1983) led to sequencing, genetic definition (Jacobs *et al,* 1985) and eventually the synthesis of these haematopoietic growth factors by the recombinant technology (Egrie *et al,* 1986, Wong *et al,* 1985).

1. 4. 1. (b) Haematopoietic Growth Factors

Proliferation of eukaryotic cells is controlled by a series of regulatory events that include the expression and redistribution of enzymes and other proteins associated with DNA synthesis occurring during the G1 phase of the cell cycle (Pardee, 1989). Colony Stimulating Factors (CSF) act mainly on the myeloid cell lines as survival determinants, leading to expansion and terminal differentiation of the progenitor cell pool. Growth factors and other cytokines regulate these proliferative events through specific receptors located on cell membranes (Jones &

Millar, 1989). These stimulating proteins lead to the activation of the signaling pathways in the nucleus, necessary for cell division (Pouyssegur & Seuwen, 1992).

Erythropoietin (Epo) is a 39 Kd glycoprotein produced by the peritubular cells of the kidney and its secretion appears to be controlled by tissue oxygen saturation (Spivak, 1992). However, bone marrow macrophages also contain m-RNA for Epo, although their contribution to erythropoiesis still remains unclear (Wang, *et al* 1992). This hormone stimulates committed erythroid progenitors to initiate division and differentiation. However, optimal clonal expansion is only achieved in the presence of other potentiating molecule(s), loosely termed as Burst-Promoting Activity (BPA).

Interleukin 3 (IL-3) a 20 Kd molecule is a multi CSF that stimulates all the myeloid cell lines, and is also the main source for erythroid BPA (Saeland *et al,* 1988). Together with granulocyte-macrophage (GM), granulocyte (G) and macrophage (M) CSF acts on the myeloid progenitors enhancing their function and terminal differentiation (Jones & Millar, 1989).

Cells of the monocyte/macrophage lineage are among the most prominent sources of G-CSF, but this factor can also be produced by endothelial, fibroblastic and mesothelial cells. Its secretion can be stimulated by endotoxin, IL-1, tumor necrosis factor (TNF), GM-CSF, IL-3, IL-4 and interferon gamma (INF-γ) (Demetri & Griffin, 1991). Through high and low affinity receptors on target cells this cytokine stimulates mainly the myeloid lineage, with neutrophils possessing the highest receptor density. Additionally, it also interacts with early multipotent precursors directly or through accessory cell populations with synergistic properties with IL-3 and GM-CSF.

GM-CSF was the first cytokine to be cloned in this group. The gene is situated on chromosome 5 (5q21-q32), not far apart from the genes for IL-3, IL-4, IL-5, M-CSF and its receptor (c-*fms*). It is secreted by T cells, mast cells, macrophages, endothelial cells and fibroblasts that have been activated during an immune response or by monokines such as IL-1 and TNF. It also interacts with high and

low affinity receptors on membranes of multipotent and lineage restricted target cells (BFU-e in combination with erythropoietin and CFU-gm). It primes neutrophils enhancing their ability to respond to triggering stimuli for super oxide generation and phagocytosis, but inhibits the migration of polymorphs (Gasson, 1991).

Stem cell factor (SCF) is the product of the steel locus (*S*) and is the ligand for the c-kit proto-oncogene tyrosine kinase receptor, encoded by the white spotting (*W*) locus of the mouse. Mutations at the S^l and *W* loci have been associated with developmental disturbances and haematopoietic defects which include macrocytic anaemia and mast cell deficiency (Zebo *et al,* 1990 a and b). This cytokine is a multipotent factor that acts on cells of the myeloid, mast cell and lymphoid lineages, with synergistic properties with IL-7, Epo and other CSF, on multipotent and lineage restricted progenitors.

It is reasonably clear now that in the haemopoietin family of GF (IL-3, G-CSF, GM-CSF, and Epo), interaction between receptors and their ligands leads to activation of phospholipase C, formation of diacylglycerol and inositol triphosphate resulting in intracellular Ca^{++} release and in activation of calmodulin. This calcium binding protein is reported to regulate cyclic nucleotides (c-AMP, c-GMP) and protein phosphorylation that are crucial for the growth-factor-dependent transition of cells from G1 to S phase (Evans *et al,* 1987).

Alternatively, the tyrosine kinase type of receptors transduce signals for another variety of growth factors. These include Epidermoid GF, Transforming GF α, platelet derived GF, CSF-1, insulin, c-kit ligand etc. They possess a large extracellular ligand binding domain, a single hydrophobic transmembrane segment and a cytoplasmic portion that contains the tyrosine kinase catalytic domain. Upon activation, following tyrosine auto-phosphorylation, oligomerization of the receptor occurs leading to the activation of intracellular second messenger proteins such as phosphoinositol 3 kinase and phospholipase C resulting in the proliferative responses (Pouyssegur & Seuwen, 1992).

It is, however, possible that convergence and synergy of these signals between both pathways exist, leading to stronger mitogenic responses from cooperative signals (Pouyssegur & Seuwen, 1992), relayed through switch kinases such as mitogen activated protein-kinases (MAP-kinases) in kinase-kinase cascades (Payne *et al,* 1991). In this area, knowledge is incomplete, and interest in the definition of the controlling sequences for cell division is keenly shared not only by cell biologists but also by clinicians dealing with disorders such as aplastic anaemia and leukaemias.

1. 4. 1. (c).- Organization of the Haematopoietic System

Haematopoietic stem cells (HSC) are considered to possess an extensive capacity to give rise to new stem cells (self-renewal) and generate progenitors that are committed to expression in single lineages (differentiation). This process of self-renewal and differentiation continues throughout the life-span of the subject, with little evidence for "ageing" of the system.

Based on the analysis of frequency distribution of spleen-colony-forming units (CFU-S), it was concluded that the replication of the HSC follows a stochastic model (Till & McCulloch, 1964). Furthermore, the regulation of this compartment may be achieved by changes in the distributional parameter for the probability of self-renewal (Humphries *et al,* 1981). Moreover, commitment also seems to follow a progressive and stochastic restriction in cell lineages (Suda *et al,* 1983).

Multipotent progenitors differentiate in semisolid cultures (Johnson & Metcalf, 1977, Hara & Ogawa, 1978, Fauser & Messner, 1979) in the absence of a structurally intact microenvironment, suggesting that bone marrow or splenic stroma are not important for stem-cell commitment. However, the long-term maintenance of CFU-S has been reported only in the presence of myeloid stromal monolayers (Gartner & Kaplan, 1981). This suggests a crucial supporting role of the bone marrow microenvironment in the self-renewal of the HSC.

Indefinite serial re-transplantation is not possible. After 4-5 repopulations it eventually leads to HSC depletion with bone marrow failure (Cudkowitz *et al,*

1982) and this is not the result of stromal injury (Gardner *et al,* 1988). Although haemopoiesis is maintained through a massive amplification, stem cell replicating potential is finite as originally indicated by the "clonal succession" model (Kay, 1965), where "founder cells" have the greatest capacity only while they have not entered into cell cycle. However, once cells divide, they will increasingly develop a more limited proliferative aptitude and become exhausted (Botnick *et al,* 1982).

Clinical experience and animal studies confirm that late haemopoietic failure has been noticed long after radiation exposure (Seed & Kaspar, 1990). When reproduced *in vitro*, this reduction in culture life was unrelated to radiation damage to stromal cells but associated with a decline of the number of HSC. This limitation in the repopulation potential seems more intense in blood derived progenitors than spleen or marrow derived CFU-S. This indicates that there is a clear role for the stroma in protecting "stemness", and resulted in the term "haematopoietic niches" (Tavassoli & Crosby, 1968; Schofield *et al,* 1978).

Following marrow transplantation, founder cells freed from this stromal protection will be lost into differentiation, while clonal succession ensures repopulation of the HSC pool and will project cells that have divided, even closer into commitment. Further support for the "niche" model has come from long-term bone marrow cultures (LTBMC) (Dexter *et al,* 1984). In this novel approach, bone marrow stroma is allowed to become confluent with the concomitant production of early myeloid progenitors.

In this system, CFU-S in the stromal layer (SL) appear to have a similar clonogenic capacity to that of normal bone marrow resident cells, while within the supernatant, among the non-adherent progenitor this is greatly reduced (Dexter *et al,* 1984). Furthermore, transfer of the stroma residing precursors onto another fresh SL decreases markedly its proliferative capacity (Moore and Dexter, 1978, Schofield *et al,* 1980) in secondary cultures, mimicking this the *in vivo* experience.

In vitro, the LTBMC proved to be the most physiological of myeloid culture assays (Gartner & Kaplan 1981) where haematopoietic progenitors proliferate and

differentiate in close contact with the stromal population for many weeks. Here, a marked diversity in the cell population forming the marrow stroma is observed (Castro-Malaspina *et al,* 1980).

Once confluent monolayers are formed, fibroblasts, endothelial cells, monocytes, blanket cells, adventitial reticular cells and their progeny the fat cells (Bianco *et al,* 1988) form a tight structure where populations of the immune system such as the B lymphocytes (McGuinnes *et al,* 1991) and those of the T lineage (Shibata *et al,* 1986) are well represented.

In this assay, haemopoietic output seems to be the product of a balance between growth factors and negative regulators or "restrictins" (Zipori & Tamir, 1989). Among the GF identified in the SL granulocyte (G), macrophage (M) and granulocyte-macrophage (GM) CSF, tumor necrosis factor alpha (TNFα), IL-1, IL-4, IL-5, IL-6, IL-7 (Zipori & Tamir, 1989, Mc Guines *et al,* 1991, Gualtieri *et al,* 1984, Gualtieri *et al,* 1987) and c-kit ligand are included, as well as the m-RNA for IL-3. These findings have been confirmed by the inhibition of the clonogenic development of growth factor (GF) dependent cell lines in the presence of target GF neutralizing antibodies (Kittler *et al,* 1992).

In the stroma, non-haemopoietic cells also secrete a variety of products that form an extra cellular matrix (ECM). These are broadly divided into three categories: collagen, proteoglycans and glycoproteins. Fibroblasts are associated with granulopoiesis; in co-culture they seem to control myeloid proliferation (Nagao *et al,* 1986) and secrete type III collagen. Blanket cells produce fibrinectin and laminin while endothelial cells produce type I and IV collagen. Glycosaminoglycans (GAGs) which interact with cellular adhesion molecules on cell membranes, also bind soluble haematopoietic growth factors making them directly available to progenitor cells (Gordon *et al,* 1987 b, Gordon, 1991). This would explain the low concentrations of some of these GF found in the culture supernatants and why clonogenic growth is maintained despite the addition of anti-CSF antibodies to LTBMC (Lipschitz *et al,* 1987). Further, confirmation of their importance in growth regulation is exemplified by a significant reduction in the HSC output, upon inhibition of collagen secretion by *cis*-hydroxyproline (Campbell

et al, 1988).

Through specific membrane cell adhesion molecules (CAM) as CD 44 (Lewinsohn *et al,* 1990, Tsai *et al,* 1987, Vuillet-Gaugler *et al,* 1990) or CD 54 (Arkin *et al,* 1991) respectively, immature erythroid precursors and granulocytic progenitors are anchored to the polypeptides fibrinectin and haemonectin. This adhesiveness appears to be down regulated and lost upon differentiation, allowing cells to be released into the culture supernatant, or *in vivo,* to the circulation (Tsai *et al,* 1987).

This culture method has been tested in a variety of haematopoietic diseases, including myeloproliferative disorders (Hotta *et al,* 1986), acute and chronic leukaemias (Verfaille *et al,* 1992) as well as in bone marrow aplasia (Boyd *et al,* 1986, Marsh *et al,* 1990) providing reproducible data.

A modification of this system has been described, where monocyte depleted myeloid progenitors are incubated on preformed bone marrow stroma and allowed to adhere, then immobilized with agar and scored for the development of 4-hydroperoxycyclophosphamide resistant blastic colonies (Gordon *et al,* 1985).

These stroma-adhesive progenitors also form secondary colonies, with a repopulating potential similar to umbilical cord precursors (Nakahata *et al,* 1982). In this assay, the adhesive properties of the earliest clonogenic cells, their growth potential as well as the growth-promoting properties of the stroma are tested without the need of external GF supplementation.

1. 4. 2.- Haematopoiesis in Aplastic Anaemia

In aplastic anaemia the data remain incomplete and are often conflicting. These results reflect the diversity in both the clinical expression of the disease and in the different research techniques employed by the investigators. However, more

recently certain new concepts have helped clarify some of these issues.

With standard culture techniques, employing various conditioned media, semi-solid clonogenic assays of the bone marrow indicated a significant reduction in the clonable progenitors (Haak, 1978). This reduction was seen even in those responding to immunosuppression or androgenic steroids, despite normalization of the peripheral blood counts. These findings contrast with the experience from those undergoing bone marrow transplantation, where *in vitro* colony formation recovered after the procedure (Haak, 1978). To explain such findings, quantitative (Heimpel & Kubanek, 1975) or qualitative abnormalities (Heimpel & Kubanek, 1975, Boggs and Boggs, 1976) of the progenitor cell compartment have been postulated in patients treated with immunomodulation.

Alternatively, and based on *in vitro* observations of cell-mediated inhibition of the myeloid and erythroid progenitors by a population with growth suppressor properties (Ascensao *et al,* 1976, Gorski *et al,* 1979, *et al,* 1980), an immunological down regulation of clonogenic growth was postulated. This was confirmed by reports that T-lymphocytes with the E+ Fcγ+ OKT3+ Ia- OKM1- phenotype or a soluble factor from their conditioned medium could mediate this inhibition (Bacigalupo *et al,* 1984, Viale *et al,* 1991). Although control bone marrow also contained immunocytes that belonged to such a population and displayed similar suppressor activities, this required pre-activation with lectins, which in patients with aplasia, was found to be spontaneous. Of interest, in another subgroup of patients expressing similar autoreactive CD4+/CD8+ lymphocytes, monoclonality of this population was documented by gene rearrangement studies for the Beta chain of the T cell receptor, (Moebius *et al,* 1991).

Further research into the immune system in these patients on presentation or in those that responded to ALG, confirmed that lymphoid cells collected and cryopreserved before therapy and later exposed, in co-cultures, to autologous marrow obtained after response to immunosuppression, in a dose dependent fashion inhibited CFU-GM proliferation. This was reversed by anti-interferon gamma (INF-γ) antibodies. Here again, effector cells were identified as CD 8+ and this activity was reversed by immunosuppressive therapy with ALG (Laver *et al,*

1988).

Although controversial, in aplasia, serum INF-γ levels appear not to be increased (Torok-Storb *et al,* 1987). Unexpectedly, excessive secretion of this cytokine was demonstrated from peripheral blood and bone marrow lymphocytes in these patients (Zoumbos *et al,* 1985), even before becoming sensitized by transfusions with blood products (Hintenberger *et al,* 1988). Nevertheless, pre-incubation of the bone marrow progenitors with anti-INF-γ antibodies did not improve colony formation. Although, when exposed to incremental concentrations of ALG, GM-CSF secretion was induced in a linear pattern by these bone marrow accessory cells present in the culture (Hintenberger *et al,* 1988).

Cells of the immune system are an active part of the bone marrow stroma (McGuinnes *et al,* 1991, Shibata *et al,* 1986), producing a variety of positive and negative cell function regulating cytokines (Pantel & Nakeff, 1989). In aplasia, experimental data have shown that INF-γ and TNFα are secreted excessively by bone marrow cells, spontaneously or after exposure to mitogens (Bacigalupo *et al,* 1984, Zoumbos *et al,* 1985, Hintenberger *et al,* 1988, Viale *et al,* 1991). Both these cytokines are known to exert antiproliferative effects on myeloid precursors (Tracey *et al,* 1989). Although this inhibition was reversed by specific neutralising antibodies (Zoumbos *et al,* 1985), in untreated subjects, excessive production was again indirectly suggested by enhanced neopterin secretion, which reversed after the infusion of ALG (Hinterberger *et al,* 1988).

Clinically, equine anti-lymphocyte globulin appears to have a equivalent effect to these neutralizing antibodies (Platanias *et al,* 1985, Tong *et al,* 1989). Moreover it seems to induce the release of various haemopoietic growth factors (Nimer *et al,* 1991) and to exert antiproliferative effects on B lymphocytes (Bonnefoy-Berard *et al,* 1992).

Of similar interest is that monocytes that are widely represented in the stroma, have also been found to be deranged, with alterations in surface membrane glycoproteins, their development to macrophages impaired (Andreesen *et al,*

1989). These findings persisted despite successful ALG therapy. Furthermore, in a subsequent study in untreated patients, IL-1 production was also significantly reduced (Gascon & Scala 1988, Nakao *et al,* 1989), while clinical response to horse immunoglobulin led to recovery in the secretion of this cytokine (Gascon & Scala 1988). It was therefore not surprising that inhibition of erythroid bursts (BFU-E) has also been reported when these adherent mononuclear cells were cocultured with normal or patient myeloid progenitors (Torok-Storb *et al,* 1980, Merchav *et al,* 1988).

Macrophages form an integral part of the bone marrow microenvironment and are a source of both haematopoietic growth factors and inhibitory proteins (Feldman *et al,* 1986). Therefore, functional alterations in this population may lead to subnormal progenitor cell stimulation. Alternatively, damage to the HSC pool can be manifested as derangements in these mature elements. Indeed, there is a considerable volume of data documenting morphological and functional abnormalities of all the differentiated haemopoietic progeny, including granulocytes, erythrocytes, platelets (de Planque *et al,* 1989 [a] and Chapter 2) and B lymphocytes (Lum *et al,* 1987). Furthermore, development of PNH in aplastic anemia is well documented (Rotoli & Luzzatto 1989, Rosse, 1982, Rosse, 1990), as well as transformations to other clonal disorders such as myelodysplastic syndromes and acute leukaemias (Tichelli *et al,* 1988, Orlandi *et al,* 1988, de Planque *et al,* 1988).

However, following the description that in this disease one half of syngeneic bone marrow transplants are unsuccessful without preconditioning (Appelbaum *et al,* 1980) and the evidence provided by the Steel (*Sl/Sl^d*) murine congenital anaemic model where the myeloid microenvironment is abnormal (Dexter & Moore, 1977, Anklesaria *et al,* 1991), research was also directed to the marrow stoma. Results of stromal cultures showed derangements in the adherent layer, with fibroblasts displaying atypical growth in multiple layers (Juneja *et al,* 1984). Contrary to normal, in some patients these failed to support CFU-gm (Hotta *et al,* 1985). Here, of further interest, the expression of genes for INF-γ in myeloid mononuclear cells appeared to predict for a favorable outcome in those treated with cyclosporine (Nakao *et al,* 1992).

To investigate this controversy further, based on the LTBMC assay, Marsh employed confluent irradiated stroma from patients responsive to ALG treatment and compared their behaviour to control stroma. She concluded that the aplastic layers did not differ morphologically from normal and in their ability to sustain normal haematopoiesis.

However, although care was taken to seed predominantly with haematopoietic progenitors, rigorous selection for this population was not attempted, allowing normal bone marrow accessory cells, including those of the immune system to "engraft" and interact with the aplastic stroma. This has confused somewhat the final conclusions with regard to the aplastic stromal integrity (Marsh *et al,* 1990).

In a follow-up study, selected myeloid precursors for the CD 34+ antigen from patients with aplasia were compared to controls in cross culture experiments on normal and aplastic stroma. Here, it was demonstrated that in the patients, progenitors were markedly decreased in numbers and had a significantly lower plating efficiency, therefore suggesting that the defect resided predominantly within the haemopoietic stem cell compartment (Marsh *et al,* 1991). This writer has also reached the same conclusions, in a similar patient population, employing the blastic colony assay (Gordon *et al,* 1987 b) with CD 34+ selected progenitors on aplastic and control stroma. Furthermore, when equal numbers of CD 34 + cells from patients with treated aplasia were exposed to rh-GM-CSF or IL-3 in the presence of erythropoietin, the plating efficiency was decreased 10 fold compared to controls (Novitzky *et al,* 1991). A mechanism for this sub-optimal response was not apparent.

1. 4. 3 Rationale for the Study and Concluding Remarks

The experimental data reviewed, indicate that an initial aggression to the myeloid tissues as a result of either alterations in the immune system or within the bone marrow microenvironment lead to a marked depletion of the haematopoietic precursors and to clinical bone marrow failure. However, contrary to the experience with intensive chemotherapy or radiotherapy, a spontaneous recovery fails to follow this initial injury.

The objectives of the present study were to define more clearly the net effect of the bone marrow stroma in down-regulating the normal proliferative signals in aplastic anaemia. Initially, bone marrow stromal layers from patients with aplasia and from controls were cultured *in vitro* and studied morphologically, cytochemically and in their ability to support the growth of myeloid progenitors. One problem identified, was that light density mononuclear populations of the marrow that contained the earliest progenitors and expressing the CD 34 antigen were also contaminated by multiple accessory cells with regulatory functions. To avoid these interactions, positive selection for these precursor cells was undertaken allowing a more clear comparison of the clonogenic potential between the diseased and the normal marrows, when grown in cross-culture on control or aplastic stroma.

The limitations of this methodology become clear during the pilot study as, due to the low selected progenitor cell yields obtained in the untreated patients, more comprehensive studies on the HSC could not be performed. With the clear understanding that the pathological processes might not be similar to those occurring during the initial presentation, this analysis was then confined to individuals that had responded to the immunosuppressive therapy. Here, as already described, despite the normalization of the blood values, morphological and functional derangements in the blood elements persisted and were associated with a significant reduction in the clonogenic progenitors.

If these initial observations confirmed abnormalities in the stem cell population, in a second step, these selected progenitors would be exposed to increasing concentrations of appropriate combinations of recombinant growth factors, and their clonogenic responses compared to the results obtained in a normal group.

In this thesis, firstly, a review of the clinical and laboratory data of patients with aplastic anaemia attending the Department of haematology at Groote Schuur Hospital is presented in Chapter 2. Here, the haematopoiesis of subjects who had responded to immunosuppressive treatments was defined by a systematic morphological, cytogenetic and functional study of the various blood elements. In Chapter 3 a description of the selection techniques employed for the CD 34+ population is given and two different methods compared. In Chapter 4 a pilot

study is described, where normal and aplastic stroma are compared in their ability to support CFU-bl, the CD 34+ populations quantitated and cultured *in vitro* in the presence of recombinant growth factors.

In Chapter 5 a detailed description of the normal and aplastic bone marrow stroma is presented, and an analysis of the interactions of the adherent layers with the selected CD 34+ adherent and non-adhesive progenitors discussed. Here, it becomes clear that the aplastic stroma does not suppress normal colony formation and that the clonogenic defect resides within the HSC. Following this logical sequence, selected normal and aplastic haematopoietic cells were exposed to growth factors belonging to the two super-families of receptors and results are presented in Chapter 6. Lastly, in Chapter 7 a summary of the findings is presented and the final conclusions discussed.

CHAPTER 2

QUALITATIVE ABNORMALITIES CHARACTERISE HAEMATOPOIESIS THAT RESTORES MARROW FUNCTION FOLLOWING THERAPY WITH ANTILYMPHOCYTE GLOBULIN (ALG) AND HIGH DOSE METHYL PREDNISOLONE (HDMP) IN APLASTIC ANAEMIA

2. 1 INTRODUCTION

Aplastic anaemia is characterized by irreversible bone marrow failure, resulting in profound pancytopenia (Camitta & Thomas, 1978). Although the causes of this syndrome are largely unknown, some of the described associations include recent exposure to certain drugs (Appelbaum *et al,* 1981), pregnancy (Aitchison *et al,* 1989; Van Besien *et al,* 1991), jaundice (Hagler *et al,* 1975, Rosenfeld *et al,* 1991) and atypical viral infections such as Dengue and Parvovirus (Rosenfeld, 1991).

The clinical expression is variable, reflecting the intensity of the haematopoietic damage, ranging from mild or moderate hypoplasia responsive to androgenic steroids (Sanchez Medal *et al*, 1969), to severe aplasia where this hormonal therapy has been of no benefit (Camitta & Thomas, 1978). In contrast, allogeneic bone marrow transplantation improves survival, particularly in younger patients (Storb *et al*, 1983), and those who have not been sensitised by previous transfusions or pregnancy (Anasetti *et al*, 1986). However, only the minority will have a suitable donor.

Based on the observations that immune mechanisms may suppress haematopoiesis (Cline & Golde, 1978), anti-lymphocyte globulin (ALG) and cyclosporine, alone or in combination with corticosteroids, have been employed with responses in over 50% of patients (Speck *et al*, 1986, Esperou *et al*, 1989, Novitzky *et al*, 1991). Extended follow-up has demonstrated that relapses may occur. In others, despite adequate recovery or even normal peripheral blood values, variable degrees of dysplasia occur (de Planque *et al*, 1989 b and c), and

platelet functions are deranged (de Planque *et al*, 1989 a), with alterations in T-lymphocyte subsets as well as reduction in the serum immunoglobulins (Lum *et al*, 1987).

These findings are reminiscent of some of the abnormalities described in myelodysplastic syndromes. These observations apply particularly in the subgroup that presents with hypoplastic bone marrow and improves following appropriate immunosuppressive therapy (Tichelli *et al*, 1988 [c]), making reliable separation from aplasia difficult. Compounding this complexity, a subgroup of patients with marrow aplasia may evolve not only to paroxysmal nocturnal haemoglobinuria and acute leukaemias, but also to myelodysplastic syndromes (Tichelli *et al*, 1988 [a and b]).

To address this issue we have studied a cohort of patients that presented with typical features of severe aplasia and received a uniform immunosuppressive treatment. We have attempted to define the morphologic, cytogenetic and functional characteristics of their haematopoiesis and to describe these abnormalities that have persisted even after eight years following therapy.

2. 2 STUDY PATIENT POPULATION

From January 1985 to December 1991, 34 individuals, with severe aplastic anaemia (Camitta & Thomas, 1978), 15 of whom have been previously reported (Novitzky *et al*, 1991) and one with hypoplasia, were referred for therapy. Their median age was 25,5 years (range 13-72; SD 15.66) and 17 were female. Only one had a donor suitable for allogeneic bone marrow transplantation. Two had convalescent serology for hepatitis B, one was pregnant and recovered partially following therapeutic abortion. One was exposed to chloramphenicol in eye drops and another received chlorquine for rheumatoid arthritis and neither recovered after drug discontinuation.

On presentation, the median values for haemoglobin (Hb) were 66 g/L (SD 20.9), the reticulocyte count 0.3% (SD 0.29), mean red cell volume 102 fL (SD

54.76), granulocyte count 0.50 x 10^9/L (SD 0.44) and platelets 11.5 x 10^9/L (SD 5.7). All had normal serum B_{12} and red cell folate levels. For this group, the median bone marrow cellularity was 15% (SD 5.4) of normal.

2. 2. Aplastic Anaemia. Therapy

All patients received uniform supportive care that included leukocyte poor packed red cells, allogeneic platelets transfusions and appropriate antibiotics. After the diagnosis had been confirmed, subjects were entered into a standard protocol with ALG and high dose-methyl prednisolone (HDMP), approved by the University of Cape Town and Groote Schuur Hospital Ethics and Research Committees. The treatment consisted of ALG (horse immunoglobulin without thrombocyte adsorption; Swiss Serum Institute, Bern, Switzerland) 50 mg/kg and HDMP 500 mg daily on each of five consecutive days, followed by 30 mg daily of prednisone for one month (Novitzky *et al*, 1991).

Patients were assessed at six months and again one year after therapy. Response was defined as reversal of symptoms, freedom from transfusions, a stable rise in the platelet count above 40 x 10^9/L and an absolute granulocyte count greater than 1 x 10^9/ L. Complete remission required Hb greater than 120 g/L, granulocytes > 2.5 x 10^9/ L and platelets of > 150 x 10^9/ L. Patients with inadequate responses or with fluctuating blood counts received oxymetholone two mg/kg for six to nine months with careful monitoring of the liver functions.

2. 2. 2 Laboratory Investigations

2. 2. 2. (a) Morphological Assessment of the Haematopoiesis in Aplastic Anaemia.

Peripheral blood

Complete blood counts, red cell indices, platelet size and distribution width were generated on a Technicon H1 Analyzer (Tarrytown, New York). Peripheral blood slides were stained with May-Grunewald-Giemsa (Lewis *et al*,

1986) and differential counts derived manually. Complement sensitivity of red cells was determined by sucrose lysis and acidified tests.

Bone Marrow Examination

Aspirates were air-dried and morphology was defined on slides stained with Romanowsky dyes (Lewis *et al*, 1986). Iron content was determined in normoblasts and reticuloendothelial cells with a special search for pathological inclusions, ringed sideroblasts or the presence of dysplastic features according to the French-American-British criteria (Bennett *et al*, 1982).

Trephine biopsies were obtained with the Jamshidi needle (Bird & Jacobs 1983), processed, sectioned and stained with haematoxilin and eosin and evaluated for inflammatory infiltrates, abnormalities in the stroma or abnormal localization of immature precursors (ALIP) (Mangi *et al*, 1992).

2. 2. 2. (b) Cytogenetics Studies of the Bone Marrow in Patients with Aplastic Anaemia

Bone marrow was collected into RPMI containing 20% foetal calf serum (Gibco, UK) and heparin (Novo, Nordisk; Johannesburg, 100 U/mL). Chromosome analysis was performed on trypsin (Dice Michigan, USA) giemsa banded metaphases obtained from direct harvest or methotrexate (0.5 μg/mL; Lederle) synchronisation of bone marrow cells. When necessary, cytogenetics on phytohaemagglutinin (PHA) (Gibco, Grand Island NY) stimulated peripheral blood cultures were also carried out to establish the constitutional cell line.

2. 2. 2.(c) Granulocyte Functions in Patients with Aplastic Anaemia

Peripheral blood leukocytes were obtained from thirteen patients with aplastic anaemia and from nine concurrent controls by Dextran sedimentation (Dextran 6% w/v in 0.9% Na Cl) of heparinized blood (20 U/mL) and divided in aliquots for the various functional tests.

Superoxide Generation

Determination of super oxide generation was done following a previously described method (Markert *et al*, 1984). Patient and control leukocytes were washed at 4° C in RPMI, re-suspended in PBS, and maintained at 4° C. To 10^6 cells, 0.01 mL of distilled water (reference control) or super oxide desmutase (Sigma St. Louis; 4200 U/mg, 0.4 mg/mL) (test sample) was added. The sample was incubated at 37° C for 2 minutes followed by the rapid addition of 0.05 mL horse heart cytochrome C (30 mg/mL MG Ca 12500, Boehringer-Mannheim W. Germany) and then of pre-warmed phorbol myristate acetate 0.75 mL (Sigma, 1 μg/mL), followed by another ten minutes' incubation at 37° C under continuous rotation, when the reaction was stopped by rapid cooling on ice. Cells were then washed at 4° C, cytochrome reduction measured in a double beam spectrophotometer at 550 ηm, and super oxide generation calculated by the formula:

$$O_2^- \ (\eta M/L) = 47.7 \times \text{absorption } O_2^-$$

$$O_2^-(\eta M/\text{min}/10^6 \text{ cells}) = \frac{(S - R)\ 47.7}{\text{cell concentration factor} \times 10} \div 10$$

S: ***sample***
R: ***reference***
47.7: ***extinction coefficient***

Phagocytosis and Killing

The combined measurement for phagocytosis and killing (Yamamura *et al*, 1977) was determined on the buffy (1 x 10^6/mL) suspended in 0.5 mL of medium containing 20% serum (Western Province Blood Transfusion Service WPBTS, Cape, S. Africa). Leukocytes were mixed in triplicates with 6 x 10^6/mL Candida Albicans organisms and incubated at 37° C under constant rotation for 30 minutes. Tubes containing 20% serum and C. Albicans or cells alone were used as controls.

For the quantitation of phagocytosis, aliquots with 0.1 mL of the cell suspension were added to wells containing 0.2 μCi of ^{3}H-uridine (Amersham). For the killing assay, polymorphs were also incubated for 30 minutes in 1.2 g Na deoxycholate (Hopkin & Williams, Essex; UK) in 0.01 mL distilled water containing 5 μg DNAase (Sigma, bovine pancreas). Radioactivity in the suspension was collected onto glass fibre disks with a cell harvester (Skatron Titertek, AS-3401 LIER, Norway), placed in scintillation fluid and counted in a scintillation spectrometer (Beckman LS 1801, USA).

Phagocytosis (PI) and killing indices (KI) were calculated as :

$$100 - \frac{\text{CPM Candida + PMN - CPM PMN}}{\text{CPM Candida alone}} \times 100$$

(The proportion of C. Albicans killed can be determined by: KI ÷ PI x 100)

Migration and Chemotaxis

Chemotaxis was studied according to Addison with slight modifications (Addison, 1976). Using Luckham Lp3 tubes (Luckham, Sussex; UK) 0.4 mL normal or aplastic neutrophils (2 x 10^{6}/mL) were placed on cellulose nitrate 3 μm filters (Sartorius 25G, type 70) prewarmed to 37° C, wet in autologous plasma or RPMI + Hepes (control) for random migration or *f*-MLP 10^{-8} M (Sigma Pharmaceuticals, Missouri, USA) for chemotaxis. These were then placed on Whatman's N° 54 filter paper, soaked in appropriate medium, incubated at 37° C for 30 minutes, rinsed in water, fixed in propan 2-ol for five minutes, stained with Harris haematoxylin and the migration distance read on a Leitz microscope.

2. 2. 2. (d) Lymphocyte Functions and Serum Immunoglobulin Levels

Peripheral blood mononuclear cells from patients with aplasia and controls were obtained from a 1.077 g/mL density gradient (Hystopaque, Sigma). Cells were washed twice in RPMI (Sigma) and 0.1 mL of the suspension at a concentration of 5 x 10^{5}/mL were cultured for three days at 37° C in 96 well microtiter plates (Cel-Cult, Sterilin Ltd. Hounslow UK) in the presence of 0.05 mL of

concavalin A (Calbiochem La Jolla Ca, USA), phytohemagglutinin, pokeweed mitogen (Gibco) or heat inactivated AB serum (WPBTS). Cells were then pulsed with 1 μCi ^{3}H-Thymidine (Amersham, 1:200) at 37° C, harvested eighteen hours later on to dry filter discs and the radioactivity counted.

For the mixed lymphocyte reaction, stimulating allogeneic control lymphocytes were thawed, their viability determined by trypan blue exclusion, and incubated at 37° C for 20 minutes in mitomycin (Sigma, Mo 0503; 1:8 dilution). After being washed twice in medium, 0.1 mL of the cell suspension (5 x 10^5/mL) was cultured in triplicates with patient or control lymphocytes for five days and pulsed with 1 μCi ^{3}H Thymidine (Amersham) for 18 hours. Cells were then harvested on to filter discs and radioactivity was measured on a Beta scintillation counter (Beckman).

Immunoglobulin levels were evaluated by agar electrophoresis. Quantification of IgG, IgA and IgM was performed by single radial immunodiffusion using immunodiffusion plates and compared to a normal range (Fahey *et al*, 1965).

2. 2. 2. (e) Determination of the Platelet Functions in Aplastic Anaemia

Bleeding Time and Platelet Aggregometry

Platelet functional activity was determined only on patients with platelet counts >100 x 10^9 /L (n=14) and compared to controls. Both groups were instructed to avoid medications that could interfere with platelet function for two weeks preceding the study. Bleeding time was determined by the Ivy method, and at the same time blood was collected for aggregometry into siliconised vacutainer tubes containing sodium citrate. Comparisons were carried out in platelet-rich plasma, using a Chronolog aggregometer with results registered on a chart recorder (Chronolog Corporation Haverton Pennsylvania; model 560vs and model 707).

The system was calibrated using platelet-poor or rich plasma at 100% and 0% respectively. Measurements were taken at the maximum point of the aggregation. These were expressed as per cent variation in light transmission

compared with baseline values in response to adenosine diphosphate (ADP Sigma, Missouri) at final concentrations of 100, 10 and 5 μg/mL, (0.2-0.01 mMol), collagen (Sigma) at a concentration of 2 μg/mL, ristocetin 1.5 mg/mL (Sigma) and epinephrine (May Baker, South Africa) 0.01 mM.

2. 2. 3 Statistical Analysis

Patient survival was measured by Kaplan-Meier. Median values and confidence intervals (confidence coefficient: .95) of the patient results were obtained, compared with control results and significances tested by two-way analysis of median values.

2. 3 RESULTS OF THE CLINICAL, MORPHOLOGICAL AND FUNCTIONAL STUDIES PERFORMED ON THE BLOOD ELEMENTS IN APLASTIC ANAEMIA

2. 3.1 Response To Therapy and Long-Term Follow Up

The patient with hypoplasia remitted after a therapeutic abortion, and as she was not treated, was excluded from analysis of response to therapy. For the entire group, the median time from initial symptoms of pancytopenia to therapy was sixty days (SD 94.3). Response was seen in 24 of the 34 subjects (71%), and this was complete in eight. At a median follow-up of 1320 days (SD 803) 22 (65%) are alive in response (Fig N° 2. 1, page 38). One patient required two courses. He is currently in complete remission. Five patients developed immune complex disease following the ALG infusion, and in one plasma exchange was needed to reverse the intense vasculitis. In the remainder, an increment of the prednisone dose to 1 mg/kg was sufficient to control these manifestations within two weeks. Although haematological response was seen at a median of 110 days (60-300), improvement in the blood counts continued for up to four years after therapy. Six of 24 patients (25%) relapsed. In one, disease recurred at 180 days. This patient subsequently died of sepsis and pulmonary embolization shortly after receiving a second course. Another reached response criteria values, but relapsed sixty days later. As she had a compatible sibling, she underwent T cell

depleted (CAMPATH 1M) allogeneic bone marrow transplantation, but failed to engraft and died at 270 days. One female relapsed at 620 days in the third trimester of her second pregnancy. She was supported until term and improved her blood counts following the delivery of a normal infant. Three patients required further immunosuppression, and are on their second (one patient) or third response (two patients). In one, cyclosporine maintenance is necessary for a sustained response.

Patients who had responded were re-evaluated at one year when their Karnovsky rating was 100% (Table N°2. 2, page 41). Median Hb was 115.0 g/L (SD 25.3), MCV 102 fL (SD 9.51), Granulocytes 1.7 x 10^9/L (SD 1.65) and platelets 88 x 10^9 /L (SD 80.0). None of the presentation clinical findings including sex, age, length of aplasia, or development of immune complex disease were predictive for response. Among the pre-treatment laboratory findings, only a raised MCV was significantly associated with response (responders, median 109 fL, SD 10.1 vs non-responders 91 fL, SD 7.6, median test p= 0.015, exact two tailed) (Marsh *et al,* 1987; Novitzky *et al,* 1991).

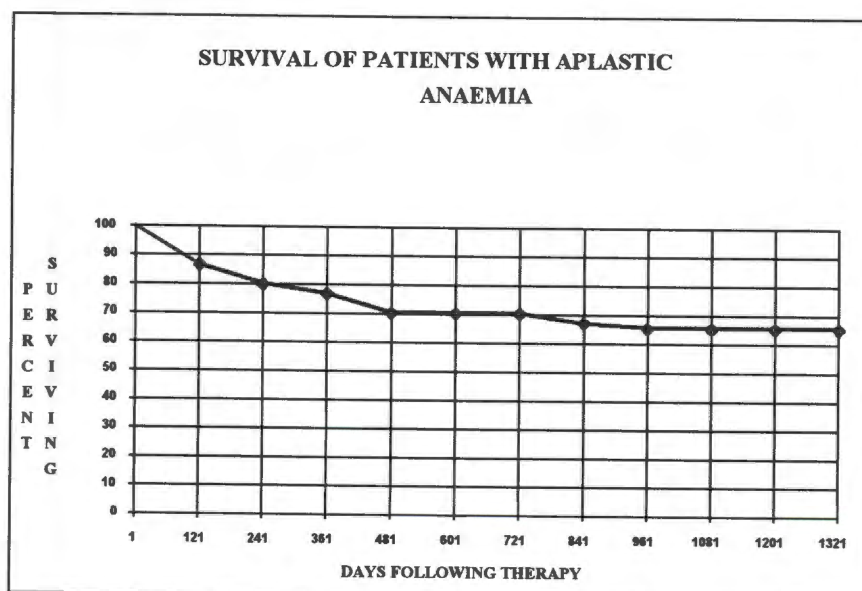

Fig N° 2. 1: Follow up of patients with acute aplasia treated with ALG+HDMP.

2. 3. 2 Study Patient Population (Table N° 2. 2)

Of 22 patients alive in response after immunosuppressive therapy and one following therapeutic abortion, sixteen were available and agreed to undergo the present study (Table N° 2. 1 and 2. 2; pages 40 and 41). On presentation, all fulfilled the diagnostic criteria for severe aplasia (Camitta & Thomas, 1978). Morphology in thirteen patients revealed mild megaloblastosis limited to the red cell precursors, while in another, in addition mild dysplasia in the granulocytic series with giant bands was also observed. Morphology was entirely normal in one, and in two patients there were no analyzable haematopoietic tissue.

Reassessment of 16 patients belonging to this group was performed at a median of four years from therapy (range 1-8). The median haemoglobin was 134.5 g/L (SD 27.2), MCV 98 fL (SD 7.17), Granulocytes 2.21 x 10^9/L (SD 1.89), lymphocytes of 1.53 x 10^9/L (SD 0.71), monocytes of 0.23 x 10^9/L (SD 0.18) and platelets 127 x 10^9/L (SD 80.44). Two patients developed a positive Ham's test with clinical features of PNH. Another four had increased sucrose haemolysis, but on blood chemistry and serological testing showed no evidence of intravascular red cell destruction (Table N° 2. 2, page 41).

2. 3. 2.(a) Morphological Assessment of the Haematopoiesis in Patients with Aplastic Anaemia (Table 2. 3)

Morphology was entirely normal in three subjects only. On the peripheral blood, erythrocyte dysplasia (mainly as megaloblastic morphology) was present in 11 patients, while in six it also affected the granylocytic series(in the form of hypersegmentation or Pelger-Huet neutrophils). In the bone marrow, megaloblastic erythroid maturation was present in 13 patients. It was accompanied by myeloid dysplasia in 8 with additional megakarycytic dysplastic morphology in 3. Iron stores were absent in one patient while pathological or ringed sideroblasts were not seen in any. No excess of blasts, inflamatory infiltrates or increase in mast cells were seen. On the biopsy, median cellularity was 40%. Morphologically, tri-lineage dysplasia with abnormal localization of immature precursors (ALIP) was present in 3 patients, and in two, a patchy increase in reticulin fibrosis was observed (Table 2.3, page 42).

TABLE 2.1

POST - ACUTE APLASTIC ANAEMIA

Patient N°	Age	Sex	Haemoglobin g/L	Mean Corpuscular Volume fL	Granulocytes x 10^9/L	Platelets x 10^9/L	Follow up Years	Observations
1	42	F	134	85	2.10	103	4.5	Response N°2
2	48	F	91	115	2.59	28	5.2	On CSA
3	18	M	128	106	2.13	100	1.3	On CSA
4	68	F	136	107	1.48	136	3.6	
5	32	F	135	100	1.98	111	1.0	Post-abortion
6	32	F	143	94	1.57	176	5.4	
7	48	M	144	94	5.76	181	5.0	
8	47	F	137	94	2.62	159	6.3	
9	19	F	117	99	1.76	216	5.0	Hepatitis
10	36	F	85	103	2.30	102	6.4	PNH
11	28	M	84	98	0.75	29	4.9	Relapsed x 3
12	24	M	163	98	5.08	180	5.2	ALG x 2
13	47	F	123	90	4.04	375	4.1	Quinine tox.
14	29	M	66	103	1.77	118	7.7	PNH
15	25	M	139	98	2.92	116	4.0	
16	29	M	145	95	1.63	143	5.2	Hepatitis

Description of the study population and the haematological parameters. CSA: Cyclosporin A therapy.

Due to the lack of features of bone marrow failure, none fulfilled the diagnostic criteria for myelodysplasia (Bennett 1982).

2. 3. 2. (b) Cytogenetic studies

Metaphases suitable for analysis were obtained in fifteen of sixteen individuals. One patient had an abnormal karyotype expressed as 47,XX +C in eight of twelve metaphases, and the remainder showed random chromosomal loss. PHA stimulated peripheral blood analysis confirmed the constitutional nature of the trisomy X. Chromosomal breaks and or gaps were present in five of fifteen patients, ranging from 13 to 40% of cells tested. No other cytogenetic abnormality was detected.

2. 3. 2. (c) Results of the Granulocyte Function Studies (Table N° 2. 3)

Only one patient of thirteen had normal granulocyte functions; in three all assays were deranged. In the remaining subjects results were below control values in at least one assay. As a group these were significantly reduced for

super oxide generation (p= 0.007) and phagocytosis (p= 0.002). Variations in migration and killing were present in eleven patients, but for the group these did not reach significance (Table N° 2. 4, page 44). No correlation was found between blood leukocyte counts and *in vitro* granulocyte functions, or any other clinical or laboratory data, including age, sex or time from therapy to these investigations.

TABLE 2.2
QUANTITATIVE EVALUATION

Peripheral Blood (n= 16)	Observation Period			
	One year		Four years	
	Median	Standard Deviation	Median	Standard Deviation
Haemoglobin g/L	115.00	25.30	134.50	27.21
Granulocytes x 10^9/L	1.70	1.65	2.21	1.89
Lymphocytes x 10^9/L	0.92	1.65	1.53	0.72
Monocytes x 10^9/L	0.15	0.12	0.23	0.18
Platelets x 10^9/L	88.00	80.00	127.00	80.44

Peripheral blood values of the study population with aplastic anaemia evaluated at 1 and 4 years following ALG therapy.

3. 2. 2 (d) Results of Lymphocyte Function Studies and Serum Immunoglobulin Levels

All measurements were normal in twelve patients, but subnormal in both patients on cyclosporine therapy. Serum immunoglobulins were determined in fourteen subjects and values were found within normal range in all, including both patients on maintenance immunosuppression.

2. 3. 2. (e) Functional Studies of the Platelets. Results

Bleeding Time

The platelet count was greater than 100 x 10^9/L (median 127; SD 80.44 x 10^9/L) in fourteen patients. However the Ivy time was normal in only five of

the individuals. For all patients the median bleeding time was 8.5 minutes (SD 3.4) (laboratory normal range 3-7 minutes), and in three was greater than fifteen minutes.

TABLE 2. 3

MORPHOLOGICAL EVALUATION

Bone Marrow Morphology Cellularity Percentage: Median 40 (SD 21)	**Number of Patients**
Normal	3
Abnormal	13
Dysplasia	
One lineage	2
Two lineage	6
Three lineage	5
Megaloblastosis	
One lineage	4
Two lineage	6
Three lineage	2
Abnormal localization of immature precursors (ALIP)	3

The bone marrow was studied in patients with aplastic anaemia at 4 years following ALG therapy.

Platelet Aggregation Studies. Results (Table N° 2. 5)

Aggregation studies were abnormal in all patients and this derangement applied to each of the agonists tested with significantly sub-optimal values for ADP 100 μg ($p = 0.001$); 10 μg ($p = 0.0012$); 5 μg ($p = 0.08$), Adrenaline ($p = 0.001$), Collagen ($p = 0.003$) and Ristocetin ($p = 0.0002$) (Table N° 2. 5, page 45).

2. 4.- DISCUSSION OF THE FINDINGS

This study confirms the effectiveness of anti-lymphocyte globulin in reversing the haematological abnormalities associated with severe aplasia. The response-rate and long-term survival of 71% and 65% respectively are consistent with

other reports (Speck *et al*, 1986). However, when the various therapeutic options are examined, several points must be considered. Selection in the patient population, differences in the schedules employed (Doney *et al*, 1992, Ozsoylu *et al*, 1984) as well as variability in the biological product (Novitzky *et al*, 1991, Smith *et al*, 1985) may all lead to fluctuations in the results reported. Young children (Locasciulli *et al*, 1990) and patients with the very severe variety (Marsh *et al*, 1987, Gluckman *et al*, 1992) have lower response rates, and this should be a clear consideration when entertaining therapeutic alternatives such as matched unrelated donors for this high-risk population (Hows, 1991).

Retrospective studies indicate that dosage of horse immunoglobulin may be of importance, with consistently higher response rates when values over 100 mg/kg are infused (Coiffier *et al*, 1984). In vitro, results appear to correlate clinical response to proliferation of lymphocyte subsets (Abe *et al*, 1991) and the release of CSF (Kawano *et al*, 1988), when these cells are exposed to ALG in culture, following a clear dose response (Tong *et al*, 1991). Clinically, more intense therapeutic schedules may combine profound immunosuppression with activation of the various growth factors *in vivo*, balancing in favor of stem cell recovery (Kawano *et al*, 1988, Tong *et al*, 1991).

Endorsements for this hypothesis are the results of recent controlled trials showing that, while corticosteroids with cyclosporine or ALG appear of similar therapeutic potency (Doney *et al*, 1992), significantly better responses were obtained when cyclosporine was added to ALG with steroids (Frickhofften *et al*, 1992), which in the very severe group resulted in a significant survival advantage.

Presentation macrocytosis was the only feature significantly associated with Favourable response to this therapeutic regimen (Marsh *et al*, 1987, Novitzky *et al*, 1991). However, mild megaloblastic changes progressed to widespread dysplasia, increased deposition of reticulin and even abnormal localisation of immature precursors over the follow up-period. Nevertheless, in no case did this progress meet the diagnostic criteria for myelodysplasia (Bennett *et al*, 1982).

TABLE 2. 4

GRANULOCYTE FUNCTIONS

FUNCTIONS TESTED	PATIENTS (n = 12)			CONTROLS (n = 10)	
	Median	Standard Deviation	Number of Patients Abnormal	Median	Standard Deviation
Generation of Peroxide (mMol/min/10^6 cells)	2.79**	0.60	4	4.66	0.85
Chemotaxis (μm)	83.00	33.97	7	97.50	20.68
Migration (μm)	65.00	17.38	10	51.50	17.02
Phagocytosis (Percentage)	53.10**	12.89	1	87.00	6.59
Killing (Percentage)	32.00	18.61	7	46.00	9.36
* *p<0.05*					

Functional study of the plymorph leukocytes of patients with aplastic anaemia tested at a median of 4 years following ALG therapy and compared to concurrent controls. ** denotes significance ($p < 0.05$).

Karyotypic abnormalities may provide a clue to the possible link between aplasia and myelodysplasia (Appelbaum *et al*, 1987) and the present demonstration that 13 to 40% of metaphases in five of fifteen individuals were abnormal, supports the concept that disturbed DNA repair mechanisms (Hashimoto *et al*, 1975, Turner *et al*, 1981) may presage evolution to the preleukaemic syndromes (Tichelli *et al*, 1988 [b]).

Abnormalities in the granulocyte functions have also been detected in the study group, with significant reduction in the generation of hydrogen peroxide and phagocytosis. Similar observations were described in patients with severe congenital neutropenia where alterations in the cellular signalling pathways leading to the activation of granulocyte functions had been demonstrated (Kyas *et al*, 1992; Kurtzberg *et al*, 1992, Roesler *et al*, 1992). Of note, however, is

that in our patients the incidence of cutaneous or systemic infections was not increased.

TABLE 2. 5

PLATELET FUNCTIONS

Aggregation Studies	PATIENTS (n = 14)		CONTROLS (n = 14)	
	Median	Standard Deviation	Median	Standard Deviation
ADP 100	56.00**	19.94	85.00	10.75
ADP 10	47.50**	20.07	74.50	12.74
ADP 5	31.00	15.98	70.00	20.94
Adrenaline	10.00**	20.13	68.00	25.16
Collagen	35.00**	31.70	80.00	12.74
Ristocetin	69.50**	19.28	88.00	9.10
** $p<0.05$				

Platelet Count: Median 127 (SD 80.44) $x10^9/L$.

Platelet aggregation studies performed on patients with aplastic anaemia at a median of 4 years following ALG therapy.** denotes significance ($p < 0.05$).

However, these findings vary from another report on untreated subjects with this disorder, where granulocyte super oxide production was increased and further enhanced by *in vitro* incubation with GM-CSF (Oshaka *et al*, 1992). This discrepancy can be explained in that during early disease the immune system appears activated, with increased generation of certain cytokines such as INFγ and TNF (Hinterberger *et al*, 1987, Zoumbos *et al*, 1985, Tong *et al*, 1991). These cytokines have been described to up modulate granulocyte responses.

Contrary to previous reports (Lum *et al*, 1987), lymphocyte functions and immunoglobulin blood levels were uniformly normal in those not receiving immunosuppression. This disparity may be related to a recovery in the immune

system that might have occurred with time, or to differences associated with sample size. Nonetheless, wide variations in the immune response have been described in some patients, with excessive production of interferon gamma and tumor necrosis factor (Hinterberger *et al*, 1987, Zoumbos *et al*, 1985, Tong *et al*, 1991) and sub-optimal generation of IL-1, stressing the heterogeneity of both the populations studied and the methodologies employed.

During sequential testing, despite continuous improvement in the bone marrow cellularity, mild thrombocytopenia has persisted in the majority. As in this disease, platelet survivals were previously shown to be normal (de Planque *et al*, 1989), this persistent reduction in the thrombocyte numbers is likely to reflect decreased production or ineffective thrombopoiesis (de Planque *et al*, 1989). However, despite platelet counts exceeding 100 x 10^9/L in fourteen, a value that normally is not associated with a bleeding tendency, the bleeding time was prolonged in seven patients, and was over fifteen minutes in three. Not surprisingly, all had aggregation abnormalities of variable degree and as a group these were significant. Similar defects have been previously described, although the interpretation of those results was compounded by significant thrombocytopenia in the population under scrutiny (de Planque *et al*, 1989). There again, however, despite improvement in the platelet values, aggregation studies remained subnormal.

As the pathogenesis of the disease still remains unclear, it could be interpreted that the initial insult irreversibly damages the haematopoietic stem cells and this continues to be reflected as qualitative defects in the mature progeny. Alternatively, it is conceivable that the progenitor cell pool and the differentiating population are subject to continuous aggression by a sub-clinical disease within an abnormal haematopoietic microenvironment (Ershler *et al*, 1980, Juneja *et al*, 1984, Hotta *et al*, 1985, Fujiwara *et al*, 1990).

In conclusion, following immunosuppressive therapy for acute aplasia, even after a long period of quantitative recovery in the various blood elements, persistent chromosomic abnormalities, together with tri-lineage morphological and functional derangements are observed. These derangements are mainly sub-

clinical, but due to the possibility of disease recurrence and progression towards clonal disorders, further research into the cellular interactions that occur within the bone marrow microenvironment is needed.

CHAPTER 3

BONE MARROW PURIFICATION AND SELECTION FOR THE PROGENITOR CELL POPULATION. RESPONSE TO RECOMBINANT GROWTH FACTORS

3. 1 INTRODUCTION

In mature mammals, the production of the blood occurs predominantly in the bone marrow which is composed of heterogeneous populations of cells of haematopoietic origin, stromal cells and lymphoid tissue. In this environment the stem cell is able to regenerate its own pool, commit into the various cell lines and sustain a continuous output of terminally differentiated elements (Dexter 1977).

Clonogenic assays are useful tools in the research of the proliferation and differentiation of haematopoietic progenitors. However, the presence of multiple accessory cells in the light density marrow fraction, interacting through autocrine loops with the various regulatory pathways controlling growth regulation of myeloid precursors, makes the interpretation of these assays difficult (Iscove 1971, Feldman 1986). Therefore, when specifically studying the progenitor cell pool in disorders of the haematopoiesis, the definition of the clonogenic efficiency is as relevant as the exclusion from the culture system of unpredictable interactions with contaminating accessory cells. This situation makes the selection for the populations under scrutiny highly desirable.

For this purpose, research on cell membrane surface determinants in enriched populations of bone marrow progenitors resulted in the identification of a 110 KD membrane glycoprotein present only in 1-2% of the bone marrow population, but in the majority of the haematopoietic precursors that form colonies *in vitro*. This membrane structure has been molecularly defined, its genetic profile sequenced (Xing-Yue 1992) and integrated into the Leukocyte Differentiation listing as CD 34 (Civin 1989). The development of monoclonal antibodies against epitopes on

this structure resulted in the description of methodologies for the identification and selection of these cells to a greater purity (Greenberg 1985, Ungelstad 1987).

Positive selection of specific cell populations has been reported with several techniques, including "panning" (Greenberg 1985), immunomagnetic selection (Ungelstad 1987) or flow cytometry with cell sorting (Visser 1990). When these techniques are employed in the selection of bone marrow cells carrying the CD34 antigen, a significant enhancement of the haematopoietic colony formation is observed *in vitro*, with marked reduction in clonogenic growth in the depleted fraction (Ungelstad 1987). These data and the reports of successful bone marrow reconstitution with grafts containing CD 34 enriched populations in humans and other primates (Berenson 1988, 1991), confirmed the hypothesis that this antigen was broadly represented on the membranes of the haematopoietic stem cells.

To define the optimal methodology with regards to cell homogeneity and clonogenic yields, two purification techniques were compared. Additionally, the clonogenic behaviour of the selected population was characterised in dose response studies to four haematopoietic growth factors constructed in specific combinations.

3. 2 METHODS FOR THE PURIFICATION AND SELECTION OF THE PROGENITOR CELL POPULATION. RESPONSE TO RECOMBINANT GROWTH FACTORS

Bone marrow was obtained by multiple sternal aspirates from haematologically normal patients undergoing sternotomy under general anaesthesia for heart valve replacement or coronary artery cardiac by-pass surgery. All marrow donors had given written informed consent according to the guidelines of the Ethics Committee of the University of Cape Town and the Groote Schuur Hospital.

Bone marrow rich blood was collected into 50 mL tubes containing 20 U/mL preservative fee heparin (Pularin, 10 U/mL; Nordisk, Johannesburg), in Iscove's modified Dulbecco's medium (IMDM), (Gibco Life Technologies, UK). Light

density cells were collected from the interface of a polysucrose diatrizoate density gradient (1.077 g/mL, Hystopaque, Sigma, USA) after 30 minutes centrifugation at 400 G at room temperature. Subsequently, cells were washed twice in medium and aliquots taken for morphological assessment, indirect immunofluorescence and quantitative determinations with an electronic particle counter (Multisizer, Culter Electronics, USA).

The light density population was re-suspended in IMDM containing 2% foetal calf serum (FCS, Gibco, Life Technologies; UK) placed into 200 mm plastic Petri dishes (Bibby, UK) and incubated for two hours at 37° C to remove adherent cells. Non-plastic adhesive cells were decanted, washed twice and lymphocyte depleted by incubating in 15 mL of the same medium containing 0.1 mL CAMPATH-1M (6.25 mg/mL; 0.01 mL antibody / 5 x10^6 cells/mL) (1983) for 50 minutes at 37°C in the presence of 30% AB serum as a source of complement (Western Province Blood Transfusion Service, C. Town).

3. 2. 1 Progenitor Cell Selection

The resulting lymphocyte and monocyte-poor progenitor cell concentrates were washed three times in IMDM, re-suspended in 0.2 mL of cold (4° C) phosphate buffered saline (PBS) containing 0.02 mL/10^7 cells of anti human CD 34 antigen murine monoclonal antibody (Anti HPCA-1, My 10, Becton and Dickinson, Sunnyvale, California), and incubated on ice for 30 minutes. Labelled cells were then washed 3 times in phosphate buffered saline (PBS) containing 2% AB serum (complement inactivated, Western Province Blood Transfusion Service, Cape Town, RSA). On the labelled cells, two selection techniques were compared: antibody-mediated plate binding (Greenberg 1985) and immunomagnetic separation (Ungelstad 1987).

3. 2. 1. (a) Progenitor Cell Antibody-Mediated Plate Binding (Greenberg 1986)

Plastic petri dishes (Falcon, 75 cm^2) were coated with 0.1 mL high affinity goat anti-mouse polyclonal immunoglobulin (Dakopatts z 420, Denmark; 2 mg/mL) in

9.9 mL of tris buffer (pH 10) for one hour at 4°C. Dishes were decanted and washed gently with PBS containing 2% AB serum. Labelled cells were re-suspended into three mL of the same medium and layered on antibody coated dishes for 70 minutes. Non-adherent cells were washed off with PBS containing 2% AB serum (complement inactivated). The labelled population was then detached with jets of PBS and collected into 50 mL tubes, centrifuged at 400 G for ten minutes, counted in a haemocytometer, re-suspended in IMDM at a cell concentration of 1 x 10^5/ mL, and cultured in the mixed colony assay. Aliquots were also taken for trypan blue exclusion determinations, immunofluorescence, cytospin preparations and stained with May-Grunewald-Giemsa and α-naphthyl butirate esterase for morphological and cytochemical determinations.

3. 2. 1. (b) Antibody-Coated Magnetic Bead Progenitor Cell Selection

Polystyrene paramagnetic beads covalently bound to affinity purified goat IgG against all mouse IgG subclasses (M-450, prod No 125.01, Dynal, Oslo) at a concentration of 5-10 beads per target cell population (one gram of dry beads contains 1.4 x10^{10} particles or 4 x10^8 /mL suspension, with a total surface area of 3-5 m^2) were washed four times in IMDM and exposed in continuous rotation at 4° C to the antibody labelled population at a cell concentration of 5-10 x10^9/L for 30 minutes (Ugelstad 1987).

Target cells were then harvested with a magnet (MPC-1, Dynal) applied to the sides of the tube and free cells decanted. Incubation with the paramagnetic particles and magnetic separation was repeated once on decanted cells. To remove the magnetic beads, selected cells were re suspended in 0.1 mL of PBS containing 0.01 mL of a polyclonal murine immunoglobulin (Detachabead, Dynal) set at room temperature for 60 minutes in a continuous rocking motion and exposed to the same magnetic conditions (Rasmussen 1992). Cells freed from spheres were selected, viability determined by trypan blue exclusion, counted in a haemocytometer and numbers adjusted to a concentration of 1-5 x10^5/ mL.

3. 2. 2 Immunofluorescence Studies of the Selected Populations

Aliquots of cells obtained from the initial density gradients and of the final separations were incubated overnight at 37° C in medium containing 15% FCS, then re-suspended, washed twice, dripped on multitest slides (Highveld Biologicals), air dried over 4-6 hours and frozen to -80°C for immunophenotyping, or employed for cytological determination after staining with Romanowsky dyes (Lewis 1986) and non-specific esterase.

For immunophenotyping, frozen Multiwell slides (Highveld Biologicals, Transvaal; S. Africa) containing light density and selected cells were thawed and dried overnight, fixed in 100% acetone for 5 minutes, washed with PBS and labelled for 45 minutes with FITC-conjugated CD3 (0.005 mL UCHT-1 Dako, Denmark), CD19 (0.005 mL Dako HD-37) antibodies, or CD 14 (0.01 mL CD 14, Mo 2; Culter; USA) and CD34 (0.01 mL Anti-HPCA, My 10, Becton Dickinson, USA) for indirect studies. With each case, positive (Class II antibody, Barnstable 1978) and negative (directed against β-galactosidase, Department of Clinical Sciences, Prof. E. Dowdle, University of Cape Town) controls were always included. Where relevant (wells containing CD 14 and CD 34), a fluoresceine conjugated second rabbit antibody (Dakopatts) was added for 30 minutes.

All excess reagents were washed with PBS, and nuclei counter-stained with 1 μg/mL ethidium bromide for one minute, washed, mounted and read under ultraviolet illumination at 50 ηm with a mercury gas lamp on a Nikon microscope.

3. 2. 3 Definition of the Progenitor Cell Population. Clonogenic Assays

3. 2. 3. (a) Growth Factors

Recombinant human cytokines (Amgen Thousand Oaks, California); rh-IL 3 (specific activity of 10^7 U/mg), rh GM-CSF, G-CSF(specific activity 1 x10^8 U/mg respectively) and c-kit ligand, at protein concentration of 0.5, 0.5 and 2 mg/mL each were diluted to 10^3 ηg/mL and kept at 4° C until used. Erythropoietin (Epo)

was donated by Bioclones (Sandton, Transvaal), once reconstituted kept in 2% FCS at 4°C and a concentration of 1000 U/mL.

3. 2. 3. (b).Clonogenic Assays of the Selected Populations

Positively selected CD 34 progenitors were cultured in multi-cluster dishes (Nunclon Delta, UK) at a cell density of 1×10^4 in IMDM, containing L-Glutamine, supplemented with 30% FCS, 5×10^{-5} 2-mercaptoethanol, in the presence of haematopoietic growth factors in dose response studies.

Granulocyte-Macrophage Colony-Forming Units (GM-CFUc) were defined as myeloid aggregates (granulocytic, monocyte-macrophage or mixed) containing more than 40 cells. CFU-e were described as erythroid clusters of 4-100 cells, while BFUe were expressed as colonies containing more than 100 cells or when aggregates contained three or more subclusters.

3. 2. 3. (c) Dose Response to Growth Factor Combinations

Further analysis of the clonogenic growth of the CD 34+ population was assessed in the mixed colony assay, by determining the response to incremental concentrations of growth factors. Six normal subjects were studied by dose response curves that were constructed in various concentrations for Epo (0.05, 0.2, 2, 10 and 100 U/mL) in the presence of IL-3 10 ηg/mL; GM-CSF (0.1, 10 and 50 ηg/mL), combined with Epo 2 U/mL; IL-3 (0.05, 0.1, 1, 10 and 50 ηg/mL) together with Epo 2 U/mL and c-kit ligand (1, 20, 40, 100 and 200 ηg/mL) in combination with Epo 2 U and IL-3 10 ηg/mL.

3. 3 RESULTS OF THE PROGENITOR CELL SELECTION AND OF THE CULTURE STUDIES

3. 3. 1.- Progenitor Cell Selection: Morphology and Immunofluorescence Studies

3. 3. 1. (a) Selection by Antibody-Mediated Plate Binding (Table 3. 1)

Five normal subjects were studied. Monocyte adherence, lymphocyte depletion and panning led to a marked enrichment for morphologically immature blast cells, from a median of 7% (2-13) in the light density cell fraction, to a 71% (57-88). Immunophenotyping of four specimens showed that 62% (44-76) expressed the CD 34 antigen, leading, therefore, to a 51.7 fold enrichment of this population, while the viability was of 72% (59-88) as determined by trypan blue exclusion.

3. 3. 1. (b) Selection by Antibody-Coated Magnetic Beads

Accessory cell depletion and positive selection with goat anti-mouse IgG coated magnetic beads also led to a significant enrichment of morphological blasts to a median of 85% (72-98). In 3 studies the selected population expressed fluorescence for CD 34 antibody in 80% (67-84) of cells. In this group none was reactive with non-specific esterase (Fig. N° 3. 1 and 2, page N° 55). The total clonogenic enrichment by this procedure was 53.3 times and the viability, by the dye exclusion method, of 97% (Table 3. 1, page 56). Semi-solid cultures in the mixed colony assay confirmed a significant enrichment in clonogenic precursors, for both selection methods with increments in the numbers of erythroid (BFU-e, CFU-e) and myeloid (CFU-GM) colonies. Although the immunomagnetic method was superior, this was not significant for colony types ($p > 0.05$). Significantly, more cells taking up trypan blue were found with the panning technique ($p < 0.05$).

3. 3. 2 Clonogenic Assays. Dose Response (Table 3. 2)

Dose response studies were performed on six controls having at least three studies on each GF concentration. Cells selected by the immunomagnetic procedure were cultured at a cell concentration of 1×10^4. For Epo, increments in erythroid colony formation reached a plateau at 10 U/mL, while CFU-gm colonies were not influenced by increments in this hormone. At top concentrations macroscopic mixed colonies, immature BFU-e (more than seven sub colonies of blasts) as well as massive bursts were elicited (Fig. N° 3. 3, page 58).

GM-CSF stimulated erythroid, myeloid and mixed colony development with

plateau values for BFU-e and CFU-GM colonies at 10 ηg/mL. Again, macroscopic myeloid colonies were obvious (Figs N° 3. 4, 3. 6, 3. 7 and 3. 8, pages 58-61).

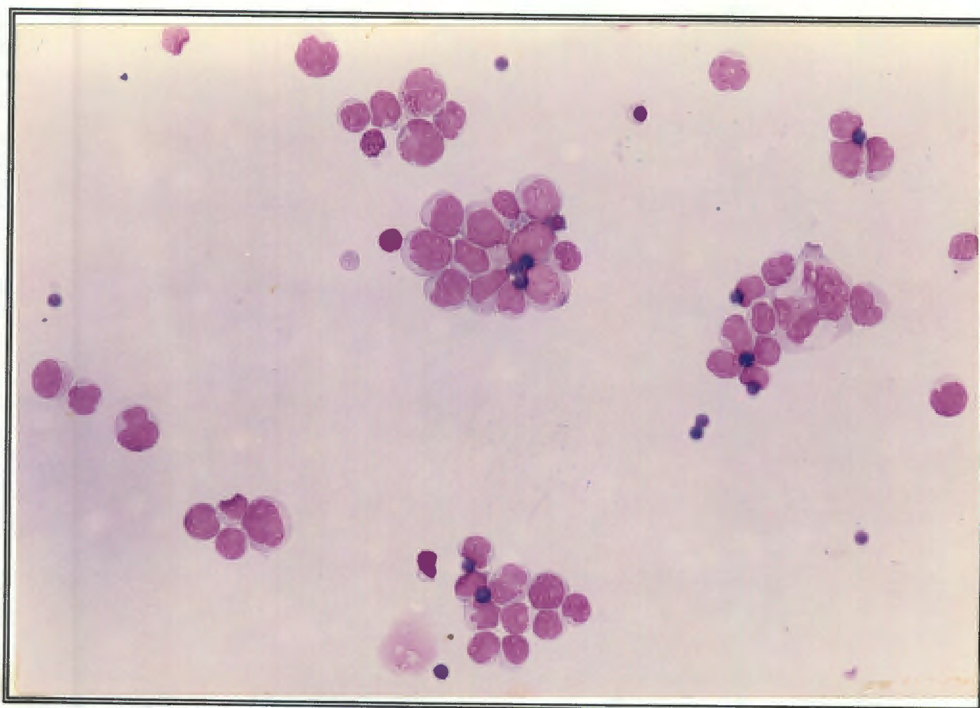

Fig. N° 3. 1: Immunomagnetic selection. Cells labelled with CD 34 antibody were magnetically selected to a median of 85% blasts (range 72-79) (May Grunewald Giemsa; x 200 magnification).

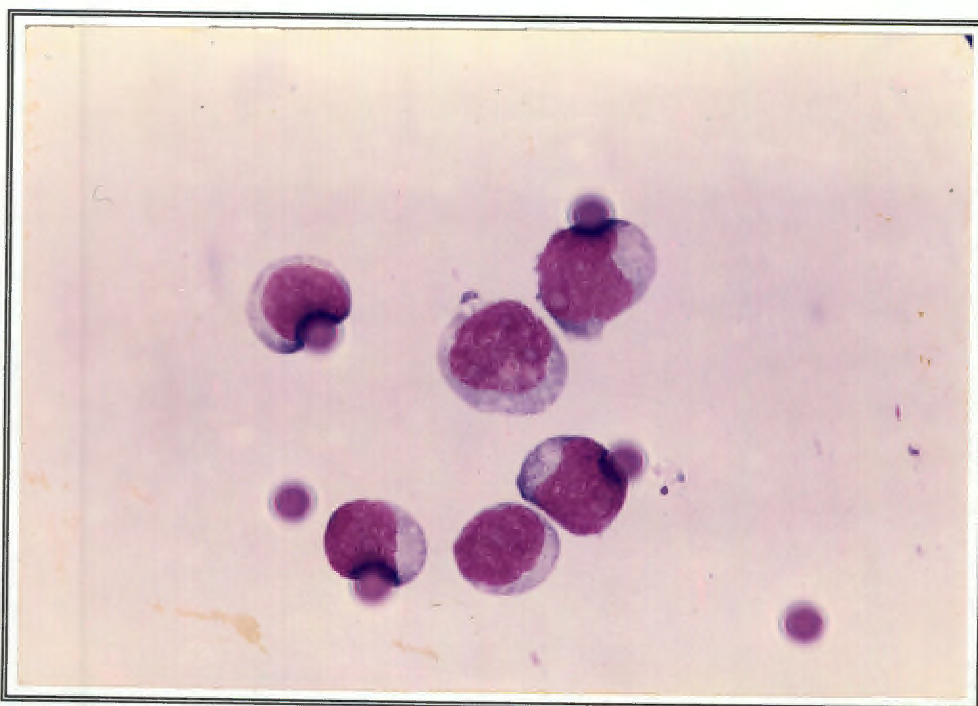

Fig. N° 3. 2: Morphological details of the positively selected population. Occasional paramagnetic beads are still visible. (May Grunewald Giemsa; x 400 magnification).

TABLE 3.1

PROGENITOR CELL SELECTION COMPARATIVE STUDY

COMPARATIVE ANALYSIS	ANTIBODY MEDIATED PLATE BIDING				IMMUNO MAGNETIC SELECTION			
	LIGHT DENSITY CELLS		FINAL SELECTION		LIGHT DENSITY CELLS		FINAL SELECTION	
MORPHOLOGICAL ASSESSMENT	n=5				n=5			
Percentage	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
Blasts	7	2-13	71	57-88	8	5-17	85	72-98
Lymphocytes	7	11-24	12	5-16	12	6-18	5	1-18
Monocytes	7	3-17	4	1-4	9	4-10	1	1-3
NSE	8	8-11	<2	0-2	12	4-13	0	0-1
IMMUNO-FLUORESCENCE STUDIES	n=4				n=3			
Percentage	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
CD 34	1.2	0.7-1.6	62	44-76	1.5	0.9-1.7	80	67-84
CD 3	7	1-15	<5	2-8	5	4-12	<5	0-5
CD 19	4	4-1-	7	3-7	7	5-13	5	0-7
Cell viability	95	88-100	72	59-88	95	85-98	97	92-100
MIXED COLONY ASSAY	n=5				n=3		n=5	
Colonies 10^{5}	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
CFU-mix	13	0-19	22	12-22	0	0-2	70	10-120
BFU-e	51	12-61	231	51-461	39	12-75	380	140-680
CFU-e	291	231-593	1050	783-3653	72	40-133	928	260-2180
CFU-GM	70	42-106	166	135-210	29	17-63	372	40-928

Comparative study for the selection of CD 34+ cells between the antibody mediated plate binding and immunomagnetic selection. Assessment was performed on morphological, immuno-phenotypic and clonogenic results obtained by both methods on medians of 3 experiments.

Response to IL-3 was seen in all type of colonies and was maximal between 1 and 10 ηg/mL. Stem Cell Factor (c-kit ligand) was tested in 3 normal subjects; its clonogenic effect favored predominantly erythroid production, with BFU-e numbers reaching a plateau growth between 100 and 200 ηg/mL (Fig. N° 3. 4, page 58), while comparing to the baseline factors present (Epo and IL-3), little activity was detected at 1 ηg/mL.

TABLE 3. 2

DOSE RESPONSE

GROWTH FACTORS COMBINATIONS	CFU-mix		BFU-e		CFU-e		CFU-GM	
	Median	Range	Median	Range	Median	Range	Median	Range
IL3 10ηg+ERYTHROPOIETIN								
EPO:								
0.05 U/mL	0	0	2	1-2	36	19-41	71	31-76
0.2 U/mL	2	1-5	48	12-55	52	19-66	48	22-51
2 U/mL	7	7-11	60	29-71	82	59-92	67	23-69
10 U/mL	7	5-14	68	39-98	120	78-122	87	54-91
100 U/mL	19	11-29	60	27-61	104	59-117	71	39-73
Epo 2 U/mL + GM-CSF								
GM-CSF:								
0.1 ηg/mL	0	0	21	12-29	66	21-91	12	4-29
1 ηg/mL	0	0-3	45	19-51	86	42-105	35	21-36
10 ηg/mL	7	1-9	105	39-112	110	68-119	59	17-66
50 ηg/mL	15	3-16	79	63-82	144	38-151	71	34-75
Epo 2U/mL+INTERLEUKIN3								
IL-3:								
0.05 ηg/mL	0	0-1	19	5-22	95	39-98	16	7-22
0.1 ηg/mL	0	1-6	27	9-31	138	77-143	34	29-62
1 η/mL	2	1-5	67	32-75	124	79-125	29	11-43
10 ηg/mL	3	3-9	39	21-65	134	37-144	125	69-144
50 ηg/mL	9	3-22	57	29-97	136	98-143	113	92-166
Epo 2 U/mL+IL-3 10 ηg/mL + STEM CELL FACTOR								
SCF:								
1 ηg/mL	0	0	40	18-51	38	33-69	6	3-16
20 ηg/mL	2	2-7	61	43-75	49	37-89	21	12-49
40 ηg/mL	2	2-16	99	54-119	41	32-87	26	23-69
100 ηg/mL	9	8-19	118	68-145	38	32-69	80	27-101
200 ηg/mL	2	1-16	121	77-148	39	22-57	93	22-139

Dose response of normal selected CD 34 progenitors to combinations of growth factors in the mixed colony assay. Results are expressed as colonies / 10.000 cells.

At optimal concentrations c-kit ligand induced massive clones of macroscopic bursts of mixed erythroid and myeloid growth. Immature bursts (more than seven subclusters, little haemoglobinization) were obvious at 100 and 200 ηg/mL.

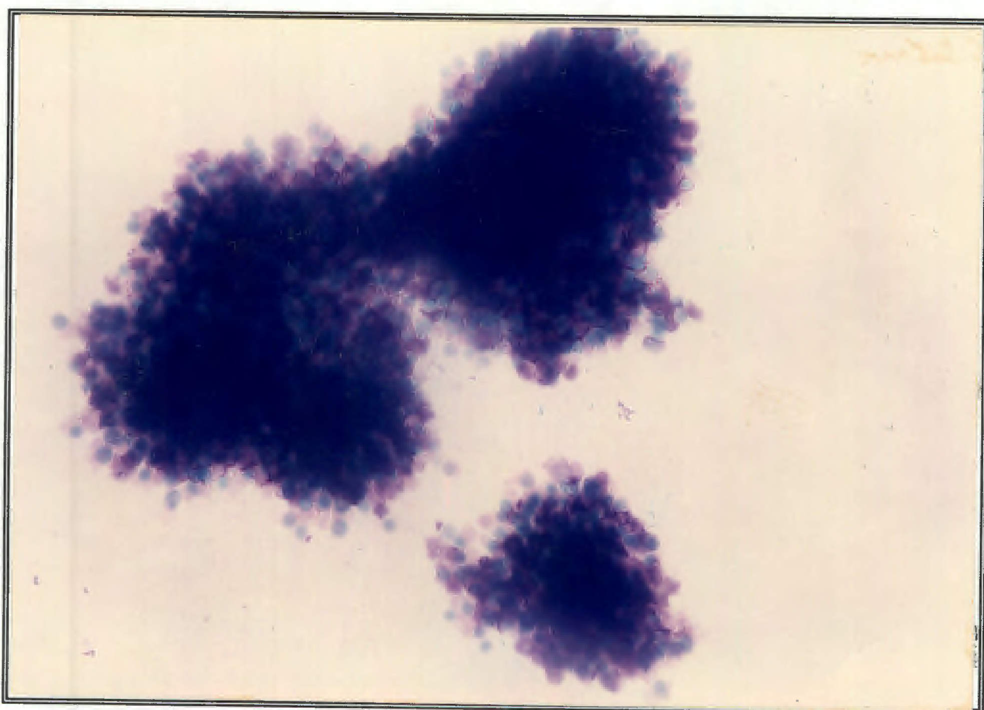

Fig. N° 3. 3.:Morphological details of a BFU-e colony at plateau concentrations of growth factors (Epo 100 U/mL and IL-3 10 ng/mL), (May Grunewald Giemsa; x 100 magnification).

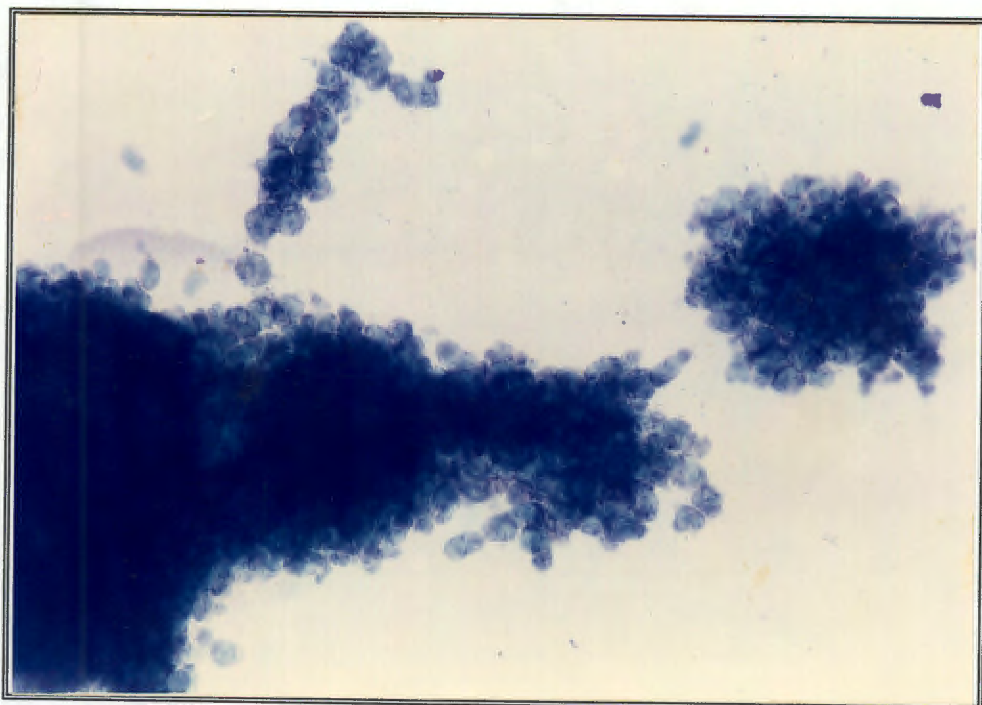

Fig. N° 3. 4: Morphological details of an immature BFU-e colony, cultured at plateau concentrations of c-kit ligand (Epo 2U/mL and IL-3, 10 ng/mL). Note the immature appearance of the cells and lack of haemoglobinization (May Grunewald Giemsa; x 100 Magnification).

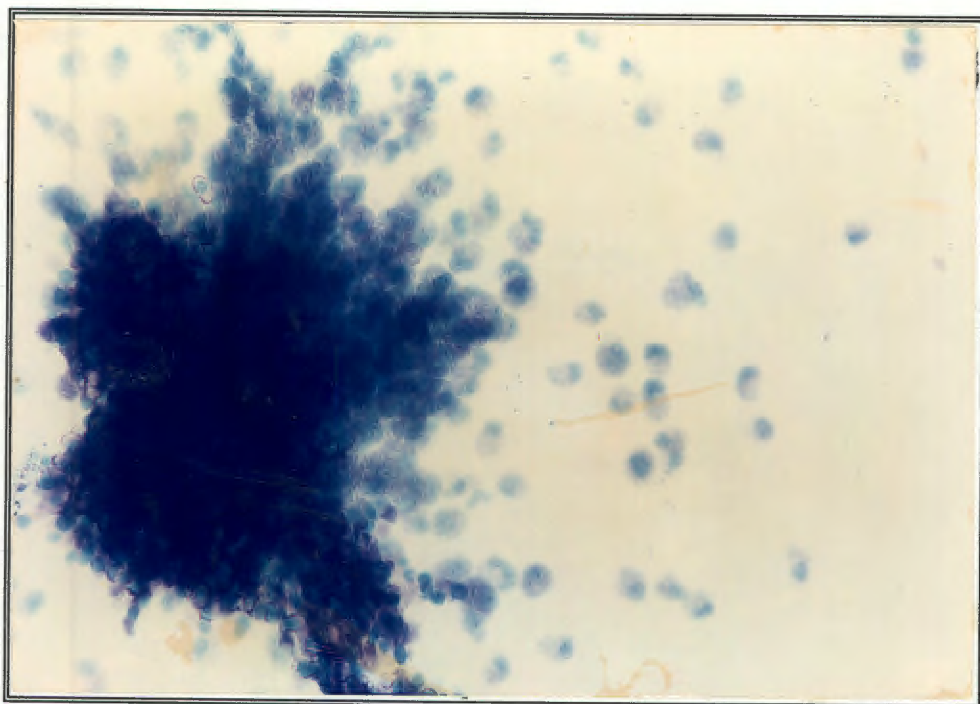

Fig. N° 3. 5: Morphological details of a mixed erythroid and myeloid colony cultured at plateau concentrations of GM-CSF (Epo 2 U/mL), (May Grunewald Giemsa; x 100 magnification).

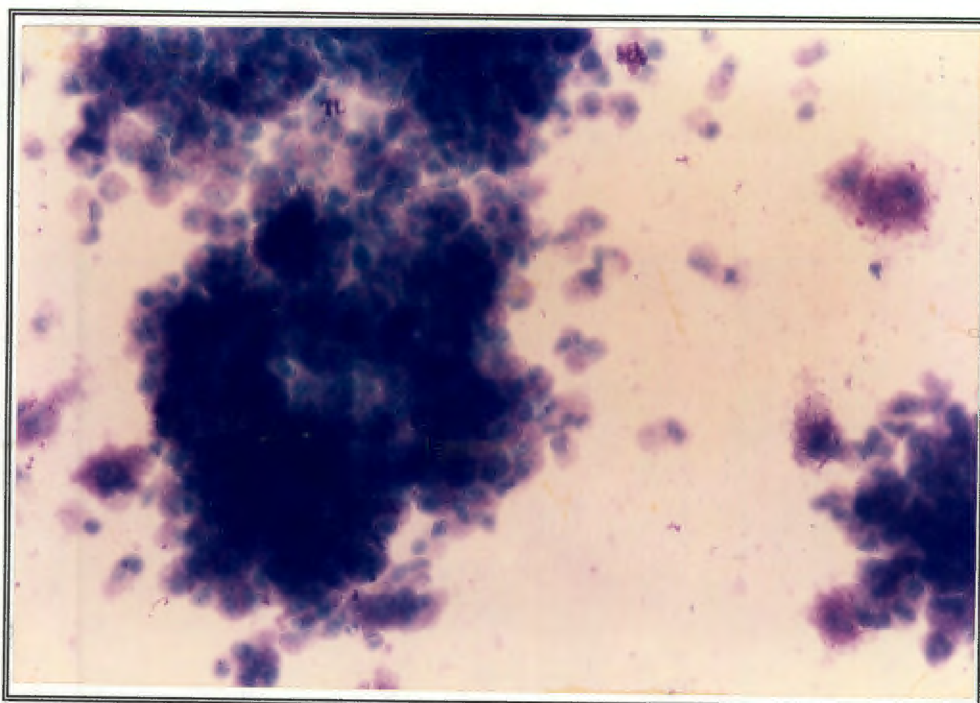

Fig. N°3. 6: Mixed granulocytic and eosinophilic colony cultured in the presence of IL-3 10 ηg/mL and Epo 2 U/mL (May Grunewald Giemsa; x 100 Magnification).

3. 4 DISCUSSION OF THE RESULTS

In humans, most bone marrow progenitors capable of forming colonies have been found to reside within the CD 34+ fraction. This heterogeneous population which represents 1-3 % (Civin 1984, 1987, 1990) of the marrow mononuclear cells, includes most of the primitive myeloid and lymphoid progenitors (Verfaille 1990), capable of reconstituting *in vivo* human and non-human primate bone marrow (Berenson 1988, 1991).

As the selection for the progenitor cell was considered advantageous in the definition of the stem cell function in haematological disorders, it was considered to be of importance to develop some experience in this field, and compare two methods where selection techniques had been well described and no specialized equipment was necessary. Panning has been reported as an efficient method for both positive selection of specific cells, or as a complement to other purification procedures such as fluorescence activated cell sorting, specifically for the initial depletions of undesired populations (Strife 1987).

Similarly, immunomagnetic methods have also been extensively described using products with wide variations in the architecture and particle sizes (Advanced Magnetics Boston, USA; Dynal, Oslo). In an initial pilot study paramagnetic particles of 1-2 μm adsorbed with high affinity goat anti-mouse IgG (Advanced Magnetics) were tested. Although this proved to be an efficient system, it was cumbersome as it required long selection times. This resulted in the decision to study the present magnetic spheres (Novitzky 1991 and unpublished observations).

Dynal particles, originally developed by Ugelstad (Ugelstad 1987), consist of uniform polystyrene spheres of 4.5 μm in diameter with paramagnetic properties produced by maghaemite, dispersed throughout the volume of the bead to a final composition of 22% w/w. The outer surface of the particle is smooth and hydrophobic. It contains hydroxyl groups that can be used for the chemical attachment of proteins. Several techniques have been described to attach antibodies chemically to the bead surface, of which tosylation is the most widely used (Danielson 1986).

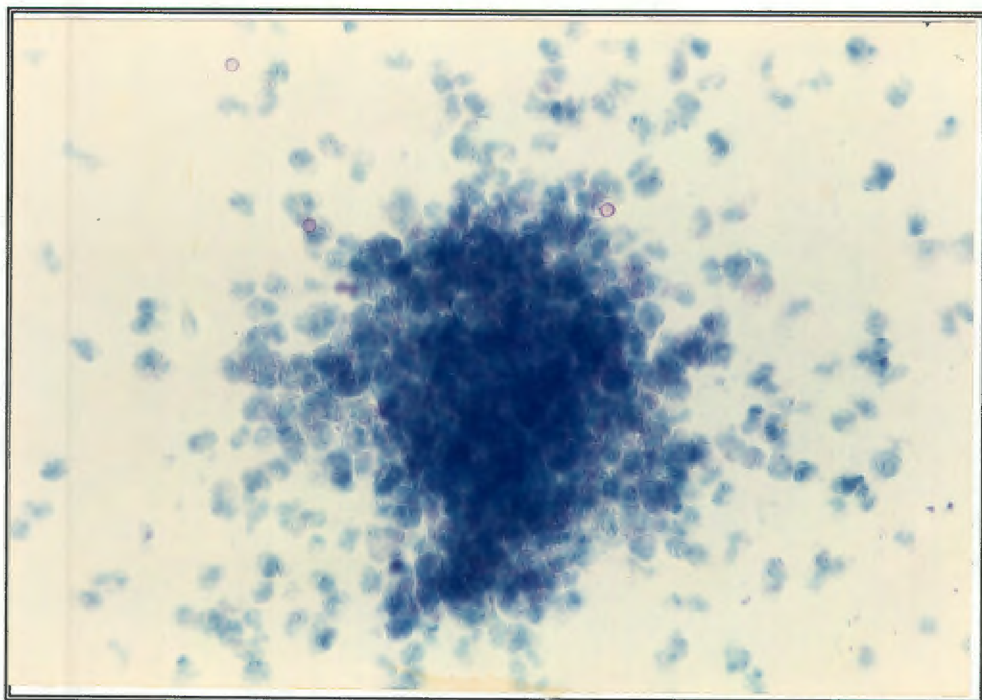

Fig. N° 3. 7: Granulocyte-Macrophage colony cultured in the presence of GM-CSF 10 ηg/mL and Epo 2 U/mL (May Grunewald Giemsa; x 100 magnification).

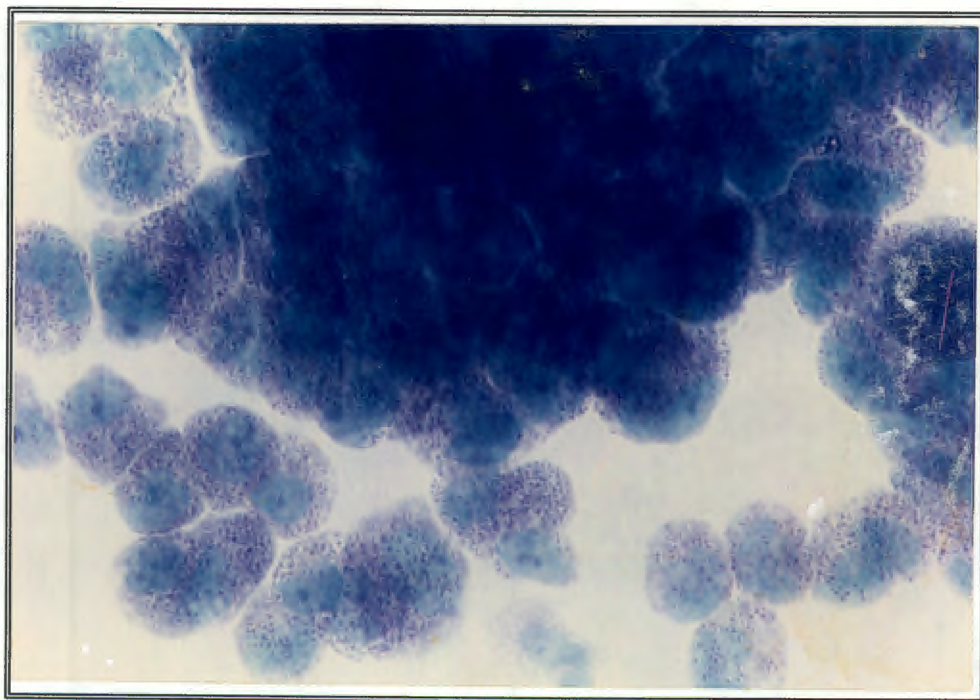

Fig. N° 3. 8: Morphological details of a granulocytic colony cultured in the presence of GM-CSF 10ηg/mL and Epo 2 U/mL (May Grunewald Giemsa; x 100 magnification).

At present pre-activated tosyl beads with various monoclonal antibodies are

commercially available, making the selection procedures feasible for routine use.

Although there was no marked difference in the cell yields between the two described methods, with panning a significantly higher population of cells retained trypan blue compared to the magnetic procedure, reflecting the high shear forces necessary to dislodge the attached cells from the antibody coated plastic surface leading to cell damage.

Both selection techniques resulted in a marked enrichment for the clonogenic cells, approaching a the clonal efficiency of 5-10% for the total number of cells plated. Dose response results of the various combinations of cytokines were consistent with other reports (Ottman 1989), and indicated that maximal colony formation are reached with Epo between 2-10 U/mL, IL-3 10-50 ηg/mL, GM-CSF 10-50 ηg/mL and SCF 40-100 ηg/mL. This data will prove helpful to study the behaviour of the selected progenitors in disorders where the interaction of progenitors and GF may be affected such as in myeloproliferation, myelodysplasia and plastic anaemia.

In conclusion, this initial study confirmed that the selection of CD 34+ haematopoietic progenitors is feasible with both tested methods, leading to a marked enrichment in the colony formation. The magnetic separation was preferred as greater cell purities were obtained and selected cells had significantly better viability.

CHAPTER 4

BONE MARROW STROMA AND STEM CELL FUNCTION IN UNTREATED PATIENTS AND PATIENTS WITH APLASTIC ANAEMIA RESPONDING TO ANTI-LYMPHOCYTE GLOBULIN. PILOT STUDY

4. 1 INTRODUCTION

Abnormalities in the bone marrow microenvironment (Hotta *et al,* 1985, Hintenberger *et al,* 1988) and of the haematopoietic stem cell (Marsh *et al,* 1990) have both been implicated as the probable mechanisms of bone marrow failure in aplastic anaemia.

To further clarify the pathogenesis of this disorder, Marsh studied in cross culture experiments, non-adherent normal and aplastic marrow mononuclear cells. Progenitors were seeded on to pre-formed irradiated stroma from control or patients with non-acute aplastic anaemia, in the LTBMC assay (Marsh *et al,* 1990). In this study, although the clonogenic output of normal cells was adequately supported by the microenvironment from the patients' stroma, target cells were not specifically selected for the early progenitor population. The possibility of normal non-adherent accessory cells contaminating the suspension thus reconstituting and correcting the aplastic LTMC, was not completely ruled out.

To further define the mechanisms of the bone marrow failure and indicate the direction of future investigations, a pilot study was initiated to test the clonogenic potential of aplastic progenitors. These mononuclear cell fractions were compared with control precursors in cross-culture experiments on preformed bone marrow stroma employing a short-term stromal culture system (blastic colony assay, CFU-bl, Gordon *et al,* 1987). In a further step, these control and aplastic CD 34+

progenitors were quantified and cultured in the presence of recombinant growth factors in the mixed colony assay and their clonogenic potential compared.

This initial study would also allow the writer to establish the technical problems associated with such methodology in patients with severe pancytopenia and intense marrow aplasia, specifically as seen during the acute presentation of the disease.

4. 2 PATIENTS WITH APLASTIC ANAEMIA AND METHODS

4. 2. 1 Description of the Patient Population

In this pilot study, two patients with untreated aplastic anaemia, two in response after ALG therapy and another in early clinical relapse after this immunosuppressive therapy were studied and compared with five haematologically normal individuals. Their blood and bone marrow parameters are described in table N° 4. 1.

TABLE 4. 1

STUDY POPULATION
CLINICAL AND LABORATORY DATA

PATIENTS		PERIPHERAL BLOOD COUNTS			BONE MARROW CD34+ PERCENT	TOTAL NUMBER CD34+ SELECTED x 10^4
N°	STATUS	Hb g/dL	WCC $x10^9/L$	Plat $x10^9/L$		
1	Response	10.22	2.9	104.0	0.5	3.3
2	Response	9.7	3.1	97.0	0.3	2.2
3	Relapse	7.4	1.8	49.0	0.0	0.5
4	Untreated	8.8	0.6	19.0	0.0	<0.5
5	Untreated	9.1	0.4	11.0	0.0	<0.5

Peripheral blood values in patients with aplastic anaemia treated with ALG+HDMP, or in those studied before receiving this therapy.

4. 2. 2 Bone Marrow Cells

After informed consent had been obtained, bone marrow from patients with aplasia was aspirated under analgesia provided by intramuscular pethidine hydrochloride (1 mg/kg,) injected 30 minutes before the local anaesthetic. Control marrow was drawn under general anaesthesia from haematologically normal individuals during routine sternotomy for cardiovascular surgery and placed into tubes containing minimal essential medium (MEM, Gibco, Life Technologies, UK) and heparin (Pularin, 20 U/mL Nordisk, Johannesburg). Mononuclear light density cells collected from a polysucrose diatrizoate density gradient (1.077 g/mL, Hystopaque, Sigma, USA) were washed twice in αMEM and divided into two fractions, one for the formation of stromal layers and the second for the selection of the clonogenic progenitor cells.

4. 2. 2. (a) Cultures of the Bone Marrow Stroma

Stromal cultures were established from normal or aplastic subjects by suspending 5 x 10^5/mL light density marrow cells in αMEM containing 12.5% each of horse serum and fetal calf serum, supplemented by 2 x 10^{-6} hydrocortisone. One milliliter of the suspension was cultured in 33 mm petri dishes (Falcon) at 37° C in 5% CO_2 in high humidity. The medium was changed weekly and upon confluence, the cultures were irradiated at 0,75 Gy/min to a total of 15 Gy. At regular intervals adherent layers were inspected under phase contrast microscopy and analyzed morphologically for gross anomalies in the development of the stroma.

4. 2. 2. (b) Blastic Colony Assay (CFU-bl)

The second fraction was suspended in αMEM containing 2% FCS and monocyte depleted by two-hour incubation in 15 x 220 mm plastic dishes (Bibby, UK). Aliquots were taken for cytochemical and immunophenotypic determinations, and 5 x 10^5 selected cells were layered over the preformed normal and aplastic stromal cultures for two hours at 37° C, for adhesion to take place. The stroma non-adherent population was decanted by standardized washing with tissue culture medium. Adhesive precursors were covered with 0.3% agar and cultured for five days at 37° C in 5% CO_2 and 90% humidity. Blastic colonies (CFU-bl) (Gordon *et al,* 1987) were scored as aggregates containing more than twenty cells.

4. 2. 2. (c) Immunomagnetic Selection of the Haematopoietic Progenitor Cells

The remainder of the monocyte-poor marrow was lymphocyte-depleted by incubating cells with CAMPATH 1 M (6.25 mg/mL; 0.01 mL/10^6 cells) (Hale *et al,* 1983) for 50 minutes at 37° C in the presence of 10% human AB serum as a source of complement (Western Province Blood Transfusion Services; Cape Town, South Africa). Accessory cell-free fraction was re-suspended in 0.2 mL PBS (pH 7.4) and incubated with anti-CD 34 (MY 10 Becton-Dickinson) at 4° C for 30 minutes in continuous rotation. Excess antibody was washed off, and labelled cells exposed to 5-15 x 10^6 paramagnetic particles coated with high affinity goat anti-mouse immunoglobulin (Magnetics Institute, Boston Mass). A positive selection was achieved with a magnetic field applied to the side of the tube. As described in the previous chapter, aliquots of the initial light density and selected populations were taken for morphological, cytochemical, immunophenotypic characterizations and cell viability studies.

4. 2. 2. (d) Clonogenic Tests. Mixed Colony Assay

Selected cells were re-suspended in IMDM, and 1-10 x 10^3 cells cultured for fourteen days in the mixed colony assay in IMDM (with L-Glutamine, Gibco) in the presence of 2 U erythropoietin and rh-IL 3 10 ηg/mL or rh GM-CSF 10 ηg/mL; supplemented by 5 x10^{-5} mercaptoethanol and 30% FCS in 0.3% agar. All results were corrected and expressed as colonies / 10^5 cells. Erythropoietin was generously donated by Bioclones (Sandton, Transvaal). Once reconstituted, it was kept at 4° C at a concentration of 1000 U/mL. IL-3 and GM-CSF were gifts from Amgen (Amgen Thousand Oaks, California) with specific activities of 10^7 and 10^8 U/mg respectively.

4. 3 RESULTS

4. 3. 1 Cultures of the Bone Marrow Stroma

Both normal and aplastic stroma became confluent between three and four weeks from initiation. On inspection, there was no morphological difference between normal and chronic aplastic stromal cultures, while a reduction in the macrophage numbers was evident in the cultures derived from untreated patients.

4. 3. 2 Blastic Colony Assay (Fig. N° 4. 1)

Both normal and aplastic stromal layers supported the formation of CFU-bl to an equal extent. There was, however, a marked reduction in the number and size of colonies generated from the marrow of patients with treated aplasia when grown over normal stroma. These aggregates were often poorly circumscribed. No clonogenic growth occurred from progenitors derived from patients with untreated aplasia.

4. 3. 3 Immunomagnetic Progenitor Cell Selection and Mixed Colony Assay

In patients with aplastic anaemia following immunosuppressive treatment, the CD 34 positive population in the bone marrow was quantitatively decreased to 0.1 and 0.2% of mononuclear cells. Absolute numbers of the selected populations were also markedly reduced in the treated group (mean 2.0×10^4, range 0.5-3.3) while in those with the untreated disease, this population was practically undetectable (fewer than 0.5×10^4 /mL cells).

Furthermore, cultures of accessory-free population defined as containing fewer than 5% of CD 3 and CD 14 positive cells, unreactive with α-naphtyl butyrate esterase and positively selected to a purity of greater than 80%, showed a marked reduction in the colony growth when compared with normal precursors in response to IL3 and GM-CSF (Table 4. 2 and Fig N° 4. 2, page 69). Here again, no growth was elicited from cells derived from patients with untreated aplasia.

4. 4 DISCUSSION OF THE RESULTS

The blastic colony assay (CFU-bl) exploits the natural adhesive properties of early bone marrow progenitors to allogeneic myeloid stroma, supporting the development of colonies of undifferentiated blasts. These precursors have been shown to form secondary colonies, and have cell kinetics similar to fetal cord blood derived progenitors (Gordon *et al,* 1985, 1987).

Morphological inspection of the diseased stroma failed to reveal any obvious

differences with the control cultures. The only feature observed was fewer macrophages in the stroma derived from the untreated patients. Functionally, however, no differences in the CFU-bl numbers were elicited compared with control layers, seeded with normal light density cells. These data suggest that the aplastic stroma has no significant inhibitory action on the normal cells studied.

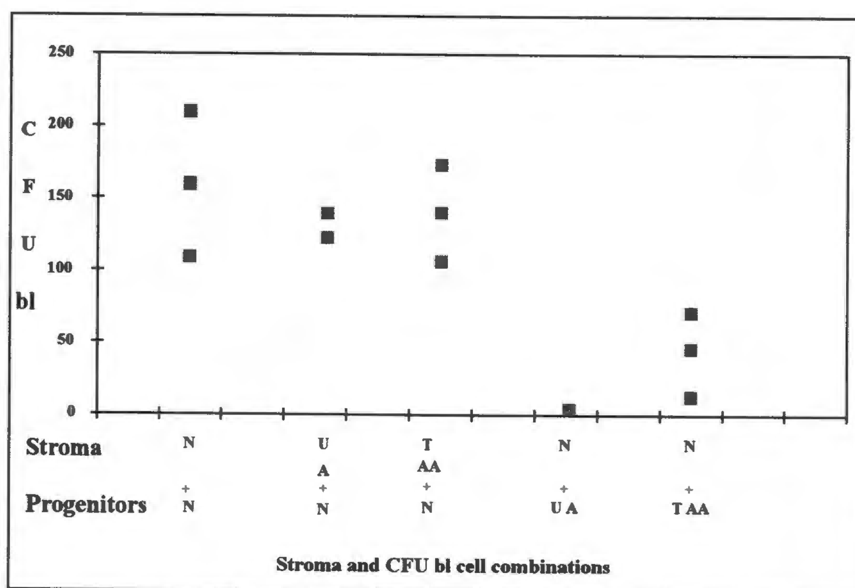

Fig. N° 4. 1: Stromal blast cell colony assay. N = Marrow obtained form normal volunteers. UA = Marrow from patients with untreated aplastic anaemia. TAA = Marrow from patients who have previously received antilymphocyte globulin.

Compared to control cultures, the number of blastic colonies in patients responding to immunosuppressive therapy, was markedly decreased and absent in those studied during the acute phase of the disease. This is consistent with the immunofluorescence studies that showed a striking reduction in the CD 34 positive progenitors from aplastic marrows. This observation would suggest that with the current methodology, extensive studies on the bone marrow cells of patients with untreated severe aplasia would be unsuccessful.

As expected, when positively selected progenitors were cultured in the mixed colony assay in the presence of recombinant growth factors, despite equal numbers of CD 34+ cells seeded, resulted again in no growth in the untreated group.

TABLE 4. 2

SELECTED POPULATIONS

STUDY GROUP	COLONIES x 10^5					
	IL3 50 ηg/mL			GM-CSF 50 ηg/mL		
	BFU-e	CFU-e	CFU-gm	BFU-e	CFU-e	CFU-gm
PATIENTS						
1	110	107	21	60	290	100
2	210	220	98	110	120	150
3	67	100	52	49	73	58
4	0	3	0	0	9	0
5	0	1	0	0	2	0
NORMAL	482	2032	231	380	928	372

Results of the clonogenic growth of CD 34+ progenitors in the mixed colony assay from patients with treated (N° 1, 2 and 3), untreated aplastic anaemia (4 and 5) and compared to median colony numbers obtained in 5 controls.

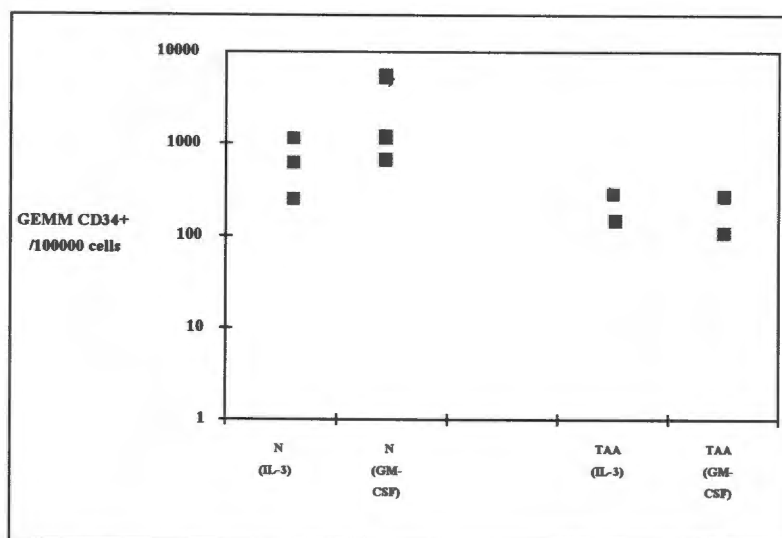

Fig. 4. 2: GEMM: CFU assay results. CD 34+ cells from normals (N) compared with those derived from patients with treated aplastic anaemia (T AA) cultured in the presence of rh Epo with IL-3 or GM-CSF.

However, in those recovering after immunosuppression, the clonogenic output was detectable but appeared markedly reduced. This result is consistent with the

postulate that the haematopoietic abnormalities reside mainly within the HSC. The possible alternatives include, firstly, that stem cells that survive the initial attack (such as immunological or irradiation) and repopulate the bone marrow are inherently hypoproliferative and the normal balance between positive and negative regulators favours sub-optimal colony formation (Young, 1992). Alternatively, continuing immunological suppression by the microenvironment (Merchav, 1988, Hotta *et al,* 1986, Bacigalupo *et al,* 1984, Zoumbos *et al,* 1985, Hintenberger *et al,* 1988) or a deranged interaction between growth factor receptors and ligands within the bone marrow milieu leads to a reduction in the clonogenic output.

In the current pilot study it is concluded that aplastic stroma is not suppressive to the development of normal progenitors. This implies that the primary defect probably resides with the aplastic stem cell population. To confirm this hypothesis a more extended study was initiated, where a purified population of progenitors positively selected for the CD 34 membrane antigen and devoid of accessory cells was cultured on preformed stroma or subjected to incremental concentrations of various growth factor combinations.

Finally, although irradiation with 15 Gy does not affect normal or aplastic stroma, it remains possible that this leads to damage of the of resident radiosensitive alloreactive accessory cells, allowing normal proliferation of the control progenitors. Evidence also exists that following this physical insult, release of GFs from stromal cells is detected (Tsuboi *et al,* 1991), altering further the interpretation of the results. To avoid these technical variations, future studies would employ unmanipilated stromal layers as negative controls.

CHAPTER 5

IN APLASTIC ANAEMIA THE BONE MARROW STROMA DOES NOT INHIBIT NORMAL CLONOGENIC GROWTH

5. 1 INTRODUCTION

Under steady state conditions, commitment, differentiation and maturation of the haematopoietic stem cell occur in the bone marrow. Although the activation of programs for cell division appears to be of a stochastic nature (Suda *et al,* 1983), it is generally accepted that the amplification of the various cell lines and signalling towards maturation are controlled by stromal cells, extracellular matrices and growth factors present in this organization that are named collectively as the bone marrow microenvironment (Gualtieri *et al,* 1987). Elements in the stroma appear to provide both structural adherence sites and to secrete positive and negative growth-regulating molecules that allow early self-replicating progenitors to sustain a stable haematopoiesis (Gordon *et al,* 1987).

In aplastic anaemia, the nature of the haematopoietic defect remains unknown but as reviewed in Chapter 1 abnormalities in the myeloid microenvironment (Hotta *et al,* 1985, Hintenberger *et al,* 1988, Juneja *et al,* 1984) or in the stem cell pool (Aoki *et al,* 1990) have been documented. In addition, well defined animal models exist of genetically anaemic mice where the stroma (*S/S^{l}*) or the haematopoietic stem cells (*W/W^{v}*) are defective (Mc Culloch *et al,* 1965; Bernstein *et al,* 1968).

Since an integral part of the bone marrow microenvironment are cells of the immune system (McGuiness *et al,* 1991, Shibata *et al,* 1986) and of monocyte origin (Strobel *et al,* 1986) that are known to secrete growth modulating molecules, alterations in the immunity may indeed affect haematopoiesis. Furthermore, as described in Chapter 2, immunomodulation with anti-lymphocyte globulin or cyclosporine have led to successful reversal of this syndrome in the

majority. Nevertheless, some patients who respond to these therapies, on more detailed inspection have derangement in their haematopoiesis. Here, in addition, the function of the differentiated progeny appears abnormal and of more concern is that, in a subgroup, evolution to myelodysplasia, PNH and acute leukaemia has been described (Tichelli *et al,* 1988 [a and b]).

To define the relevance of each pathogenetic mechanism, Marsh studied patients with aplasia after immunosuppressive therapy, with the LTBMC system. In cross-over experiments, marrow cultures of adherent-cell-free light density cells were seeded onto irradiated preformed stroma. She concluded that, in all patients tested, a primary stem cell defect was present. Of importance was that, regardless of the degree of response or treatments received, significant reductions in the CFU-GM output were found. In addition, in one patient a combined stromal defect was also demonstrated (Marsh *et al,* 1990).

In this work, however, "engraftment" with normal elements present in the unselected plastic non-adherent population correcting a latent abnormality was not excluded. This point was partly addressed in a limited follow-up study when purified populations of aplastic or control CD 34+ cells were cultured over preformed stromal layers (SL). Results of this work confirmed the initial findings (Marsh *et al,* 1991).

In a modification of this system that exploits the adhesive properties of clonogenic cells, it is possible to examine an early population of undifferentiated blasts, (CFU-bl) (Dowding *et al,* 1992, Gordon *et al,* 1987). These resemble murine spleen colony-forming cells in their resistance to cycle specific cytotoxics (Gordon *et al,* 1985). They are able to produce secondary blastic colonies with a repopulating potential similar to, or exceeding that of umbilical cord blasts (Gordon *et al,* 1987).

Applying this model, the writer has previously reported that both the stroma derived from untreated aplastic patients or from those responding to immunosuppression support growth of normal precursors effectively. However, aplastic haematopoietic progenitors were defective (Novitzky *et al,* 1991). We

have now extended these studies and confirm that the marrow stroma in aplasia allows the development of normal blastic colonies adequately, while the clonogenic growth of stroma adherent and non-adherent CD34 positive progenitors is significantly reduced.

5. 2 Study Population (Table 5. 1)

Sixteen patients and seven control subjects gave written consent according to Institutional guidelines to donate bone marrow for the clonogenic studies.

5. 2. 1 Patients with Aplastic Anaemia

Between February 1984 and March 1991, thirty-five patients with severe acute aplastic anaemia were referred to our Institution for further evaluations. One subject had a rapid improvement following a therapeutic abortion, while the rest received a uniform protocol of anti-lymphocyte globulin and high-dose methyl-prednisolone, followed by oxymetholone for six to nine months (Novitzky *et al,* 1991, Chapter 2). Twenty-three fulfilled the criteria for response (freedom from transfusions, granulocyte count of >1 x 10^9, and platelets of >40 x 10^9) and at a median of 1320 days, 66% are alive.

Their median time in response was four years (1-8). Three required further immunosuppression, and two are currently receiving cyclosporine and androgenic corticosteroid maintenance. Two patients developed haemolysis, haemoglobinuria and a positive Ham's test with clinical features of paroxysmal nocturnal haemoglobinuria (PNH). The median age is 33 (17-61), nine are females and for the whole group the median performance status is 0 (WHO).

5. 2. 2 Control Population

Progenitor cells in the control population derived from haematologically normal individuals undergoing sternotomy for cardiac surgery under general anaesthesia.

5. 3 METHODOLOGY FOR THE CULTURE OF THE BONE MARROW STROMA AND THE BLASTIC COLONY ASSAY

5. 3. 1 Bone Marrow Cells

In the study population, bone marrow was aspirated under pethidine HCl (1 mg/kg IM) sedation and local anaesthesia. Both normal and aplastic marrow-rich blood was placed into tubes containing preservative-free heparin (Pluralin, 10 U/mL; Nordisk, Johannesburg), and a monocellular suspension was achieved by forcing marrow cells through 23 gauge needles diluted 1/5 in Minimal Essential Medium (aMEM) (Gibco, Life Technologies, UK) and layered to on a polysucrose diatrizoate density gradient (1.077 g/mL, Hystopaque, Sigma, USA).

Light density cells obtained from the interface were washed three times in the same medium and re-suspended in 10 mL. Aliquots were taken for morphological assessment, indirect immunofluorescence and quantitative determinations with an electronic particle counter (Multisizer, Coulter Electronics, USA). Cell suspension was then divided into two fractions, one to establish the adherent stroma and the other for the selection of the progenitor cells.

5. 3. 2.- Cultures of the Bone Marrow Stroma

Throughout the study sequential stromal cultures from three haematologically normal males aged 34, 41 and 42, who had undergone surgery for coronary artery by-pass grafting were employed. In morphological and functional studies their stroma was compared with aplastic SL. Neither the patients nor the controls had a history of recent inflammatory or infectious disorders.

Normal or aplastic light density bone marrow cells were suspended in aMEM containing 12% of horse serum (HS; GIBCO; Life Technologies, UK) and foetal calf serum (FCS; GIBCO), supplemented with hydrocortisone 2 x 10^{-6} and gentamycin 5 mg/mL. Mononuclear cells were cultured in 33 mm Petri dishes (T. C. Dishes; Bibby-2500 B, UK) at a cell concentration of 0.5 x 10^{6} per dish at 37°C

in 90% humidity and gassed with 5% carbon dioxide until stroma became confluent. The medium was changed weekly throughout the culture life.

5. 3. 3 Selection of the Haematopoietic Progenitor Cells

The methodology for CD 34+ cell selection has been detailed in chapter three. Briefly, lymphocyte and monocyte-poor progenitor cell concentrates were re-suspended in 0.2 mL of cold (4° C) phosphate buffered saline (PBS) containing 0.02 mL/10^7 cells of anti human CD 34 antigen murine monoclonal antibody and incubated at 4° C for 30 minutes. Labelled cells were then washed three times in PBS containing 2% AB serum and exposed at 4° C to polystyrene coated paramagnetic beads at a cell concentration of 5-10 x 10^9/L, for 30 minutes in continuous rotation. Incubation with the paramagnetic particles and magnetic separation were performed twice on labeled cells.

To free cells from beads, selected cells were re-suspended in 0.1 mL of PBS containing 0.01 mL of a polyclonal murine immunoglobulin (Detachabead, Dynal) at room temperature for 60 minutes in a continuous rocking motion and exposed to the magnetic forces again. Cells freed from beads were recovered, aliquots taken for immunofluorescence studies (as described in chapter three) and viability determined by trypan blue exclusion. Recovered cells were counted in a haematocytometer and numbers adjusted to a concentration of 1-5 x 10^4/ mL.

5. 3. 4 Blastic Colony Assay

Magnetically selected 1 x 10^4 CD34 positive cells obtained of the control population or patients with aplasia were layered in duplicates in cross-culture experiments on either preformed normal or aplastic SL for two hours, to ensure optimal adherence. The unattached cells were then removed by standardised washing with aMEM. Progenitors affixed to SL were covered with 1 mL of 0.3% agar (final concentration) in aMEM containing 30% FCS, and cultured for 5 days at 37° C in 5% CO_2 (Gordon *et al,* 1987).

Aggregates defined as containing more than twenty cells were scored with an inverted microscope on days five and seven of culture. Layers that had not been

seeded with CD 34+ cells served as negative controls. CD 34 positive cells from six normal subjects were also co-cultured on normal stroma. CFU-bl numbers were then determined to provide comparative values to contrast for colony numbers obtained from control progenitors layered on aplastic stroma.

Following the two-hour incubation, non-adherent CD 34 positive progenitors were washed off SL, pooled and cultured in Iscove's modified Dulbecco's medium (IMDM, with L-Glutamine, Gibco) containing 30% FCS and 15% PHA lymphocyte conditioned medium in agar 0.3% (final concentration) in cluster dishes (Nunclon, Delta, Code 176740; UK) for 14 days at 37° C in a CO_2-rich, humidified atmosphere. As morphological and cytochemical determinations of cytospin specimens from progenitors eluted from two-hour incubation on stroma showed significant contamination with stromal macrophages (absent on the CD 34+ pre-incubation specimens), aggregates of these cells were not scored. Only colonies of granulocytes and granulocyte-macrophage CFU containing more than 40 cells were counted and results of colony numbers from these duplicates were divided by 2 and expressed /10^4 cells.

5. 3. 5 Morphological and Cytochemical Assessment

To define the stromal elements, upon confluence, representative cultures were dried and stained *in situ* with May-Grunewald-Giemsa (Lewis *et al,* 1986), or incubated with 1 mL 0.1% bovine trypsin (Difco Corp, Detroit, MI) for 10 minutes at 37° C. This enzyme was then inactivated with cold FCS and washed twice in aMEM. Cytospins of the complete stromal suspension were prepared (Shandon) and stained with May Grunewald Giemsa, Sudan Black, Oil Red O, and alkaline or acid phosphatases. In this analysis of the cytochemistry of the marrow stroma, differential counts were performed, and results compared between patients and controls.

5. 3. 6 Statistical Analysis

Standard two-way analysis of variance was used to determine the significance of differences between groups of cultures, or a single culture versus control group. Student's t test was used to assess the standard error of the difference between two

means in the results of colony assay data.

5. 4 THE BONE MARROW STROMA IN APLASTIC ANAEMIA. RESULTS OF THE BLASTIC COLONY ASSAY

5. 4. 1 Morphology and Cytochemical Staining of the Stroma (Table 5. 1)

Both normal and aplastic stroma became confluent at a median of four weeks (3-4 Vs 3-5). In one patient it did not reach confluence by five weeks, when it commenced to lift off in the majority of the dishes.

When inspected under phase contrast microscopy, no obvious abnormalities were detected in the stroma from patients with marrow aplasia. Morphologically, they looked similar to normal stroma (Fig. N° 5. 1 and 5. 2, page 79 and 80). Similarly, there was no difference in the stromal architecture shown by the various stains used *in situ*. Differential counts of stained cytospin preparations from normal controls showed that Sudan black positivity was present in a median of 13% (SD 7.2) of cells, oil red O in 9% (SD 4.1) acid phosphatase 72% (SD 17.5) while alkaline phosphatase was positive in 61% (SD 17.2).

In stroma from patients with aplasia, the respective values were: Sudan black 16% (SD 7.7), oil red O 3% (SD 2.5%), alkaline phosphatase 56% (SD 11.5) and acid phosphatase 64% (SD 20). Between the two groups, none of these values differed significantly (Table N° 5. 1, page 78).

5. 4. 2 Selection of the Haematopoietic Progenitor Cells (Table N° 5. 2)

Immunofluorescence studies on unselected light density cells from patients with aplasia resulted in a median of 0.65% CD 34+ (SD 0.35) cells. These were significantly fewer progenitors than normal controls at 1.6% (SD 1.38) ($p < 0.05$).

TABLE 5.1

CYTOCHEMICAL DEFINITION OF THE BONE MARROW STROMAL LAYERS

STROMA	SUDAN BLACK		O RED OIL		ALKALINE PHOSPHATASE		ACID PHOSPHATASE	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
APLASTIC	16.00	7.76	3.06	2.52	55.56	11.43	64.31	19.95
NORMAL	13.00	7.20	9.00	4.10	61.00	17.20	72.00	17.50

Cytospins of suspensions of bone marrow stroma from normal controls and patients with aplastic anaemia were stained with cytochemical stains and mean % (SD) positivity determined on 300 cells.

When corrected to an initial cell number of 100 x 10^6 light density cells, the total median number of selected progenitors harvested was higher within the control population at 0.539 x 10^6 vs 0.116 x 10^6 from the study group ($p= 0.18$).

Upon morphological or immunophenotypic examination of the selected fraction, there was no obvious difference in the cell content (Table N° 5. 2, page 82). Morphologically, undifferentiated blasts represented a median of 80 and 82.5% in each group. The majority of contaminating cells were of the early granulocytic series, while monocytic cells were always < 2% in both selected groups.

5. 4. 3 Blastic Colony Assay (Table N° 5. 3)

Normal progenitor cells selected for the CD 34 antigen and cultured for five days on aplastic stroma gave a mean of 103.1 colonies/10^4 cells (Median 104; SD 30.4). Two types of colonies were obvious: the majority belonged to colonies that were adherent and well circumscribed, compact, round or more often elongated, following the body of fibroblastic cells in the stroma (Fig N° 5.3 to 5.5, pages 81 and 84), while the second group appeared to detach from the stroma and rise into to the agar .

The mean number of 6 normal CFU-bl on control stroma was 117 colonies/10^4 cells (mean 109, SD 20.4) ($p > 0.05$) on day five, and this number did not change

significantly on day seven, when some clones became looser and started detaching from the adherent layer. None of the SL that were not seeded with selected progenitors showed any CFU-bl formation.

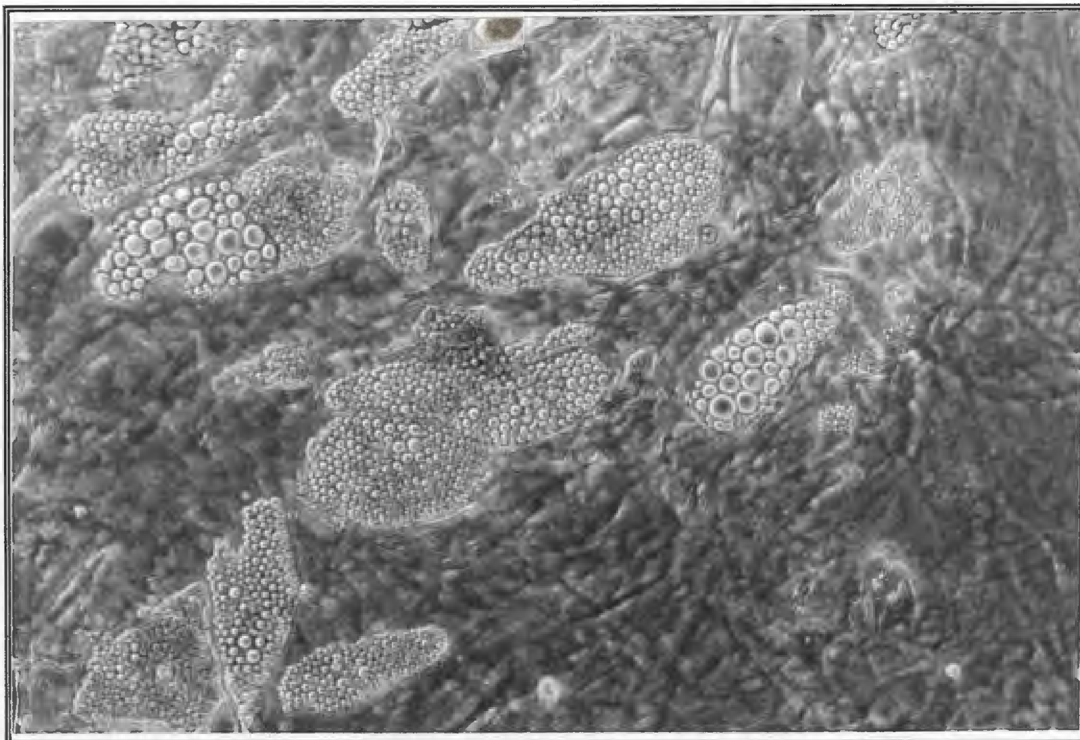

Fig. N° 5. 1.- Normal confluent bone marrow stroma as observed with contrast microscopy (x 100 magnification). Fat cells are prominent and surrounded by fibroblasts.

When aplastic CD 34+ precursors were incubated on normal stromal cultures, a mean of 41.06 colonies /10^4 cells (median 29, SE 42.9) was scored (Fig N° 5. 7, page 86). This was significantly lower than colony numbers obtained from normal CFU-bl on aplastic SL ($p= 0.0002$ exact one tailed T test). The majority of the colonies were small, loose, with many scattered cells (Fig. N° 5. 6, page 85). When observed on day seven, although the colony number scored did not change significantly, some aggregates continued to increase in size even until day ten, when all cultures were terminated.

5. 4. 4 Stroma Non-Adherent Clonogenic Growth (Table 5.3)

When cultures from stroma non-adherent cells decanted after a two-hour incubation and grown for fourteen days in the presence of PHA-LCM were scored for clonogenic growth, results revealed that both normal and aplastic CD 34+ had

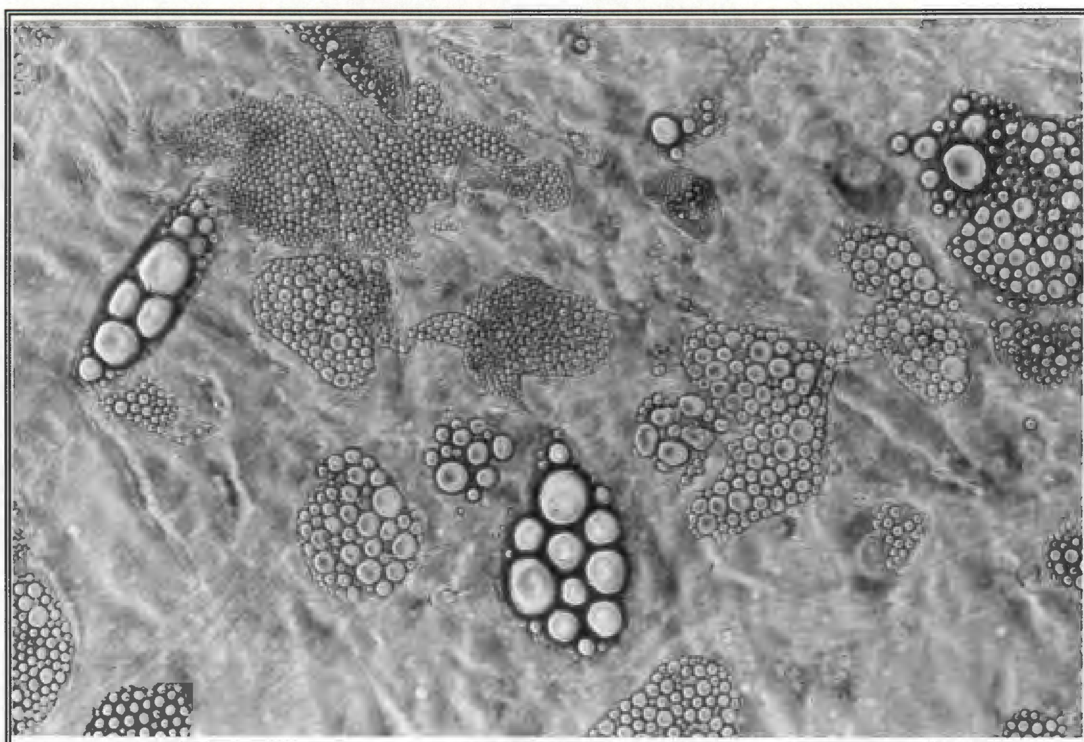

Fig. N° 5. 2.-Confluent bone marrow stroma from a patient with aplastic anaemia in partial remission. Contrast microscopy (x 100 magnification).

proportionately similar numbers of stroma adherent and non-adherent precursors. Specifically, when aplastic CD 34+ were incubated on normal stroma, the mean CFU-bl/CFU-gm ratio was 41.06 (SD 42.9): 142 (SD 104.8). Corresponding results from normal CD 34+ on aplastic stroma were 103.1 (SD 30.4): 361.7 (SD 91.3) and similar to control progenitors off normal adherent layers at 117 (SD 20.4): 335 (SD 56.8) (Fig. N° 5. 7, page 86).

Furthermore, when results of adherent and non-adherent aplastic colonies were combined as a total clonogenic potential and compared with the normal composite, diseased CD 34 positive cells had significantly lower colony output (combined mean 185.8 vs 447.1; $p= 0.0009$, exact two-tailed). These data indicate that both stroma adherent and non-adherent progenitors are decreased in aplastic anaemia, and alterations in the adhesion to the stroma were not a significant cause of the reduction in the CFU-bl in this disease. Alternatively, these results may argue for a relative increase in the CD 34+ non-clonogenic population within the aplastic fractions. Nevertheless, because CFU-bl were generally smaller in size and took longer to develop, an intrinsically hypoproliferative population is favoured.

5. 5 DISCUSSION OF THE RESULTS

Haematopoiesis in mammals is under the control of a variety of positive and negative regulators with feed-back mechanisms (Layton, 1989) between the mature elements, the progenitor cell pool and the bone marrow microenvironment (Gordon, 1987).

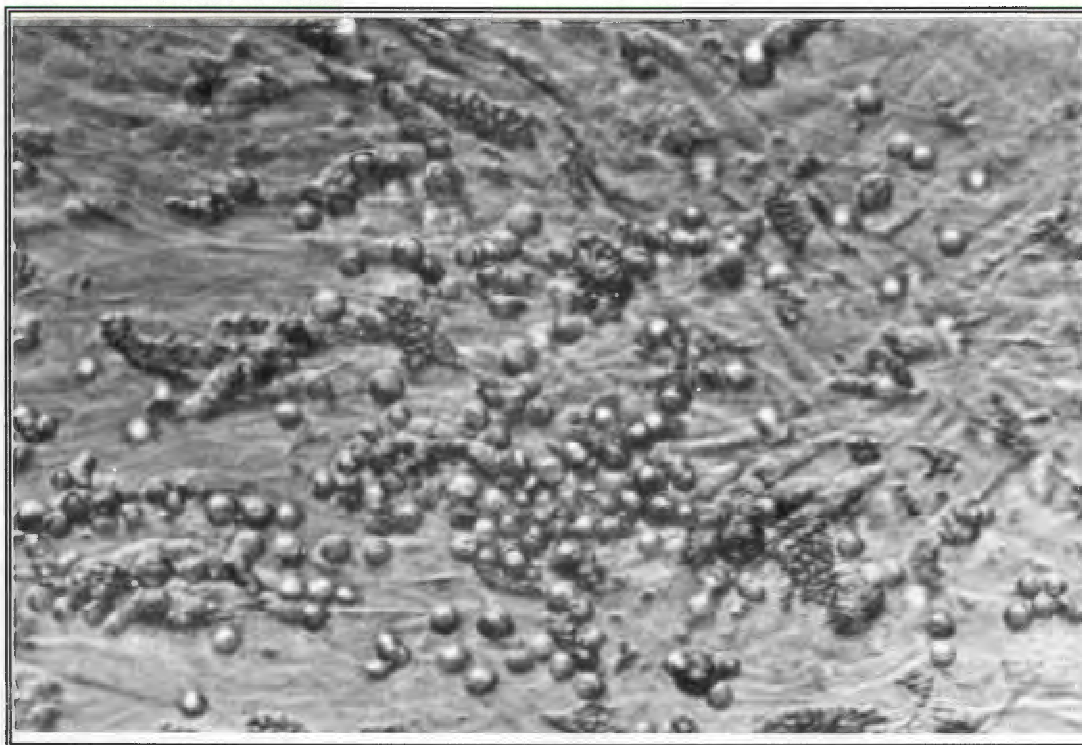

Fig. N° 5. 3: Blastic Colony Assay. Contrast Microscopy of a day 5 colony of haematopoietic progenitors derived from normal CD 34+ adherent cells (x 100 magnification).

It is likely that steady-state haematopoiesis is maintained by the bone marrow stroma, either through direct cell membrane interactions as suggested by co-cultures with formalin fixed NIH-3T3 fibroblasts (Roberts *et al,* 1987), or through the release of growth factors (Gordon *et al,* 1987 Kittler *et al,* 1992). These cytokines are secreted by the stroma, or resident cells of the immune system (lymphocytes, macrophages), which then act on receptors present on the target progenitor cell membranes (Nicola and Metcalf, 1984, 1985).

However, *in vitro* lymphocytes and monocytes can also down regulate myeloid proliferation (Irvine *et al,* 1991), independently from Interferon and IL-1 secretion. Alternatively, they can also induce TNF production by accessory cells.

TABLE 5. 2

PROGENITOR CELL SELECTION

CELL POPULATION	APLASTIC ANAEMIA		CONTROL	
	MEDIAN	SD	MEDIAN	SD
LIGHT DENSITY CELLS				
MORPHOLOGY	n =15		n=7	
(%)				
BLASTS	9.00	4.79	9.00	3.55
IMMUNOFLUORESCENCE STUDIES	n=10		n=7	
(%)				
CD 3	5.00	2.80	8.00	2.91
CD 14	9.00	2.61	8.00	3.10
CD 19	12.50	7.70	14.00	4.93
CD 34	0.65**	0.35	1.60	1.38
SELECTED POPULATION				
MORPHOLOGY	n=15		n=7	
(%)				
BLASTS	81.00	9.63	87.00	7.02
NSE	1.00	0.98	3.00	2.34
IMMUNOFLUORESCENCE STUDIES	n=9		n=6	
(%)				
CD 3	<10	-	<10	-
CD 34	80.00	4.97	82.50	5.74

Characteristics of the light density cell population of the patient and control groups. Results of purification procedures and positive selection for the CD 34+ cells for both populations are presented. ** Denotes statistical significance ($p < 0.05$).

All these effects may lead to inhibition of both erythropoiesis (Johnson *et al,* 1991) and myelopoiesis (Kobari *et al,* 1990, Klimpel *et al,* 1982), which can be reversed by increments of colony stimulating factors in the cultures (Klimpel *et al,* 1982). Therefore if the net secretion of inhibitory molecules predominates, it will result in a limited marrow output (Raefsky *et al,* 1985).

In aplastic anaemia an overall activation of the immune system has been described with increase production of IFN-g (Zoumbos *et al,* 1985), neopterin and TNF (Hintenberger *et al,* 1988), from enhanced spontaneous secretion of these cytokines and increased bone marrow suppressor cell activity from CD 8+ Fc Gamma+ lymphocytes (Bacigalupo *et al,* 1984).

TABLE 5. 3

STROMA ADHERENT AND NON-ADHERENT COLONIES

PATIENT	NORMAL STROMA APLASTIC CD 34+ CFU-bl	APLASTIC NON-ADHERENT CD 34+ CFU-gm	APLASTIC STROMA CONTROL CD 34+ CFU-bl	NORMAL NON-ADHERENT CD 34+ CFU-gm
1	15.0	99.0	82.0	214.0
2	12.0	30.5	92.0	-
3	6.0	129.0	72.0	502.5
4	27.0	306.0	-	-
5	82.0	367.0	102.0	310.0
6	20.0	38.0	104.0	384.0
7	181.0	316.0	111.0	210.0
8	49.0	64.0	143.0	369.0
9	20.0	80.0	105.0	-
10	32.0	147.0	71.0	-
11	2.0	63.0	98.0	-
12	63.0	165.0	186.0	390.0
13	38.0	141.0	108.0	471.0
14	26.0	103.0	108.0	376.0
15	50.0	-	104.0	410.0
16	34.0	91.0	61.0	344.0

Absolute values of stroma adherent/non-adherent progenitors from patients with non-acute aplastic anaemia cultured on normal stroma and of progenitors from normal subjects on aplastic stroma.

A few studies have suggested that the mechanisms of the pancytopenia originate from abnormalities in the marrow microenvironment (Knospe *et al,* 1971, Ershler, *et al,* 1980, Hotta *et al,* 1985). Specifically, marrow inhibitory effects and alterations in certain cell populations have been demonstrated both in the acute presentation and following immunosuppressive therapy (Anderseen *et al,* 1989 Hinterberger *et al,* 1988, Bacigalupo *et al,* 1984). However, when tested, aplastic stromal cells secreted adequate amounts of G-CSF, GM-CSF and IL-6, constitutively or after induction with IL-1 (Kojima *et al,* 1992). This behaviour is consistent with two recent studies that suggested that defects at this level are rare in aplasia (Marsh *et al,* 1990, 1991).

The initial pilot study demonstrated that the stroma in both acute and non-acute marrow aplasia supports normal CFU-bl adequately (Novitzky & Jacobs, 1991, Chapter 4). To examine further the interactions between the bone marrow progenitors and the adherent layers, in the current study we employed selected CD

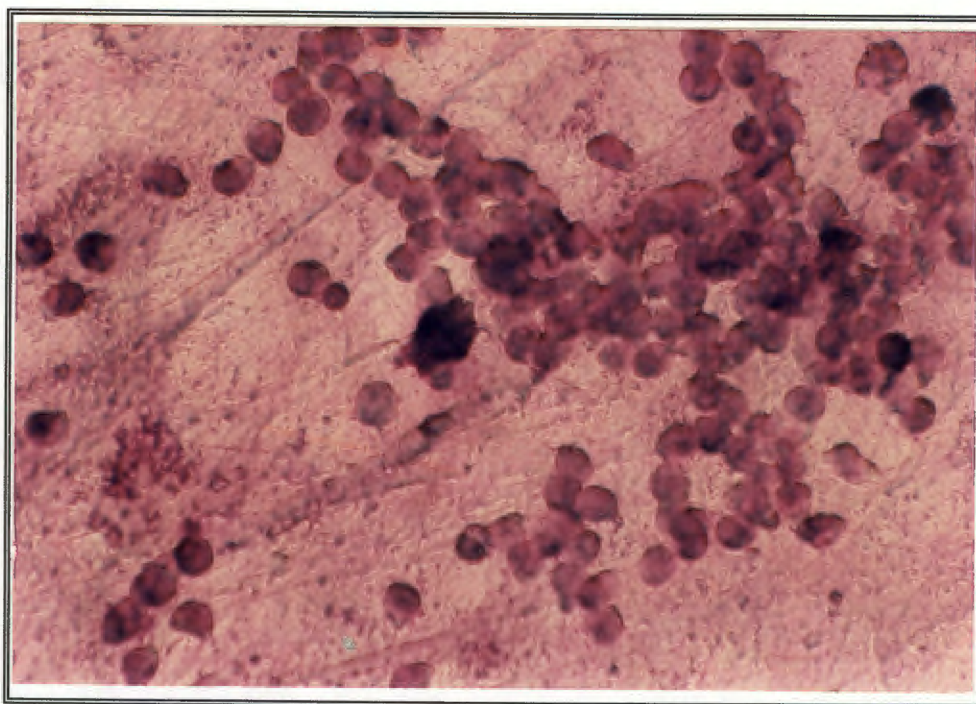

Fig. N° 5. 4.: Blastic colony assay. Photomicrograph of a day 5 stromal colony derived from normal CD 34+ progenitors (May Grunewald Giemsa; x 200 magnification).

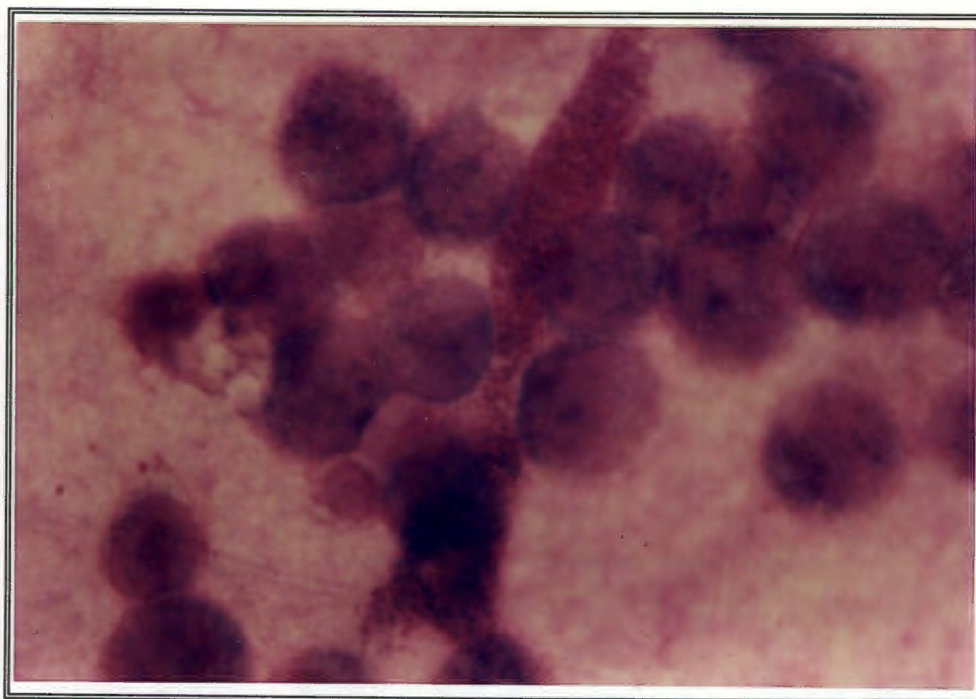

Fig. N° 5. 5: Blastic colony assay. Details of a day 5 colony. Note the conspicuous nucleoli with lack of cell differentiation (May Grunewald Giemsa; x 600 magnification).

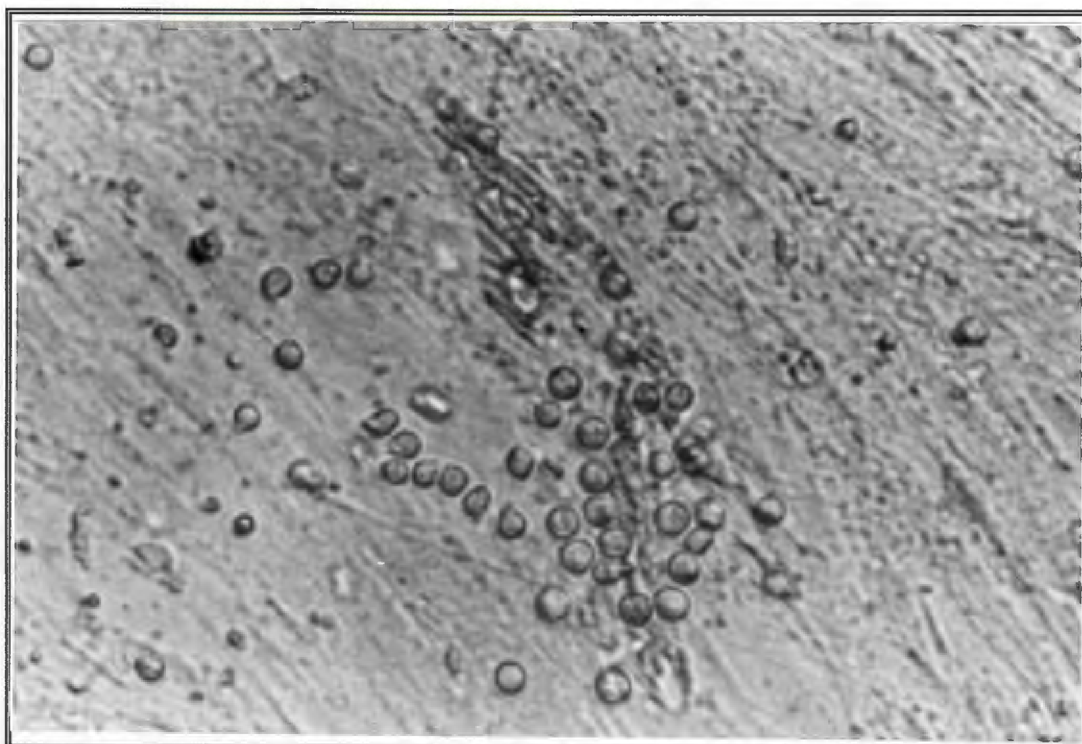

Fig. N° 5. 6: Contrast microscopy of a day 7 stromal colony derived from aplastic CD 34+ progenitors.

34+ progenitors and chose the short blast-colony assay (Gordon *et al,* 1987) for various reasons. Firstly, it provided clear end-points in the form of quantifiable CFU-bl. Secondly, the adhesive interactions of the CD 34+ population with the stroma could also be tested, analyzing in clonogenic assays the selected stroma non-adhesive population and determining their proportions. However, in this study the percentage of non-haematopoietic cells such as endothelial, fibroblastic cells or B lymphocytes in the CD 34+ selected populations were not determined (Simmons *et al,* 1989). These non-myeloid cells from normal suspensions did not appear to affect the behaviour of the aplastic stroma as demonstrated by similar growth of control CFU-bl on normal or aplastic SL. This analysis was unable to exclude completely the possibility that such populations present in the aplastic suspension could have had inhibitory effects on their own myeloid precursors. Nevertheless, this was thought improbable due to the low cell numbers remaining adherent to the stroma.

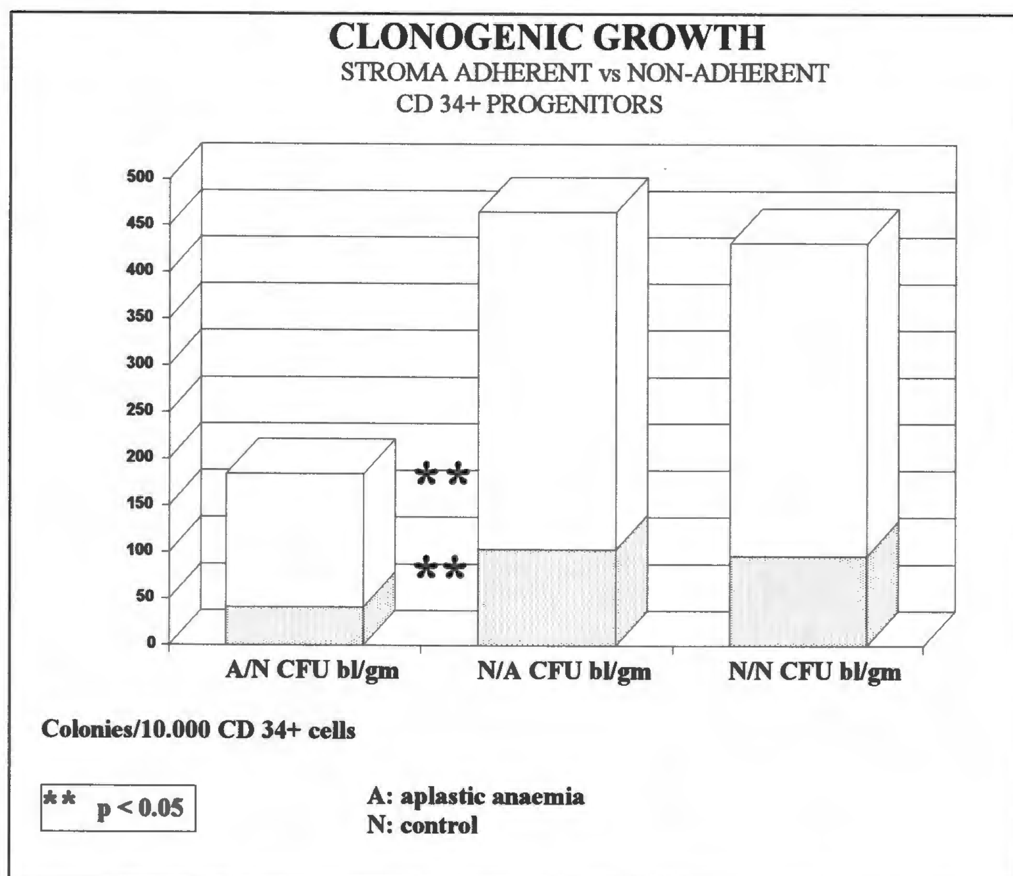

Fig. N° 5. 7: Clonogenic output of CD 34 + cells from controls and patients with aplasia on the CFU-bl and CFU-gm assays. Aplastic CD 34 + cells were incubated on control stroma (A/N) and compared with normal selected precursors cultured on aplastic stroma (N/A) or control stroma (N/N)

The original method was modified in that the irradiation of the allogeneic stroma was omitted, on the one hand to avoid damage to the possible presence of radiosensitive cells of the immune system that *in vivo* modulate the aplastic haematopoiesis, and on the other to avert the release of bursts of growth factors that follows this physical insult (Gualtieri *et al,* 1984, 1987, Tsuboi *et al,* 1991).

Upon confluence, *in situ* morphological inspection of the cultures failed to demonstrate significant differences between normal and diseased stroma. Cytochemistry of the trypsinised and fixed stromal layers also showed similarity in the cell populations staining for Sudan black (macrophages), ring red O (fat cells), and those reacting with acid or alkaline phosphatases (fibroblasts, reticular cells).

In this study it is confirmed that the bone marrow stroma in aplasia supports normal CFU-bl adequately with a clonogenic output that is no different from that

of control layers (Table N° 5.3, page 81). The data would suggest that, as tested with this assay, levels of negative regulators in aplastic stroma are not significantly higher than that of control layers and support the conclusion that defects at this level are rare (Marsh *et al,* 1990). It may still be possible that aplastic stroma is unable to sustain earlier progenitors that are not tested by this system. This is unlikely since no such abnormalities were detected in similar patients with the Long Term Bone Marrow Culture assay, where a wider range of haematopoietic progenitors are examined (Marsh *et al,* 1990).

Of greater relevance is that in this disease, selected CD34+ precursors formed significantly fewer colonies on normal stroma, and generally these showed a sub-optimal development. In addition, some required longer to develop, suggesting that clonogenic potential had not been realized even after five days as is characteristic of the normal group. Furthermore, when stroma non-adherent CD 34+ cells w--ere cultured in semi-solid systems supplemented with PHA-LCM, CFU-gm colony numbers were proportionately similar but significantly lower in the diseased group than the control (Fig N° 5. 7, page 86). These observations for the first time provide evidence that the fraction of dividing cells among the adherent and non-adherent populations is markedly reduced in this disease, and that decreased CFU-bl were not related to alterations in the adhesiveness to stroma.

These results are consistent with the hypothesis that the haematopoietic abnormality in these patients lies within the progenitor cell population. Whether the defective cells have been selected during the initial marrow damage or resulted because of ongoing immunological action remains to be clarified.

CHAPTER 6

THE BONE MARROW PROGENITOR CELLS IN APLASTIC ANAEMIA HAVE A SUB-OPTIMAL RESPONSE TO GROWTH FACTORS

6. 1 INTRODUCTION

Little is known about the various mechanisms that lead to bone marrow failure in aplastic anaemia. In this regard, while strong evidence accumulates suggesting primary stem cell abnormalities, the inhibitory roles of the immune system and the marrow microenvironment still remain controversial (Young 1992, Fujiwara *et al,* 1990).

In the previous chapter it has been shown that the bone marrow stroma in non-acute aplasia supports adequately normal CFU-bl. This is a sub-population of clonable progenitors with a very immature phenotype (Gordon *et al,* 1987 [a]), indicating that the level of negative regulating peptides in the stroma is not excessive. Similar conclusions were reached by other studies, employing in cross-culture experiments the LTBMC system, even when stroma had been seeded with CD 34 positive selected progenitors (Marsh *et al,* 1990, 1991). However, none of them provided further evidence regarding the possible mechanisms for the bone marrow dysfunction.

As reviewed in Chapter 1, steady state haematopoiesis is the result of interactions of positive and negative regulators secreted locally by the stroma (Sieff *et al,* 1987, Cannistra *et al,* 1988, Zipori *et al,* 1989, Gualtieri *et al,* 1984, 1987), acting through specific receptors on target cell membranes (Budel *et al,* 1990, D'Andrea *et al,* 1990). Additionally, autocrine modulation of the haematopoietic progeny has been described (Gasson *et al,* 1987; Rambaldi *et al,* 1987; Young & Griffin, 1986) constitutively or in response to indirect signals (Sieff, 1987), as occurs with the

release of M-CSF or G-CSF following the exposure of monocytes to IFN-g, IL 1 or endotoxin (Cannistra *et al,* 1988). Various purification steps and positive selection for haematopoietic progenitor cells markedly decrease these interacting signals from accessory cells, allowing an easier interpretation of the behavior of the populations under scrutiny.

Clinical experience indicates that some patients with aplastic anaemia respond to therapy with pharmacological doses of Epo, (Bessho *et al,* 1990) GM-CSF (Vadhan-Raj *et al,* 1988) or G-CSF (Kojima *et al,* 1991). This response suggests that despite intense pancytopenia, under appropriate stimulation, amplification of the terminal elements was still possible and resulted in improvements in blood counts of clinical significance. These were transient however, and only directed at the cell lineage under stimulation. Similar responses were observed *in vitro* after marrow mononuclear cells were exposed to erythropoietin, at concentrations that in normal cultures were well above plateau growth (Aoki *et al,* 1990). However, it remains unclear why clinical responses are not sustained.

Unresponsiveness to a specific cytokine can result from lack of target cells or be the consequence of abnormal receptor-ligand interactions. However, resistance to the actions of several of these glycoproteins can be explained by modifications to common structures or mechanisms present in the receptors or alternatively variations of the intracellular growth regulatory pathways (Reddy *et al,* 1992). In a further step and to determine the mechanisms involved in the haematopoietic defect affecting the early precursors in non-acute aplastic anaemia, positively selected progenitors were studied with specific cytokine combinations in dose response studies to growth factors belonging to the two major receptor groups: the haemopoietin superfamily (G protein) and the tyrosine kinase group.

6. 2 PATIENT POPULATION AND METHODOLOGY

The patient population involved the sixteen individuals whose clinical data were reviewed in chapter two, while their bone marrow functions were defined on the CFU-bl assay in chapter five. Briefly, fifteen subjects that presented with acute aplasia and had responded to anti-lymphocyte globulin, or, upon relapse, cyclosporine immunosuppression, and additionally one individual who had

developed hypoplasia during the first trimester of pregnancy and elicited a partial response after a therapeutic abortion formed the study population. The median follow-up for this group has been four years (1-8).

The controls involved seven haematologically normal individuals undergoing sternotomy for cardiac surgery under general anaesthesia. All gave consent to donate bone marrow for this study according to institutional guidelines of the University of Cape Town and Groote Schuur Hospital.

6. 2. 1 Selection of the Haematopoietic Progenitor Cells

Methodology for progenitor cell purification has been described in detail in chapters 3, 4 and 5. In short, a light density mononuclear population depleted of plastic adherent cells and lymphocytes (CAMPATH 1-M and complement) was incubated at 4° C with anti CD 34 murine antibody (MY 10, Beckman and Dickinson) for 30 minutes. Excess antibody was washed off and labelled cells were exposed to goat anti-mouse coated paramagnetic spheres (M-450, Dynal) at 4° C for 30 minutes. Selection was achieved after applying a magnet (MPC-1, Dynal) to the side of the tube. This procedure was repeated once on the remaining unselected fraction. Beads were detached from selected cells after incubating the cell suspension at room temperature with a murine polyclonal IgG for 50 minutes (Detachabead, Dynal). Selected cells were re-suspended in culture medium at a cell concentration of 1-5 x 10^4/mL. Viability, morphological assessment and immunofluorescence studies were performed on the final suspension.

6. 2. 2 Clonogenic Assays of the Selected Progenitor Cell Population of the Bone Marrow

6. 2. 2. (a) Haematopoietic Growth Factors

The following cytokines were employed: rh-IL 3 (specific activity of 10^7 U/mg), rh GM-CSF, G-CSF (specific activity 1 x 10^8 U/mg each), and c-kit ligand (SCF) (Amgen Thousand Oaks, California) at protein concentration of 0.5, 0.5 and 2 mg/mL respectively. They were diluted to 10^3 hg/mL and kept at 4° C until use.

Erythropoietin was donated by Bioclones (Sandton, Transvaal). Once reconstituted it was kept at 4° C at a concentration of 1000 U/mL.

6. 2. 2. (b) Clonogenic Assays. Dose Response

Normal or aplastic positively selected CD 34 progenitors were cultured *in vitro* for fourteen days at 37° C in multi-cluster dishes (Nunclon Delta-Code 176740, UK) at a cell density of 1-5 x 10^3/mL in Iscove's modified Dulbecco's medium (IMDM, with L-Glutamine, Gibco). Cultures were supplemented with 30% FCS, 5 x 10^{-5} 2-mercaptoethanol, in 0.3% agar (final concentration) and incremental concentrations of Epo in the presence of IL3 10 hg/mL; of GM-CSF in combination with Epo 2 U/mL; increments of IL3 with Epo 2 U/mL and of c-kit ligand in association with Epo 2 U/mL and IL-3 10 hg/mL.

Dose responses were constructed, based on the previously described study (chapter 3), where the starting growth factor concentration represented 25-50% of maximal clonal efficiency. The highest dose usually expressed concentrations well above plateau growth values for the cytokines tested. Additionally, G-CSF (10 hg/mL) with or without c-kit ligand (20 hg/mL) were added to IL-3 10 hg/mL, and Epo 2U/mL, to determine the effect of GF addition.

For permanent recording, cultures were fixed in 10% glutheraldehyde and stained in May-Grunewald-Giemsa. GM-CFUc were defined as myeloid aggregates (granulocytic, monocyte-macrophage or mixed) containing more than 40 cells. CFU-e were described as erythroid clusters of 4-100 cells, while BFUe were expressed as colonies containing more than 100 cells or when aggregates contained three or more sub-cultures.

6. 2. 3 Statistical Analysis

Due to the marked dispersion of the data, raw values of colony scores were subjected to logarithmic transformation with the formula $Y^1 = \ln (Y+1)$. Means, standard deviations and confidence intervals were calculated using the normalised data. A two-way analysis of variance was employed to determine the significance

of differences between groups of cultures or a single culture versus a control group. Mulivariate analysis was performed to define the correlation between sets of observations, discriminant functions and clustering of data (Morrison, 1976).

6. 3 RESULTS OF DOSE RESPONSE AND GROWTH FACTOR COMBINATION STUDIES

6. 3. 1 Selection of the Bone Marrow Progenitor Cells

Results of the selection for the CD 34+ population have been presented in Chapter 5. Briefly, aplastic mononuclear cells had significantly lower cell numbers that emitted fluorescence after being labelled with anti-CD 34 antibody (0.65%, SD 0.35 vs 1.60% SD, 1.38; $p<0.05$). The median corrected number of selected progenitors was also lower at 0.116×10^6 (normal donors: 0.539×10^6; $p= 0.18$) with cell viability in excess of 90% (Table 5. 2, page 82).

6. 3. 1. (a) Clonogenic Assays. Dose Response (Table 6. 1)

6. 3. 1. (a). i Erythropoietin (Epo) [Fig. N° 6. 1. (a)]

Incremental concentrations of Epo 0.2, 2, 10 and 100 U in the presence of IL3 10 hg/mL showed that in both the patients and controls, the number of BFU-e increased with hormone concentrations. However, in the aplastic group these were significantly lower at 0.2, 2 and 10 U/mL ($p < 0.001$, 0.001 and 0.05). In contrast with the normal group, erythroid growth in the aplastic progenitors continued improving significantly in a linear fashion from the lowest concentration to 100 U/mL ($p < 0.01$), without achieving plateau growth and approaching control values. The number of CFU-e were lower at all concentrations, but did not achieve significance ($p > 0.05$). CFU-gm were unaffected by changes in Epo concentration.

At plateau concentrations, normal and aplastic progenitors showed adequate haemoglobinization, with formation of massive macroscopic multi-lobed colonies, and stimulation of immature bursts (more than 7 subclones of mainly blastic cells

with poor haemoglobinization). Mixed colonies were also present in both aplastic and control cultures at 10 and 100 U.

6. 3. 1. (a). ii Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) [Fig. N° 6. 1. (a)]

Control and aplastic progenitors were cultured with Epo 2 U/mL and GM-CSF at concentrations of 0.1, 1, 10 and 50 hg/mL. Among normal cultures, a linear response was found for CFU-gm commencing at 0.1 hg/mL, while stimulation for BFU-e and CFU-e appeared already at a plateau.

Aplastic progenitors again displayed an elevation in the numbers of all colony types, commencing with significantly lower scores than those of normal cultures for BFU-e at 0.1 and 1 hg/mL ($p < 0.001$ and 0.001) and CFU-gm at 0.1 and 1 hg/mL ($p < 0.025$ and 0.01), but colony numbers continued increasing at 10 and 50 hg/mL up to control values. In the patients, from the starting concentration, the increment in the number of BFU-e and CFU-gm were significant at 10 ($p < 0.05$ and 0.01) and 50 hg/mL ($p < 0.01$) respectively. At top cytokine concentration, CFU-mix as well as macroscopic BFU-e and CFU-gm colonies were also present in the patient population.

6. 3. 1. (a). iii Interleukin 3 (IL3) [Fig N° 6. 1. (b)]

In the presence of Epo 2 U/mL, IL 3 gave lineal increments in the normal precursors, with maximal clonal expansion at 10 hg/mL for all colony types. At 50 hg/mL a plateau effect on CFU-gm and some inhibitory action on the erythroid colonies was noted.

In the study group, proportional increments in the three colony types were again observed and it was significant for BFU-e at the highest concentration ($p < 0.05$). Compared to control cultures, colony numbers from aplastic clones were significantly lower for BFU-e and CFU-e at 1 hg/mL ($p < 0.001$ and 0.025) and BFU-e at 10 hg/mL ($p < 0.001$). However, at 50 hg/mL, BFU-e and CFU-gm showed a combined increment in colony numbers of 109%, achieving similar

TABLE 6. 1

CLONOGENIC ASSAYS

CYTOKINE COMBINATION	TYPE OF COLONY											
	BFU-e				CFU-e				CFU-gm			
	APLASTIC		CONTROL		APLASTIC		CONTROL		APLASTIC		CONTROL	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
IL 3 10 hg + Erythropoietin Epo:	n=11		n=7		n=11		n=7		n=11		n=7	
0.2 U/mL	**0.61**	0.95	2.90	0.69	2.96	0.78	3.93	0.52	2.67	1.26	3.02	1.66
	n=15		n=7		n=15		n=7		n=15		n=7	
2 U/mL	**2.10**	1.32	3.97	0.54	3.50	1.08	4.18	0.55	2.94	0.93	3.74	0.62
10 U/mL	**3.03**	1.18	4.14	0.49	2.99	1.25	3.83	0.87	2.95	1.07	3.42	1.14
100 U/mL	3.57	0.71	3.96	0.54	3.34	0.97	3.97	0.91	3.25	0.68	3.72	0.86
Epo 2 U/mL + GM-CSF GM-CSF:	n=7		n=7		n=7		n=7		n=7		n=7	
0.1 hg/mL	**1.62**	0.95	3.66	0.71	**3.23**	1.37	3.53	1.19	**1.12**	1.26	2.28	1.24
	n=15		n=7		n=15		n=7		n=15		n=7	
1hg/mL	**1.67**	1.27	3.37	0.40	**2.87**	1.27	3.65	1.19	**1.51**	1.72	3.10	0.77
10 hg/mL	2.59	0.99	3.04	1.09	2.94	1.23	3.70	1.16	3.02	1.13	2.91	1.52
50 hg/mL	2.98	0.70	3.57	0.82	3.23	1.12	3.68	1.03	3.88	0.71	3.63	0.44
Epo 2 U/mL + Interleukin 3 IL-3:	n=15		n=7		n=15		n=7		n=15		n=7	
1 hg/mL	**1.50**	1.06	3.29	0.76	2.76	1.05	3.85	1.19	**1.62**	1.26	2.42	1.13
10 hg/mL	**2.10**	1.32	3.97	0.54	3.50	1.08	4.18	0.55	2.94	0.93	3.74	0.62
50 hg/mL	2.94	0.98	3.02	0.96	3.19	1.03	3.73	1.13	3.43	0.68	3.37	1.61
Epo 2 U/mL+IL-3 10 hg/mL + Stem Cell Factor SCF:	n=7		n=5		n=7		n=5		n=7		n=5	
1 hg/mL	**1.76**	1.69	4.13	0.42	2.61	0.92	3.71	1.55	3.11	1.08	4.21	0.53
20 hg/mL	3.65	1.01	4.57	0.54	2.84	1.26	3.26	1.37	3.81	0.61	4.75	0.47
200 hg/mL	3.99	0.50	4.26	0.71	2.68	1.43	3.36	1.17	4.29	1.24	4.72	0.89

Dose response to growth factors combinations. Bold figures in squares denote significance: $p < 0.05$. Results are expressed as mean (Mean) and standard deviations (SD) of $Y^1 = (\log Y+1)$.

values to those of control progenitors (Fig N° 6. 2, page 101). At the maximal cytokines levels, mixed clones and colonies of immature blasts were also present in the cultures of some of the patients.

6. 3. 1. (a). iv Stem Cell Factor (c-kit ligand) [Fig N° 6. 1. (b)]

Normal and aplastic progenitors were cultured with stem cell factor at concentrations of 1, 20 and 200 hg/mL in combination with Epo 2 U and IL3 10 hg/mL. In the normal assays, results showed that the addition of c-kit ligand to the basic IL3+Epo combination led to a marked enhancement in BFU-e and CFU-gm by 82.94% and 154.87% respectively (Fig N° 6. 2, page 101). This increment achieved significance for the granulocytic colonies at 200 hg/mL ($p < 0.05$).

In subjects with aplasia, increments in this cytokine up to 200 hg/mL also resulted in a significant enhancement in the BFU-e and CFU-gm to 263.3 and 300 % respectively ($p < 0.01$ and 0.05) over the basal IL-3 10 hg/mL + Epo 2 U/mL combination. Therefore, values of cultures were significantly lower only for BFU-e at 1 hg/mL ($p < 0.05$). Although at this concentration the stimulating effects were provided mainly by the basic IL-3 + Epo combination (Lowrey *et al,* 1992). At effective concentrations (^{3}20 hg/mL), results were not significantly different from those of control cultures. Similar to their normal counterparts, at the highest cytokine concentration, marked increments in colony sizes with macroscopic mixed, myeloid and erythroid clones were also observed.

In summary, results of dose responses in the mixed colony assay showed a significant reduction in the sensitivity of CD 34+ cells of patients with aplastic anaemia to Epo, Il-3 and GM-CSF. Of interest, at pharmacological concentrations the clonogenic potential improved and even matched that of controls.

In contrast, exposure of aplastic progenitors to concentrations of c-kit ligand which were effective in normals (> 20 hg/mL), led to significant clonogenic enhancement, which was superior to top concentrations of any other growth factor

combination, resulting in increments which were proportionally higher than those of normal cultures (Fig N° 6. 2, page 101). These growth improvements in the aplastic cultures led to colony numbers which were not significantly different from those of normal precursors.

6. 3. 1. (b) Growth Factors in Combinations (Table 6. 2)

The addition of fixed concentrations of G-CSF 10 hg/mL to IL 3 and Epo, or this combination with SCF at 20 hg/mL, showed conflicting results but confirmed that under appropriate stimulation, aplastic progenitors are able to respond adequately (Table 6. 2, page 97 and Fig. 6. 2, page 101).

G-CSF added to the patients' cultures had a marginal effect on erythroid growth. This growth remained significantly lower than that of control studies ($p < 0.001$), but led to an enhancement of the myeloid colonies over the basal IL-3 and Epo combination by 101.43% ($p < 0.02$), and to results not different from those of control. The four cytokine combination also gave significant improvement in the clonogenic potential up to 147 and 231 % for BFU-e and CFU-gm respectively, which was proportionately higher than the growth registered in control cultures. With this combination in the aplastic studies, massive erythroid bursts of both mature and immature BFU-e and macroscopic granulocytic colonies were obvious.

6. 3. 2 Multivariant Analysis

Studies performed on the logarithmic transformation of the aplastic mixed colony assays determined that the combination of Epo 2 U/mL with IL-3 10 hg/mL could discriminate 100% of the patients from the control population for both BFU-e and CFU-gm colonies (discriminant analysis). Multivariant analysis also segregated the results for all three colony types in cultures supplemented with c-kit ligand at biologically effective concentrations from the remaining dose response studies. Interpretation of these data would suggest that cultures containing SCF were affected by independent interactions from those induced by the other growth factor combinations.

6. 4 DISCUSSION OF THE RESULTS

The data presented in Chapter five confirmed that the bone marrow stroma in aplasia supported haematopoiesis adequately, suggesting that in this disease the primary defect lay within the haematopoietic stem cell.

TABLE 6. 2

GROWTH FACTORS IN COMBINATIONS

CYTOKINE COMBINATION	TYPE OF COLONY											
	BFU-e				CFU-e				CFU-gm			
	APLASTIC		CONTROL		APLASTIC		CONTROL		APLASTIC		CONTROL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	n=11		n=7		n=11		n=7		n=11		n=7	
EPO 0.2 U/mL IL-3 10 hg/mL	**0.61**	0.95	2.90	0.69	**2.96**	0.78	3.93	0.52	2.67	1.26	3.02	1.66
	n=15		n=7		n=15		n=7		n=15		n=7	
EPO 2 U/mL IL-3 10 hg/mL	**2.10**	1.32	3.97	0.54	3.50	1.08	4.18	0.55	2.94	0.93	3.74	0.62
	n=15		n=7		n=15		n=7		n=15		n=7	
EPO 2 U/mL IL-3 10 hg/mL G-CSF 10 hg/mL	**2.47**	1.35	4.28	0.68	3.03	1.25	3.71	1.20	3.73	0.86	4.24	0.72
	n=7		n=5		n=7		n=5		n=7		n=5	
EPO 2 U/mL IL-3 10 hg/mL SCF 20 hg/mL	3.65	1.01	4.57	0.54	2.84	1.26	3.26	1.37	3.81	0.61	4.75	0.47
	n=7		n=5		n=7		n=5		n=7		n=5	
EPO 2 U/mL IL-3 10 hg/mL SCF 20 hg/mL G-CSF 10 hg/mL	**3.00**	1.41	4.66	0.66	2.39	1.35	2.80	1.50	4.24	0.94	5.00	0.48

Results of *in vitro* culture of aplastic and control CD 34 + progenitors to the basic IL-3 10 hg/mL with Epo 0.2 U/mL or Epo 2 U/mL and the addition of G-CSF, SCF or both. Values in bold denote significance $p < 0.05$. Results are expressed as mean (Mean) and standard deviations (SD) of $Y^1 = (\log Y+1)$.

To further investigate the possible mechanisms of this lesion, and to determine if the hypoproliferative state could be reversed by more intense GF stimulation, selected CD 34+ progenitors greatly depleted of accessory cells were studied in dose responses to recombinant growth factors in various combinations. These

studies were designed to test the proliferative mechanisms activated by the two super-families of haematopoietic receptors: the G protein superfamily and those belonging to the tyrosine kinase type. Culture results showed that aplastic progenitors have a significantly lower sensitivity to Epo, IL-3, GM-CSF than do controls. Furthermore, it has been possible to document for the first time that in aplasia, the clonal potential of the haematopoietic progenitors is preserved since, at the highest cytokine concentrations, colony numbers matched control values.

This point is also stressed by those studies showing that addition of G-CSF again potentiated the actions of IL-3 at 10 hg/mL on myeloid progenitors and led to an efficient stimulation of the aplastic granulocytic colonies to levels not significantly different from those of normal precursors while BFU-e results remained unaffected.

These findings, however, appear at variance with other publications, where mononuclear cells from patients with acute aplasia, or those responding to immunosuppressive therapy were cultured in the presence of incremental concentrations of Epo (Aoki *et al,* 1988) or GM-CSF (Bacigalupo *et al,* 1991). Here results demonstrated generally poor *in vitro* proliferative responses to single cytokines. Furthermore, in a limited number of transfusion independent patients, at pharmacological concentrations of growth factors, improvements in the clonogenic growth were observed, although median values failed to match control results.

Nevertheless, as in these reports, no positive selection for the clonogenic precursors was attempted, progenitors were cultured in unknown numbers and contaminated with accessory cells. In this disease, adherent and non-adhesive accessory cell, present in the bone marrow inoculum, have been described as inhibiting normal bone marrow growth (Bacigalupo 1984, Merchav, 1988). These technical distinctions may explain the differences in the results. Alternatively, INF-g and TNF are produced excessively by aplastic accessory cells (Hintenberger, 1988). At high GM-CSF concentrations, this contaminating population may further induce the release of TNF (Cannistra & Griffin 1988), or other regulators, limiting the proliferative effects on the haematopoietic progenitors.

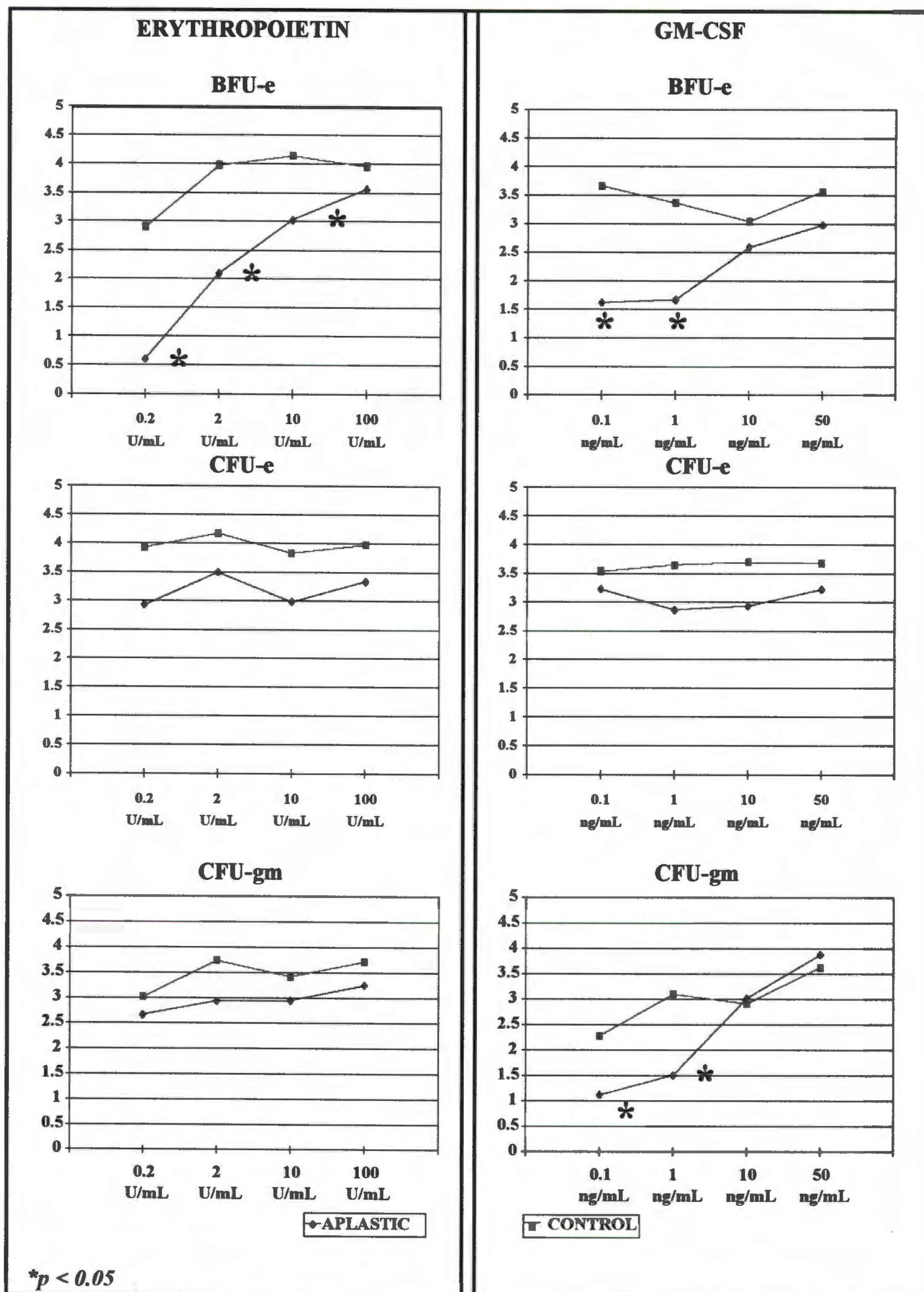

Fig. N° 6. 1 (a): Dose response of aplastic and control CD 34+ cells exposed to Epo 0.2, 2, and 10 U/mL (with IL-3 10 hg/mL) and Gm-CSF 0.1, 1, 10 and 50 hg/mL in the presence of Epo 2 U/mL. With GM-SCF differences at 0.1 and 10 hg/mL were significant for BFU-e and CFU-gm colonies, while for CFU-e only at the lowest concentration.* denotes significance of $p < 0.05$.

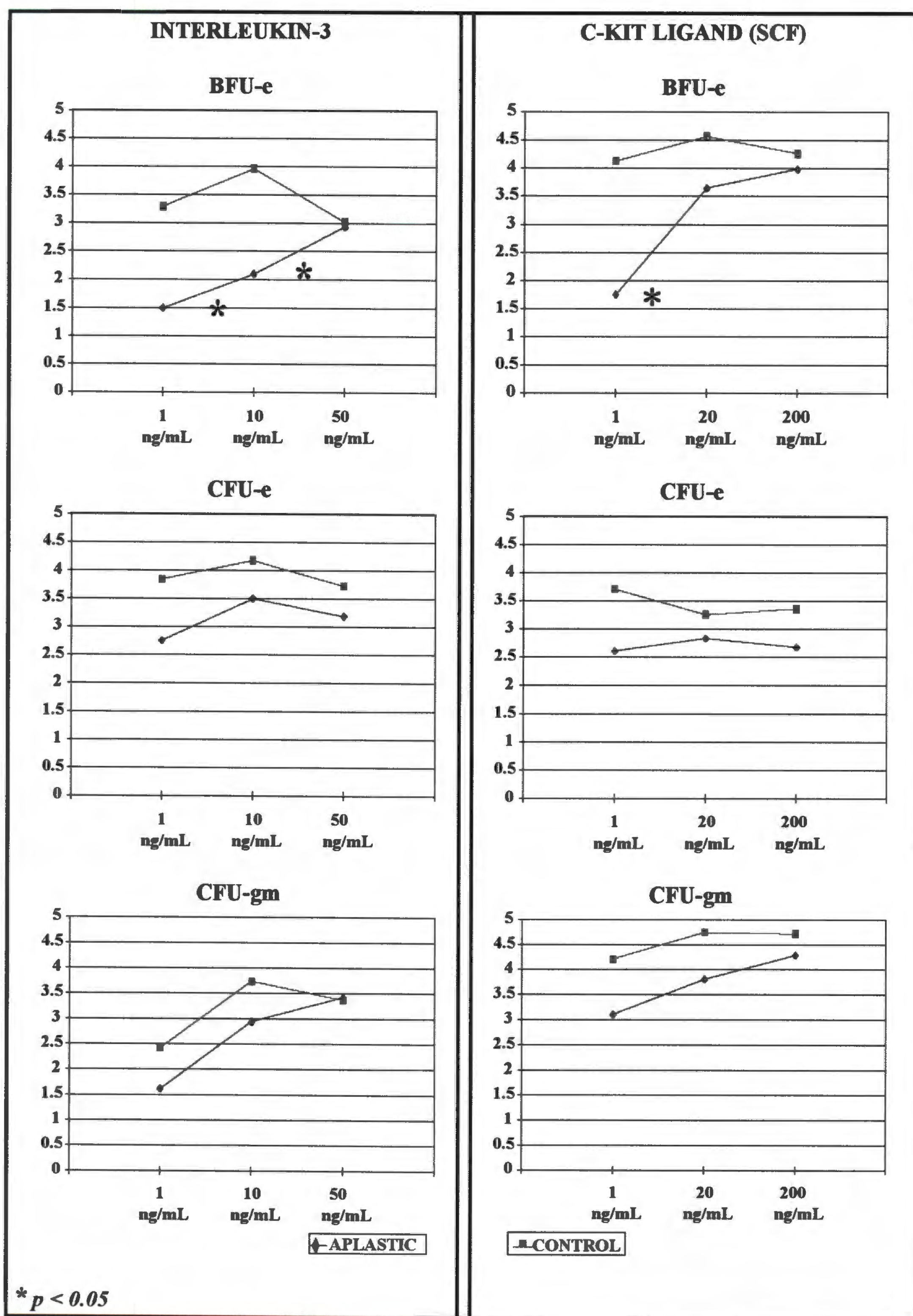

Fig. N° 6. 1 (b): Comparative results of clonogenic growth from patients with aplastic anaemia to normal individuals. Dose response to Il-3 1, 10 and 50 hg/mL (with Epo 2 U/mL) and SCF at 1, 20 and 200 hg/mL supplemented with IL-3 10 hg/mL and Epo 2 U/mL.* denotes significance of $p < 0.05$.

These experiments have also documented adequate responsiveness of aplastic precursors to c-kit ligand when cultured in the presence of Epo and IL-3. Despite a sub-optimal response to the more restricted stimulators (Epo 2 U/mL and IL-3 10 hg/mL), at effective concentrations stem cell factor significantly corrected this inferior colony development.

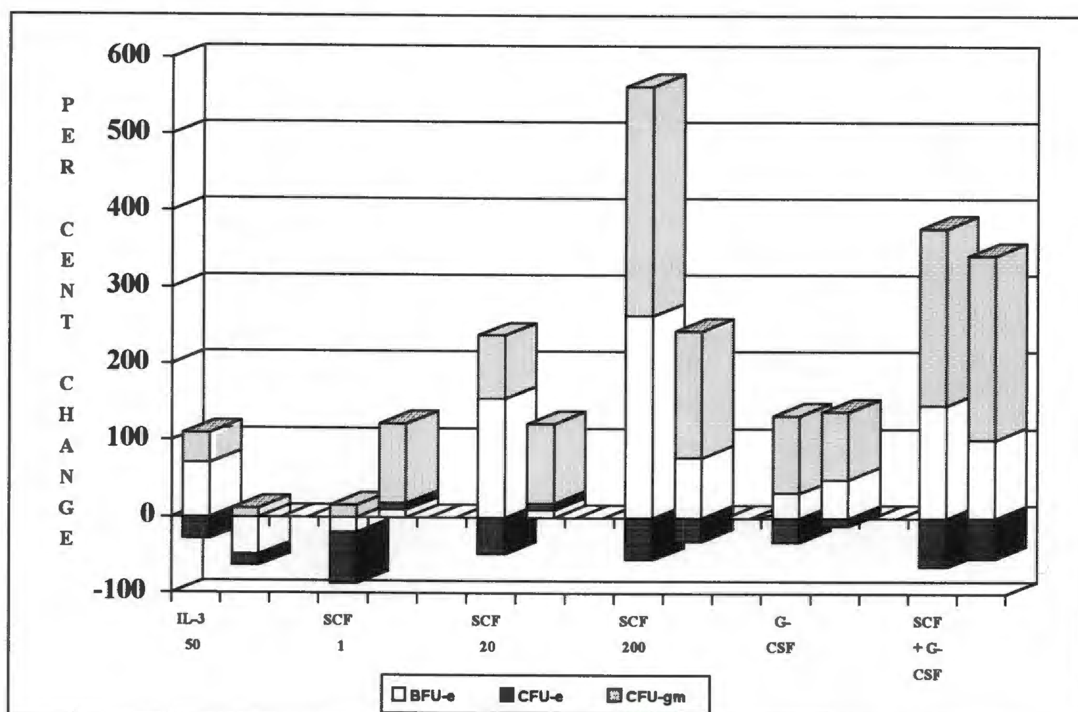

Fig. N° 6. 2: Per cent change in clonogenic growth over the basal IL-3 10 hg/mL, Epo 2 U/mL expressed as 1, by growth factor combinations. Values represent % difference for each colony type (see also Table ·6. 2). Each pair of bars represents respectively values for "Aplastic" and "Control" experiments.

C-kit ligand has little clonogenic activity on its own (Lowrey *et al,* 1992). However, as seen in this study, at appropriate concentrations it is synergistic with the actions of other early acting cytokines. In aplastic progenitors the proportional increment in the colony numbers was superior to that of control studies. It exceeded the basic IL-3 10 hg/mL + Epo 2 U/mL by 500% for erythroid and myeloid colonies together, leading to the highest scores for BFU-e and CFU-gm (Fig N° 6. 2). It resulted in clonogenic yields not significantly different from control cultures, suggesting again that the clonal potential in this disease remains preserved.

Similarly to normal progenitors, remarkable enhancements in colony size and haemoglobinization of BFU-e was observed, with macroscopic CFU-gm and mixed colonies. Multivariate analysis indicated a segregation of cultures supplemented with effective concentrations of c-kit ligand, from those grown with other GF combinations, suggesting independent interactions affecting the cultures containing this growth factor. These data appear to be in good agreement with another study in a similar patient population, employing unfractionated marrow mononuclear cells and confirmed by ^{3}H Thymidine uptake studies (Wodnar-Filipowitz *et al,* 1992).

Since the results of the CFU-bl assay show that cells in the aplastic stroma do not exert excessive inhibition on control CD 34+ progenitors (Chapter 5), while appropriate response to certain growth factors is observed only at high GF concentration or in the presence of stem cell factor, alterations in the signalling pathways for cell division are consequently favoured.

Reduction in the response to GF may result from abnormalities in the receptor densities or affinities for the ligands leading to sub-optimal clonogenic growth. Additionally, soluble forms or binding proteins of these receptors exist, secreted naturally into the microenvironment or alternatively originating as proteolytic cleavage products of transmembrane molecules. Such binding proteins can potentially function as inhibitors to the action of their ligands in a feedback regulation (Olsson *et al,* 1992).

However in this study, the described derangements in the responses to GF ranged widely over three cytokines, suggesting alterations in shared structures or pathway signalling for cell division. Receptors for IL-3, IL-4, IL-7 Gm-CSF G-CSF and Epo (but not M-CSF or c-kit which have tyrosine kinase activity) belong to a common receptor family with some structural similarities (Demetri *et al,* 1991, Gasson *et al,* 1991, Pouyssegur *et al,* 1992). They form part of the growing transmembrane GTP-binding group of proteins which control a series of second messenger molecules including adenylate cyclase, cGMP phosphodiesterase, phospholipases and ion channels (Gillman *et al,* 1989). Since cooperation and

integration of signals between pathways are important in the mitogenic response (Pouyssegur, 1992), phosphorylation of c-kit receptor by its ligand may lead to correction of the signals relayed by the abnormal G family of proteins and result in rectification of the mitogenic response. This may have important therapeutic implications.

It is concluded that in patients with aplastic anaemia treated with ALG+HDMP, intrinsic abnormalities in the CD 34+ population result in subnormal clonogenic growth. Alterations in the cytokine receptors' structures or affinities or damage to common intracellular signalling mechanisms for cell division are proposed. Whether during the initial presentation, a more profound abnormality of the same pathways leads to the severe pancytopenia, or this derangement is a characteristic of the escape population that survives the original attack, remains to be determined.

CHAPTER 7

SUMMARY OF THE OBSERVATIONS AND FINAL CONCLUSIONS

7. 1 INTRODUCTION

Aplastic anaemia has attracted considerable attention since its initial description more than 100 years ago by Ehrlich (1888). The points of greatest interest were the heterogeneous clinical presentation, multiplicity of etiologic associations, its relation to acute leukaemia (Tichelli *et al,* 1988 [a and b], Orlandi *et al,* 1988) and in those with more intense manifestations, irreversible outcome from catastrophic bleeding or overwhelming infections (Camitta & Thomas, 1978).

Based on models that showed that infusions of allogeneic marrow could rescue animals from the irreversible pancytopenia that followed supralethal doses of irradiation or cytotoxics (Till & McCalluch, 1964), clinical trials in patients with aplasia have confirmed the effectiveness of bone marrow transplantation in those with an HLA compatible sibling, particularly in patients with the very severe variety (Gordon-Smith, 1987).

However, experience demonstrated that the infusion of sibling bone marrow without preconditioning with cytotoxics resulted in graft losses in a third of syngeneic transplants (Appelbaum *et al,* 1980) even in those that had received intense preparation. Allograft failure was occasionally followed by autologous recovery of the haematopoiesis. This led to the conclusion that at least in some patients this disorder could be associated with abnormalities in the bone marrow microenvironment or of the immune system.

Further points favouring the immunological basis of this disease, were provided by the frequent finding of bone marrow inflammatory infiltrates in biopsies

during the initial presentation (Haak, 1978, de Planque *et al,* 1989). Moreover, *in vitro* studies showed that when accessory cells from the marrow of patients with aplasia were cocultured with aplastic or normal myeloid progenitors they appeared to inhibit clonogenic growth (Torok-Storb 1980, Merchav *et al,* 1988). This suppression was reversed by the removal of adherent cells and T lymphocytes from the cultures (Singer, 1989). Furthermore, overproduction of certain products derived from the immune system such as interferon gamma (Zoumbos *et al,* 1985), and neopterin (Hinterberger *et al,* 1988) appeared to reverse after ALG therapy.

In the clinical context, encouraging results were reported with anti-lymphocyte globulin as preconditioning for haploidentical bone marrow support (Mathe, 1970, Mathe & Schwarzberg, 1976, Camitta & Thomas, 1984) or without graft infusions (Champlin *et al,* 1983). However, it then became more obvious that in aplasia an immunological activation triggered by idiosyncratic reaction to drugs, chemicals, viral infections and even pregnancy (Van Besien, 1991, Aitchison 1989) resulted in intense marrow damage. Confirmation of this theory was initially provided by anecdotal reports, followed by retrospective studies (Speck *et al,* 1986, Novitzky *et al,* 1991) and validated by controlled clinical trials (Champlin *et al,* 1983).

It is noticeable that, following this therapy haemopoietic recovery is slow and often incomplete, while in 20% of the patients this disease may recur. Furthermore, in some responding to this treatment, morphological and functional abnormalities of the mature blood elements have been described (de Planque *et al,* 1989, [a and b], Chapter 2 this thesis), while extended follow-up has suggested that in 15-58 % of patients, clonal diseases such as PNH, MDS or acute leukaemia may develop (Tichelli *et al,* 1988 [a and b]).

Some recent studies that investigated the pathogenesis of this disorder concluded that in both acute and non-acute aplasia the bone marrow stroma was abnormal, with altered monocyte maturation and deranged fibroblasts and monocytic functions (Hotta *et al,* 1986, Juneja *et al,* 1984, Anderseen *et al,* 1989). This notion was further strengthened by certain similarities between this disease and

the murine model carrying the Steel mutation (S^l/S^{ld}), which is characterised by a macrocytic anaemia associated with defective stromal cell environment. Here the disorder could not be cured by infusing normal marrow stem cells, but improved by implantation of normal splenic stromal tissue (Becker & McCulloch, 1965, Wolf 1978).

This evidence suggested that at least in some systems the pancytopenia could be due to abnormal cell interactions within the microenvironment. Alternatively, it could be argued that the unrestrained action of cells of the immune system residing in the bone marrow could lead to overactive secretion of negative regulators and to marrow failure.

Due to low numbers of clonable progenitors, there are limited data on the growth potential during the acute stage of the disease. Nevertheless, in non-acute aplasia, even despite marked improvement in the blood counts, myeloid precursors appear to express sub-optimal clonogenic growth when cultured in the presence of recombinant growth factors (Marsh *et al,* 1991, Novitzky *et al,* 1991). Moreover, *in vivo* these patients may respond to pharmacological doses of colony stimulating factors, with significant expansion of the lineage under stimulation. However, these responses are unsustained after discontinuation of the cytokines (Vadham-Raj *et al,* 1989, Bessho *et al,* 1990, *et al,* Kojima *et al,* 1991).

7. 2 MAIN OBSERVATIONS OF THE THESIS

In the present study, a cohort of patients with aplastic anaemia treated with a uniform protocol of immunosuppression with anti-lymphocyte globulin and high-dose methyl-prednisolone has been followed up for a median of four years. In this homogeneously treated group it was initially noted that, despite haematological recovery, some patients continued to display various morphological and functional abnormalities. Data presented in chapter 2 confirmed widespread dysplasia in one or more myeloid cell lines. Although the number of blasts always remained normal, the biopsy material showed ALIP formation and, in some patients, significant reticulin fibrosis.

Functional studies of the blood elements showed that the bleeding time was prolonged in ten of fifteen patients, and significant abnormalities were detected in the platelet aggregation, generation of oxygen radicals by neutrophils and phagocytosis. These results are consistent with similar observations reported in patients with congenital anaemias where, despite the normal number of receptors and affinity for the ligand (Kyas *et al,* 1992; Rauprich *et al,* 1991), intracellular abnormalities in the response to G-CSF were detected in mature blood leukocytes (Kurtzberg *et al,* 1992, Roesler *et al,* 1992).

Furthermore, cytogenetic studies in 30% of patients revealed chromatid or chromosomal aberrations and breakage in a significant proportion of the bone marrow cells. All these features have characteristically been described in myelodysplastic syndromes. Nevertheless, as blood counts continued to improve significantly over the median follow-up period of four years, the diagnosis of MDS could not be sustained.

Following the initial pilot study (Chapter 4), that showed that clonable CD 34+ cells in the marrow of patients with acute aplasia were markedly reduced, it was felt that with the methodology at hand extensive investigations of this cell population would be unsuccessful. Nevertheless, these limited studies suggested that in untreated patients, their confluent bone marrow stroma supported the proliferation of normal CFU-bl adequately. On the basis of this initial assessment, further work was then directed at studying the clonogenic defect in the bone marrow that restores haematopoiesis following ALG therapy, employing enriched progenitors devoid of accessory cells.

To analyse the nature of these abnormalities and to define the relevant merits of each of the previously proposed mechanisms, a system was developed whereby aplastic and normal stroma were cultured *in vitro,* while clonogenic cells that had been enriched for the CD 34 membrane surface antigen could be tested in cross culture studies on these preformed bone marrow adherent layers (Chapters 3, 4 and 5).

7. 3 SIGNIFICANT CONCLUSIONS OF THIS THESIS

Comparative studies of the marrow stroma from sixteen subjects in partial or complete response following standard immunosuppression with control adherent layers, failed to demonstrate obvious morphological differences. On the cytochemical stains, the cell proportions between the two groups were similar, and when seeded with normal CD 34 positive cells they supported the growth of blastic colonies. This resulted in plating efficiencies that matched those of control stroma.

The obvious difference resided in the aplastic CD 34+ population. When incubated on normal stroma, these progenitors led to significantly lower CFU-bl compared to values obtained from control precursors incubated on normal or aplastic layers. These data would suggest that levels of negative regulators in aplastic stroma are not significantly higher than those of control layers since normal CFU-bl formation was well supported.

Moreover, this study was able to document for the first time that in this disease the fraction of dividing cells within the adherent and non-adherent CD 34+ populations was proportionately reduced and the clonogenic deficit appeared unrelated to abnormalities in the adhesiveness of the precursors on to stroma.

Clonogenic cells in the bone marrow possess receptors to growth factors belonging to two super families: the G protein type that includes Epo, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF and G-CSF, typically composed of multiple subunits necessary for high-affinity binding and cell signalling (Olsson 1992) and those belonging to the tyrosine kinase group, represented by platelet derived growth factor (PDGF), Macrophage-CSF (CSF-1) and c-kit (SCF).

In trying to clarify further the mechanisms of the described sub-optimal clonogenic responses in aplasia, it was decided to focus on the progenitor cells and determine their proliferative capacity by constructing dose response studies

to growth factors in specific combinations. To test the integrity of the mitotic signals, Epo, IL-3 and GM-CSF, ligands belonging to the G type of receptor were selected. For the second receptor group, c-kit ligand was chosen. This was done because M-CSF can induce both positive and negative regulatory signals and results may therefore, be difficult to interpret (Wang *et al*, 1992). It was also preferred for two additional reasons. Firstly because this growth factor interacts with an earlier precursor population affecting the development of all cell lines, and, lastly, because murine models of genetic anaemia (*S/S^l^* and *W/W^v^*) are associated with abnormalities in the expression of the SCF and its receptor.

Although SCF has little activity on its own (Lowrey, 1992), it has additive effects in combination with other GF. Since data of the basic IL-3 + Epo admixture were available, any additional effect would become obvious. Results of these dose response studies in patients with non-acute aplasia confirmed that at low cytokine concentrations sub-optimal clonogenic responses were observed. However, these were followed by significant improvements in the colony numbers at high GF levels.

In this study, it is documented for the first time that when purified populations of CD 34+ cells are used, at the highest concentrations of Epo, IL-3, and GM-CSF the clonogenic output is not significantly different from normal. Furthermore, in combinations containing effective concentrations of c-kit ligand, a significant enhancement in the clonal efficiency of 263.3 and 299,8% for BFU-e and CFU-gm over the basic Epo-IL-3 was obtained, respectively.

This improvement was proportionately higher than that of control cultures (Table 6. 2, page 97 and Fig. N° 6. 2, page 101) leading to colony numbers not significantly different from those derived from normal progenitors. Moreover, multivariate analysis demonstrated clustering of the results of cultures containing effective concentrations of c-kit ligand. These results suggest interactions (mechanisms?) that differ from those supplemented with the other growth factor combinations.

These data also demonstrate for the first time that the mitotic potential of the aplastic stem cells may not be significantly different from normal, but that the sensitivity of these progenitors to peptides interacting with the G super-family of receptors is deranged, explaining the observed clinical abnormalities.

Similarly, clonogenic responses to SCF in bone marrow cells from patients with congenital aplastic syndromes such as Diamond Blackfan or Fanconi's anaemia appeared enhanced compared to the stimulatory signals provided by IL-3 (Abkowitz *et al,* 1992, Alter *et al,* 1992). Although *in vitro* laboratory data may not be predictable for clinical responses to cytokines, these data suggest that therapeutic trials of stem cell factor in patients with these disorders should be strongly considered.

7. 4 FINAL COMMENTS.

The studies described in this thesis do not provide further data concerning the specific mechanism that lead haematopoietic progenitors to respond sub-optimally to GF. However, explanations for these observations could include altered interactions between specific cytokine receptors and their ligands, either through changes in the affinity or membrane surface density. Additionally, soluble forms (binding proteins) of these receptors exist for both families, originating, probably, from proteolytic cleavage products of transmembrane molecules or alternatively, they are produced by natural secretion. Such binding proteins can help in the protection and transport of these cytokines or to the contrary, they can potentially function as inhibitors to the action of the ligand in feedback regulation (Olsson *et al,* 1992).

Furthermore, alterations at the level of the receptor structure or oligomerization, can also lead to loss of affinity with the ligand, or increases in the production of the soluble receptor fractions resulting in sub-optimal cell stimulation that can be overcome with higher GFs concentrations.

However, as the inappropriate clonogenic responses were elicited to a wide range of GFs, future investigations directed at these activation cascades may

reveal abnormalities in the signalling for cell division. Indeed, malignant myeloproliferation with overactive responses to haematopoietic peptides (Dai *et al,* 1992) and aplastic anaemia could represent opposites of a spectrum originating from abnormalities in the activation sequences that lead to the G_1-M progression in the cell cycle and replication of the progenitor cells.

It would be attractive to suggest that abnormal responses to these growth factors have common intracellular defects resulting in the subnormal responses to these cytokines. The Haemopoietin super-family are part of the growing transmembrane GTP-binding proteins that control a series of second messenger molecules (Gilman *et al,* 1989). The activation of these proteins is associated with Ca^{++} release from intracellular stores (Spivak *et al,* 1992, Reddy *et al,* 1992), formation of diacylglycerol and inositol triphosphate, activation of calmodulin and progression into cell cycle.

Moreover, specific calmodulin binding proteins have been described associated to the intranuclear enzyme DNA polymerase α and a 68 Kd binding protein that controls the terminal events required for the onset of DNA replication. The nuclear localization of this binding protein leads to transition from G_1 to S phase in the cell cycle and has been found to be common to GM-CSF, G-CSF, IL-3 or IL-6 but not for CSF-1 (Reddy *et al,* 1992). Alterations in this calmodulin binding molecule, could represent a possible target site in the chronic hypoproliferation of aplasia.

Progression to S phase requires cooperation of multiple intracellular pathways, and less potent GF may need the combined action of several signals to induce a strong mitogenic response. This was clearly shown by the additive effect provided by G-CSF in the aplastic cultures (Fig N° 6. 2, page 101).

Pleiotropic programs are common to all growth factors as most responses (ion changes, Na^{+}-H^{+} antiporter, Na^{+}, K^{+} Cl^{-} co-transport activation) and early gene transcriptions are initiated and maintained by phosphorylation changes occurring at serine or threonine residues. Switch kinases activated by tyrosine

kinase, protein kinase-C and G-coupled receptors, appear to integrate at early points complex networks of kinase-kinase cascades leading to activation of multiple cell targets.

One such early convergence is represented by MAP-kinases that need phosphorylation of both tyrosine and threonine residues for full activation (Pouyssegur *et al,* 1992). Derangements of any of these mechanisms, would disadvantage the cell under study in the finely tuned balance between positive and negative regulators that normally control haematopoiesis. Since cooperation and integration of signals between pathways are important in the mitogenic response (Poyssegur, 1992), phosphorylation of the c-kit receptor by its ligand, may correct the signals relayed by the abnormal G family of proteins and result in the improvement of the mitogenic response.

In summary, these studies have confirmed that in aplastic anaemia the stroma does not exert excessive suppressive actions on the clonogenic cells tested (CFU-bl). They suggest conversely, that the defect lies within this progenitor population demonstrating for the first time that the dividing fractions of both adherent and non-adhesive precursors are affected.

It has also provided original data showing that despite a sub optimal clonal efficiency at physiological concentrations of growth factors, the clonogenic potential of the aplastic progenitors that repopulate the bone marrow remains preserved. It has also been able to provide new compelling data that this poor responsiveness was restricted to the ligands belonging to the G super family of receptors and demonstrates that the mechanisms involved in the murine genetic anaemia (Sl/Sl^{d} and W/W^{v}) do not operate in the acquired presentation of the human disease.

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