CHAPTER – VII
DISCUSSION
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Therapeutic development in the field of haematological malignancy is still under way along with many other diseases as mechanisms of disease progression and the factors involved in disease control still remain unexplored in many aspect. Indeed, a considerable number of attempts have been made to elucidate the mechanisms of leukaemogenesis in animals systems, but a large fraction of leukaemia in human remained unveiled due to un-approachable materials from the source on obvious ethical ground. Recent studies brought to the knowledge of involvement of immunological components in regulating disease progression apart from the defects located in the bone marrow involving stem cell disorder or mutations. It became obvious to choose an experimental animal model for the purpose of investigations at any convenient time. One of the most important aspects of the present thesis work is therapeutic approach with immunological components in terms of immunopotentiation either directly or indirectly using biological response modifiers. In some cases biomodulators have been administered in leukaemia with insignificant success which may be due to inappropriate use of those components in leukaemia. The present thesis embodied some aspects of successful therapeutic approaches with biological response modifiers namely interleukin-2, interferon-γ and sheep RBC.

Aetiological factors for incidences of haematological malignancies has not completely been elucidated. Extensive work protocol are in progress to explore the mystery, but these investigations will require an experimental animal model for obvious ethical reason and also for easy access to study intricate stages of development of leukaemia. Initial attempts with ionising radiation for the induction of leukaemia in experimental animals was not much effective and required much longer
period for the appearance of leukaemic blasts in the peripheral blood of animals. Further, the percentage induction was found to be unpredictable, but efforts to induce leukaemia in mice with N-N' ethylnitrosourea with a dose of 80mg/kg of body weight responded more readily for leukaemogenesis together with an appreciable percent yield. Such method of leukaemia induction has been successfully followed in rats Druckray (1966) (255) and Koestner et al in 1971 (291). Although around 40% of the animals died at an early period without leaving scope for investigations towards leukaemia, 60% survived until the period of 6 – 10 months when they developed leukaemia as evidenced from the appearance of leukaemic blasts (plate 1). These animals also produced a large number of malignant blast within the bone marrow. As discussed in the subsequent part of the thesis, these animals also served to provide acceptable data involving immunological parameters. Thus the mouse leukaemic model could provide a satisfactory model for scientific research especially the immunological investigations as we have done in the present context. To be precise, the experimental mouse leukaemic model has several advantages as discussed, but the demerits included an early death of animals due to acute toxicity, death due to secondary infections and requirement of a large sample volume assumed to be pooled. Histological examinations of the peripheral blood smear and the bone marrow showed the blast cells originating from lymphoid series in majority whereas a smaller number of myeloblast also appeared in the smear. It was, therefore, presumed that ENU could induce malignancy more readily into the lymphoid series rather than myeloid. The malignancy was induced, henceforth, can be considered as a mixed type of leukaemia which might be of a particular characteristic in the mice under this event. The reason for such specificity of ENU towards lymphoid progeny is still obscure and requires further study.
The leukaemic animal model has also been found to associate signs of secondary infections and external manifestations alongwith. These included foot and mouth infections chronic diarrhea, loss of hair, reduced locomotion, anurexia and significantly reduced life span. Such changes could be apprehended to alter the immunological status in the animals concerned as well as in human cases. The immunological status in terms of cellular functions and the humoral immunity are, therefore, considered to play an important role in maintaining health status under leukaemic conditions. In the present thesis special emphasis has been given to investigate into the immunological status in terms of immunocyte functions in animals with ENU induced leukaemia (Yin & Delamore, 1987) (292). The results obtained in the present course of investigations hinted at a generalised suppression of the immune status with particular reference to immunocyte functions.

The functions of the immunocytes, namely, lymphocytes, Polymorphonuclear neutrophils (PMN) and macrophages revealed a significant degree of functional reduction with respect to spontaneous ‘E’ resetting, cytotoxicity in terms of CTL mediated lysis, PMN mediated phagocytosis and finally macrophage functions as investigated through adherence index (LAI) and popliteal lymph node assay ment for antigen presenting capacity. All these results are in support that both spontaneous ‘E’ resetting lymphocytes and the cytotoxic lymphocytes (CTL) are significantly affected (P<0.001) during leukaemic development and in particular in ENU induced leukaemic mice.

The mechanism of such suppression may not directly relate the carcinogen induced toxicity to the cells as possibility of retaining the toxicity after a long interval of 6 – 8th months is far from the reality, and
further due to the spontaneous renewal of the immunocytes from the bonemarrow representing the new haematopoietic lineages. The question, however, remains whether the carcinogen can directly intervene with the haematopoietic cell renewal apparatus (Handin, 1996) (293). Nevertheless, the mechanism of such damage to the bonemarrow apparatus is yet to be explored but, cannot be ruled out. The other most important and more plausible reasoning of the immunosuppression of leukaemic mice may be the leukomogenic inhibition to the immune-aparatus mediated through antigen shedding, non-specific blocking antibody, viscious cycle in corporating antigens, antibody or tumour toxins. Ample evidences of tumour mediated inhibitions to the immune apparatus have been documented [Currie 1976, (294) Lachmann, 1993) (279) that described involvement of the above factors in various types of tumours both in mammals or in human individuals. Surprisingly, informations of immune-status in leukaemic subjects are poorly documented and calls for detailed investigations on the above score (Macdonald & Ford 1996) (9). As such investigations conducted in the present thesis work provided with some important documents regarding the immunological functions at the cellular level that constitute the overall immunological apparatus in the subjected concerned. It can be postulated from the above findings that leukaemia exerts an overall inhibitory influence on the functional activity of the immunocytes. This holds equally good in case of mice with experimentally induced mixed type of leukaemia by N–N' ethylnitrosourea (ENU).

The implication of leukaemia induced inhibition in cellular immune-status in the human individual can be considered as an important aspects as this can provoke opportunistic infections associated with the disease and, further, application of
chemotherapeutics is further supposed to reduce immune-status (Whittaker, 1987) (78) in the subject concerned. Nitrosourea being a chemotherapeutic agent by also showed immune suppression with a consequent bone marrow suppression in the mice, rat and human as well (Koestner, 1971).

These are evident from the secondary infections and associated lesions in the subject. The point of argument in this context is treatment protocol in the human individual with haematological malignancy should, therefore, be carefully monitored considering the immune-status in the individual concerned. Thus, applications of chemotherapeutic in the patient can reduce the tumour load but, on the contrary, with essentially down regulation of the immune status. An important concept regarding therapeutic approach has been introduced long back by Mathe (1971) (157). Halpern, Currie and many others (1966) (156). That included the use of components for immunopotentiation with a consequent increase of therapeutic index. Such method now broadly accepted as 'immunotherapy' has occupied a significant area of research with definite positive therapeutic approach. It has been argued that immunotherapy can be applied as an adjunct therapy along with chemotherapy. Such argument has a rational basis with respect to tumour load which can be reduced by chemotherapeutic agent (Currie 1976) (294) (Lachmann 1993) (279). But current researches have demonstrated that immunotherapy alone can exert an immunopotentiating as well as tumour killing function with a resultant reduction of tumour load. The importance of such a method remains with the considerations that it can act as a target killer as well as immunestabilizer. Recent approaches with immunotherapeutic measure has coined a name 'Biological Response modifiers' (BRMs) (Gillespe &
Mahalay 1991) (293) which includes a number of biological components that can be of endogenous and exogenous origin. In the present thesis work, cytokines like inter-leukin-2 and interferon-γ and a non-specific biomodulators, sheep erythrocytes (SRBC) have been used either in single or in combinations.

The treatment of malignant diseases with cytokines has been with us for more than hundred years (Coley 1893) (296) but it is only in the past 20 years that our understanding of the interaction between cytokines and cancer developed, and their therapeutic potential has fully developed. Interleukin-2 (IL-2) which plays a pivotal role in the induction of the immune response. It has been reported to bind to its own specific receptor (IL-2R) on B and T lymphocytes and act as an autocrine factor by inducing expression of its own cell surface receptor molecule. This interleukin induces maturation of the antigen specific cytotoxic T-lymphocytes (CTL) and natural killer cells (NK), Lymphokine Activated Killer cells (LAK) and B-cells. As major antitumour response is mediated by CD8+ (CTL), the NKs and the LAK cells, administration of IL-2 in the leukaemic animals was found to delay inhibit the blast formation and improve the survival period. The consequent immunological functions of lymphocytes, PMNs and macrophages are also triggered to build up a strong antitumour defense in the animals concerned. Although no direct effect of IL-2 on macrophages and PMNs have been reported, it can well be that IL-2 can exert indirect influences on these cells type via stimulation of the T-helper cells and consequent activation through respective cytokines. Thus IL-2 is found to increase significantly cytotoxic killing of the target tumour (Dalton's lymphoma) in vitro (P=<0.001) and moderately increase the activity of rosetting lymphocytes with CD2 molecule interactions P<0.01, PMN mediated phagocytosis and
macrophage activity as represented by adherence index (AI) and antigen presenting capacity through PLN enlargement. As already mentioned the beneficial effects of such immunological boosting has also been reflected on the survival period of the animal concerned, the survival being much improved compare to ENU induced leukaemic animals P<0.001. Interleukin-2 in leukaemic subjects has rarely been administered except certain approaches in acute leukaemia where the complete evaluation is yet to be established (Rossenberg et al 1988 (181). Subsequent studies in recent years, however, furnished that IL-2 in combinations of chemotherapy can be fruitful in some case. In the present course of investigations it has been possible to delineate a positive role of IL-2 in combating ENU induced leukaemia in mice system. The investigations, therefore, provided with invaluable indications for IL-2 therapy in leukaemia patients. The reasons for investigating the potential role of immunotherapy with IL-2/LAK cells in leukaemia stem largely from some of the above considerations and from the unfavourable natural course and overall poor prognosis of most adult acute leukaemia patients. However, the positive approaches by IL-2 therapy have been supported by some other workers, who showed that in immuno-suppressed nude mice IL-2 alone are capable of blocking the growth of human acute leukaemia cells [Alder et al 1998 (297), Lista et al 1989 (298), Foa et al 1999 (299)]. However, in very recent years (Vilate 1998) (243) reports concerning positive therapeutic approaches towards the combination of IL-2/IL-12 in a low threshold doses have indidated the use of cytokines combinations worthy of exploration in vivo.

Although a number of cytokines and cytokines antagonist are being evaluated in clinical studies for their anti-leukaemic activity, most appear to act by an antiproliferative mechanism. At present IL-2 and
Interferon-γ have been reported to be the only cytokines to be used clinically as antileukaemic immuno-modulators [Rosenberg et al 1988 (181); Gottlieb et al 1989 (183) and Blaise et al 1990 (199)]. The present course of investigations, however, represent a unique model of immuno-therapeutic approach for IFN-γ induced antileukomogenesis either alone or in combinations with IL-2 and sheep RBC (SRBC). IFN-γ has appeared as an important immuno-regulatory protein that can stimulate NK cells and CD8+ mediated lytic processes through induction of class-I and class-II MHC molecules both in vitro and in vivo. This is further indicated that interferon may turn tumour target cells more susceptible to lysis by cytotoxic T-cell which is MHC dependent. Interferon as a whole can exhibit synergie with chemotherapeutic agents such as vinblastin that provoked clinical trials of combinations therapy (Dexaus et 1989) (300). Thus interferon served purpose of the agent capable of tumour load reduction through antiproliferative activity and finally resemble a condition of joing immunochemotherapeutic measure. In the present context IFN-γ alone showed a moderately responsive therapeutic approach by increasing the spontaneous ‘E’ rosetting, CTL activity, PMN mediated phagocytosis and finally macrophage mediated adherence index and popliteal lymphnode assay indicated for antigen presenting capacity. Interferons therefore, alone promoted the immune-status towards the cellular functioning against the tumour. A critical analysis of data further revealed that such beneficial effects are even better than IL-2 mediated effects alone. Another striking property that has been reported by some workers [Nathan et 1983 (301), Schutliz and Kleinschmidt 1983 (302)] are capability of boosting the two functions of monocytes/macrophages activity most relevant to host defenses enhanced antimicrobial activity, tumour cell cytotoxicity. Thus interferon-γ imparted the most desired functions of inducing NK cells
cytotoxicity, monocyte/macrophage functions and an upregulatory approach towards the expression of both class I and Class-II MHC antigens. Furthermore, an anti-proliferative effect towards the tumour has been documented at these molecular level involving the enzymes 2’ 5’ oligoadenylate synthetase and protein kinase and further induction of interferon stimulated genes (ISG) and still further by modulated oncogene function or expression [Pfeffer et al 1986 (303); Clemens, 1988 (304). These explanations and the positive points in favour of interferon-γ directed towards the attempts of fruitful application of interferon-γ against malignant diseases. The results obtained are in support of the above discussions.

A unique biological response modifier that has been shown to provide an affective anti-tumour immunopotentiating role in mice as well as in rats, has been introduced for the first time in leukaemic mice. Sheep erythrocytes (SRBC) with the specific ligands on its surface structure has been found to activate the immune effector cells through a variety of mechanism investigated in this laboratory(Ref Chaudhuri et al 1990) 1991, 1992, 1993, 1998) (265-269). The spontaneous rosette forming capacity that has been found to suffer a sharp depression in leukaemic mice, has been significantly protected and and similar highly significant protection has also been provided to the cytotoxic lymphocytes when 1% SRBC was inoculated to the leukaemic mice. (Fig. 3). Activation of cytoltyic T-lymphocytes by the SRBC binding proteins presently designated as TITIs has been reported by Siliciano et al 1985 (305). Hunig, 1985, 1986, (306,307) who demonstrated that T11 Erythrocyte binding protein upon a receptor specific binding with the ligand present on the surface of SRBC (T11S) can modulate a number of immunophysiological functions for T-cell activations. Thus, the
treatment of T-cells with certain monoclonal anti-T11 antibodies resulted in antigen dependent polyclonal T-cell activations as assessed by proliferations and lymphokine secretions. In addition, the majority of thymocytes that have not yet acquired the T3–T1 antigen/major histocompatibility complex (MHC) receptor can be activated to express IL-2 receptors through this mechanism. [Acuto et al 1983 (308), Fox et al 1985 (309)]. These investigations also furnished that the natural killer cells can also be triggered by this mechanism. It can be emphasised under these instance that a combined activity of cytotoxic T-lymphocytes and natural killer cells (NK) might have operated an important role in tumour load reduction and consequently a higher survival rate compared to the ENU induced leukaemic mice and even with those treated with other cytokines, eg, IL-2 and IFN-γ. Studies conducted with PMNS also revealed a significant role in combating these secondary infections in leukaemic subjects. Previously in this laboratory, it has been demonstrated that SRBC could effectively promote PMN mediated phagocytic burst against the tumour targets namely, ascitic fibrosarcoma and Dalton’s lymphoma. The mechanism of such activations in PMNs may be explained using the same basis that SRBC can trigger the lymphokine secretions and thereby can initiate and activate cytokine network, where by, the neutrophil get stimulated. An increased antigen presenting capacity by macrophages as determined through popliteal lymph node assay and also adherence index, calls-forth, a similar basis for explanations whereby the lymphokines can activate the macrophage function as well as trigger generation of MHC receptor. Discussions mentained elsewhere also furnished liberation of interferon from a number of immunoeffectector cells including T-lineages with resultant antitumour response (Chaudhuri et al 1991) (266).
An overview towards results obtained involving IL-2, IFN-γ and SRBC as biological response modifiers on the leukaemic mice revealed that SRBC by all means exerted the most protective effect against leukaemia when compared with IL-2 and IFN-γ. It is considered probable in some instances where IFN-γ plays a probable down-regulatory role and IL-2 with an intermediate potentiating activity, the question of combination of any two or all the three is anticipated to bring forth a better result in turn. Surprisingly enough, no significant betterment is identified in the combinations of any two of the BRMs except the combination of three where the improvement is associated with an increased survival rate. It is apprehended that these interactions of the three BRMs might have caused a turbulent effectiveness arising out of both upregulatory and down regulatory activities with conquerent counteractions between each other and finally a resultant functional remodulation. The question of administration of BRMs therapy, therefore, required a careful monitoring before going through a clinical approach. However, the results obtained from the unique BRM SRBC revealed a highly promising approach towards the therapeutic application against leukaemia. Indeed, the curious structure T11TS on SRBC surface are now being considered with significant importance for the future development of therapeutic protocol with this splendid molecule.

An attempt to analyse the BRM mediated therapeutic effects on lymphocytes, however, showed that signal transduced through protein tyrosine Kinase activity with IL-2 and SRBC were almost equal. The combinations of all these three BRMS also represented protein tyrosine Kinase activity in PMNs in an almost equal grade compare of IL-2 alone. However, results that could be achieved, showed an increase PTK
activities in both lymphocytes and PMNs where the role of IL-2 and SRBC took a predominant role. It should be mentioned at this context that a number of other messengers might have overlapping role on the activation process through BRMs which may explain these exact mechanism(s) in detail.

The scanning electron microscopic investigations, however, represented stimulatory activity of SRBC on the architectural conformaty of the immuno-effector cells. The combinations of all the three BRMS could not represent a disstinguishable morphological changes at this level. Interleukin-2 and SRBC represented secretary features of the cells especially from the lymphocytes. The macrophages assumed aggressive appearances in SRBC treated leukaemic groups. This findings further indicated that SRBC, of all the three BRMs, played a comparatively superior role in exerting the therapeutic benefit as evident from the immune parameters and the survival period.

The investigations conducted in the present thesis work furnished that the biological response modifiers can impart a significant therapeutic measure in leukaemic mice of which SRBC took the most significant role. The combination therapy with the BRMs was more beneficial and should be studied in detail with the interacting mechanisms involved in vivo. The role of cytokines awaits important role in establishing the therapeutic acumen of these components. It is anticipated that SRBC within few years might come up to provide an interesting therapeutic component when the molecule(s) of interest could be isolated and purified. Such programme is already under progress.