ASSESSMENT AND AUGMENTATION OF IMMUNOLOGICAL STATUS DURING TUMOR DEVELOPMENT

Submitted to the University of Calicut
for the Degree of

Doctor of Philosophy

In Immunology (Faculty of Medicine)

By

K. SURESH, M. Sc.

AMALA CANCER RESEARCH CENTRE,
Trichur - 680 553

1990

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CERTIFICATE

This is to certify that the Thesis entitled "Assessment and Augmentation of Immunological Status during tumor development" herewith submitted by Mr. K. SURESH in fulfilment of the requirements of the Ph.D. Degree in Immunology (Faculty of Medicine) of the University of Calicut is an authentic record of the research work carried out by him under my supervision and guidance in Amala Cancer Research Centre, Trichur and that no part thereof has been presented before for any other degree.

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Trichur,
31.8.1990

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K. SURESH

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INTRODUCTION

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The immune system might have been evolved as a means to detect and eliminate foreign molecules or pathogens. The immune surveillance is involved in the effective destruction of transformed cells. Cancer may be a manifestation of breakdown of this immune surveillance.

Transformation towards malignancy is often accompanied by the appearance of new antigens on the surface of tumor cells. The tumor associated antigens (TAA)/tumor specific antigens (TSA) may elicit a specific or nonspecific immune response. Specific immune response may be either humoral immunity mediated by antibody secreting B lymphocytes or cell mediated immunity mediated by specifically sensitised T lymphocytes. Nonspecific immune response mechanism comprises NK cell cytotoxicity, Macrophage mediated cytotoxicity and Antibody dependent cell-mediated cytotoxicity (ADCC) by K cells, granulocytes etc. Immune reactivity involves the integrated action of all these response mechanisms and regulation of these components. Biologically reactive soluble factors released during the interaction of sensitised lymphocytes with the antigen (Macrophage Activation Factor (MAF), Migration Inhibitory Factor (MIF), Interferon (IFN), Interleukins) could also modulate the immune response mechanisms.

T lymphocytes are responsible for cell mediated immunity including allograft rejection and delayed cutaneous hypersensitivity. They are further differentiated into T helper, T suppressor, T cytotoxic, delayed hypersensitivity T cell and also into amplifier T cells. In the thymus, T cells acquire antigen specific receptors and differentiate into various T cell subpopulations. Recently a third population of lymphocytes, null cells have been identified that express neither T nor B cell surface marker. Although principal function of null cells is not known NK and LAK cells are thought to be derived from this subpopulation.

A variety of immune effector mechanisms are involved in the destruction of vascularised tissue or circulating tumor cells. Antibodies can mediate cell destruction either through binding of complement or by acting as an opsonin to facilitate phagocytosis by macrophages or other phagocytic cells bearing Fc receptors. Antibody dependent cellular cytotoxicity (ADCC) utilise the immune effector binding to Fc portion of antibody bound to target cell for lysis of the target cell. Lectin dependent cellular cytotoxicity involves the association of lytic cell with target using lectins such as PHA or Con A as cross linking agents. The T cell can interact with the target cell surface antigens by means of an interaction with the T cell receptor and class I or II MHC molecules. Lymphokine Activated Killer (LAK) cells are lymphocytes that acquire the

ability to lyse a broad array of fresh tumor targets following incubation with interleukin-2. Activated macrophages also can recognise and lyse tumor cells.

Lymphokine induced cellular cytotoxicity has emerged as an important mechanism responsible for a variety of cytotoxicities involved in immune surveillance against spontaneous neoplasms. Tumor necrosis factor (TNF) and Lymphotoxin (LT) are two cytokines capable of direct destruction of tumor cells. Many cytokines, alpha, beta and gamma Interferons (IFN), interleukin-2 (IL-2), tumor necrosis factor (TNF) and coloney stimulating factor (CSF) have reached the stage of clinical application in patients with cancer by virtue of their ability to augment the host immune response.

Biological response modifiers boost the host immune defence to mount an effective attack on the invading organisms. Early attempts at immunotherapy were using non specific immune stimulants such as BCG, Coryne bacterium parvum, Levamisole and specific immuno stimulation with tumor cells, tumor cell extracts and vaccines. The totally unpredictable nature and the often contradictory results obtained according to the type of cancer and its degree of evolution explain the progressive replacement of these 'Immuno stimulants' with better defined substances.

Recently efforts have been concentrated on the transfer to tumor bearing host of previously sensitised reagents such

as antibody or cells which have the ability to directly or indirectly mediate an antitumor activity. Monoclonal antibodies either alone or conjugated to toxins, radionucleides or drugs act as magic bullets to specifically seek and destroy tumor cells. Adoptive immuno therapy utilising in vitro stimulated sensitised lymphocytes, lymphokine activated normal lymphocytes (LAK), and tumor infiltrating lymphocytes (TIL) have yielded encouraging results. Though so many different modalities of treatment are available, still the solution to cancer problem remains elusive. It is indispensable to understand the intricacies of the immune responses to devise better strategies for therapy. In this thesis, therefore, it is attempted to study various parameters of the immune responses against the tumor cells produced by the tumor bearing host. The following aspects have been studied in detail:

1. The immune profiles (NK, ADCC, MMADCC, CTL, T cell subsets and ACC) during tumor development.

2. The kinetics of NK cell activity during tumor development using percoll purified fraction (percent active killer cells, V_{max}, maximum recycling capacity, NKCF assay and modulation with IL-2).

3. Production and standardisation of interleukin-2 from Con A stimulated rat spleen cells, and the effect of IL-2 on the generation of LAK cells.

4. Adoptive immunotherapy using in vitro generated and human recombinant interleukin-2 (rIL-2) expanded CTL.

5. The immunomodulatory effect of an indigencus ayurvedic drug Emblica officianalis on immune responses, in comparison with a positive control Levamisole.

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REVIEW OF LITERATURE

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NATURAL CELL MEDIATED CYTOTOXICITY

Two natural defense mechanisms in the body against a variety of infections are Natural Killer Cell-Mediated Cytotoxicity (NKCMC) and Antibody Dependent Cellular Cytotoxicity (ADCC) mediated by Natural Killer (NK) cells and the Killer (K) cells respectively (Herberman 1983). NK cells have a potential role in immune surveillance in the host against virally induced tumors or transformed cells and also in the regulation of normal stem cell differentiation (Herberman and Ortaldo 1981). The ADCC is a cytotoxic activity involving poly or monoclonal antitumor antibodies with Fc receptor bearing effector cells (Ralph and Nakoinz 1983). Both NK and ADCC are mediated by a subset of lymphocytes known as large granular lymphocytes (LGL) (Timonen et al 1981). Many scientists are of opinion that NK activity and ADCC are mediated by the same (Abrams and Abrahams 1988), or highly overlapping subpopulation, namely the LGL (Timonen et al 1981). The NK activity is mediated by putative NK receptors, while ADCC is mediated specificaly by means of IgG-Fc receptors in the presence of target cell specific antibody (Bradly and Bonavidá 1982; Herberman et al 1985). The LGL, responsible for NKCMC and ADCC, are considerably heterogenous. Some of them exhibit both cytotoxicity while some mediate only one type of activity (Ortaldo and Herberman 1984, Kim et al 1984).

NATURAL KILLER ACTIVITY AND ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY

Natural Killer cells were discovered in 1973 (Takasugi et al 1973). They can be defined as a morphological sub-population of large granular lymphocytes (LGL), capable of lysing a wide spectrum of tumor cells (syngeneic, allogeneic and xenogeneic) without prior sensitization (Hansson et al 1978, Nunn et al 1977) and are considered as the first level of defense against spread of tumor in vivo (Herberman et al 1981). They do not show Major Histocompatibility Complex restrictions (Ortaldo, 1979). Natural Killer Cells are non-adherent, non-phagocytic, 50% of which form low affinity rosettes with sheep RBC (West et al 1977) and they lack immunoglobulin on the surface (Herberman et al 1977, Kay et al 1977), but carry Fc receptors for IgG (Herberman et al 1975, Kay et al 1977). NK cell mediated cytotoxicity in various strains of mice have been found to have predictable relationship with age, peak levels were noted at 5 to 10 weeks and declined to low levels thereafter (Herberman et al 1975).

Recently a number of studies have demonstrated that the major cells responsible for NK and ADCC activity in human (Timonen et al 1982), rat (Reynolds et al 1981, 1982) and mouse (Nunn et al, 1981; Kumagai 1982) are large granular lymphocytes. NK enriched population can be obtained by seperation of low density LGL from other lymphocytes by

cetrifugation on discontinuous density gradents of Percoll (Leuni et al 1981; Kumagai 1982).

Mouse NK cells have been found to be moderatly resistant to the effects of in vivo X-irradiations, with little effect using 350-500 rads, but substantial reduction in activity with 850-900 rads. Murine NK cells are also temperature sensitive and are quite labile at 37^{o}C, losing much of their activity with in the first 4 hours (Herberman et al 1975).

ADCC represents a potentially powerful cytolytic mechanism in which humoral and cellular effectors co-operate. ADCC involves the killing of antibody coated target cells by Fc receptors (FcR) bearing cells from unsensitized host. The ADCC assay consists of three components (1) Target cells (2) Antibody with specificity against target cell antigen (3) Effector population with Fc receptors for antibody. Following the original observation of Moller et al 1965, it has been repeatedly shown that target cell coated with appropriate antibody may be specifically lysed in vitro in the presence of lymphocytes from normal individuals. The cells responsible mediating ADCC are the K cells which are phenotypically indistinguishable from the NK cells (Perlmann and Perlmann 1970). Other effector cells involved in mediating ADCC are monocytes/macrophages, polymorpho nuclear (PMN) leukocytes, T cells and platelets (Shaw et al 1978, Koren 1983, Herberman 1986). When erythrocytes are employed as targets in the presence of hetrologous or homologous anti erythrocyte anti-

sera lymphocytes, monocytes and PMN leukocytes may mediate lysis (Nelson et al 1976). In contrast, when most in vivo or in vitro passaged cell lines are employed as targets with heterologous anti target cell antisera, lysis is usually restricted to lymphocyte effectors.

ANTI-TARGET CELL ANTIBODY

The anti target immunoglobulin that are capable of sensitizing target for ADCC have mainly been limited to IgG class. Recent evidence however suggested that IgM antibodies could also mediate ADCC (Nelson et al 1981). The mouse monoclonal antibodies of IgG2a and IgG3 subclasses have been reported to be most effective in ADCC assays (Hellstrom et al 1985). Complement components are not required for ADCC. However their presence under certain conditions may augment cytolysis especially for erythrocyte targets (Nelson et al 1981). Concentration of monoclonal antibody (Moab) ranging between 0.01 and 10 pg/cell were shown to constitute 50% lethal doses of tumor cells mixed with human effectors of ADCC, even at a low effector target cell ratio (E:T = 25:1) in 4 hours assay (Christianson et al 1984). Mainly IgG1 and IgG3 fractions of sera with high anti HIV antibody titres were shown to mediate ADCC against HIV-1 infected BHK cells (Mathiesen et al 1988).

CHARACTERISTICS OF NK CELLS

Origin and Lineage

NK cells are derived from the stem cells in the bone marrow (Haller et al 1979). The distruction of bone-marrow involving disruption of hemopoetic cells as well as micro environment with radioactive strontium abolished NK cell activity (Haller and Wigzell 1977). They do not require thymus for development and therefore thymectomised mice and congenitally athymic mice can develop normal NK function (Herberman and Holden 1978).

NK cell lineage is one of the most controversial areas of research and there are three major opinions stating that (a) NK cells are derived from myelomonocyte lineage, (b) related to the T cell lineage and (c) they represent a distinct lineage, derived from the bone marrow stem cells directly (Ortaldo and Herberman 1984).

Association with macrophage lineage is relatively weak. The two types of cells share characteristics like IL-1 production, phagocytosis, antigen presentation and the presence of cell surface antigens OKM-1 and Mac-1 (Kay and Harawitz 1980; Abo et al 1984, Scala et al 1985). However other monoclonal antibodies reactive with antigen on macrophages do not react with LGL (Hanjan et al 1982). In addition macrophages do not possess cytolysin of LGL or the Poly perforin of CTL which is also found in LGL (Grossman and Herberman 1986).

There is relatively stronger evidence linking NK cell to the T cell lineage. These include the presence of certain T cell markers on NK cells, similar lytic mechanism in both cell types, response of NK cells to IL-2 and production of IL-2 and IFN gamma (Grossman and Herberman 1986). Cloned CTL have also been shown to develop NK like activity and NK cell markers (Hercend et al 1984). However the presence of high levels of NK activity in athymic mice (Herberman et al 1975), lack of productive T cell receptor gene rearrangement (Rambaldi et al 1985, Young et al 1986) and lack of T cell surface markers on most mature NK cells (Ortaldo et al 1982) undermine the theory of T cell lineage for NK cells. Recently Kalland (1987) has suggested that T and NK cells may share a common early progenitor. This cell is propalled towards either T or NK cell development depending on the relative concentration of interleukin-3 (IL-3), a cytokine produced by T helper cell and its receptor on the progenitor.

Tissue Distribution: Highest NK activity, or proportion of LGL is found in peripheral blood followed by spleen. Intermediate to low levels are found in lymph nodes, bone marrow, peritoneal cavity, lung and gut epithelium. Thymus and tonsils have undetectable levels (Ortaldo and Herberman 1984).

Morphology: It is possible to enrich LGL on discontinuous Percoll density gradients, the large lymphocytes have a diameter of 16-20 micrometer (um) with a characteristic morphology. they possess an indented nucleus and azurophilic granules in cytoplasm (Timonen et al 1981).

Phenotypic Markers: Murine NK cells are asialo-GM1$^+$ and Ly5$^+$. LGLs in mice also bind B.23.1 a monoclonal antibody (moab) recognizing cells of monocyte or macrophage lineage but are negative for J11d and Ly-2 antigenic markers. Murine NK cells also express NK 1.2 and Qa5/NK 1.1 antigens (Ritz et al 1981), thyl 1.2 (partial expression) (Sexena and Adler 1980) and Ia antigens (Sexana et al 1980). Human NK cells are phenotypically defined as CD3$^-$ lymphocytes and the majority express the surface antigens CD2, CD56 and CD16. (Stotter and Lotze, 1990).

TARGETS

NK cells lyse a wide variety of targets including syngeneic, allogeneic and xenogeneic tumor cells, many normal cells such as bone marrow, fibroblasts and thymocytes. The tumor target most frequently employed in these studies consisted of a mouse Moloney sacroma virus induced lymphoma of strain A origin named YAC-1 (Cikes et al 1973) which was proved to be the most sensitive target among a number of NK suspectible cell lines (Kiessling et al 1975). Of the various tumor cell lines exhibiting differential susceptibility to NK

cell lysis, the erythroleukemic cell line K-562 is markedly sensitive to NK cells (Jondal and Pross 1975, Rustoven 1985). NK resistance and metastatic ability have been correlated with cell surface sialic acid content and NK susceptibility is associated with expression of asialo GM_2GalNac (B)-7-Gal-(B_1-4) GLC (Ceramide) (Sherebelem and Mody 1986). Correlation of Glycosphingolipids and Sialic acid in YAC-1 lymphoma varients with their sensitivity to NK mediated lysis had been reported (Yogeeswaran et al 1981). Certain surface antigens have been identified, which might be involved in recognition of target cells by NK cells. Laminin, a glycoprotein was found on functionally active LGL. Treatment of cells with antibodies to Laminin has been shown to inhibit the binding of rat NK cells to targets (Chang et al 1983, Maghazache et al 1988). Expression of Laminin receptors on murine tumor targets and correlation with sensitivity to NK cell mediated cytotoxicity has been reported (Lay Bown et al 1989).

The structures on target cells recognized by NK effectors have been identified only in case of certain NK clones (Moingeon et al 1985). Differentiation or activation related antigens are believed to represent the major types of structures recognized by NK cells (Ortaldo and Herberman 1984, Moingeon et al 1985). Some observations suggest that NK cells may bind to specific glycoprotein on target cells (Ortaldo 1983). The involvement of transferrin and transferrin receptors in effector target interactions has been implicated

(Wright and Bonavida 1983, Vodenelich et al 1983) as well as contradicted (Shaw et al 1986). Experiments involving transfection with DNA coding for class I MHC antigens on sensitive targets have shown that quantitive expressions of MHC class I antigen may be inversely related to NK susceptibility of target cell (Storkus et al 1987).

NK cells utilize a variety of membrane associated molecules to interact with their targets. A family of heterodimeric adhesion structures comprising LFA-1 (CD11a), OKMI/Mac-1 (CD11b) and Leu5 (CD11c) have been reported to contribute to NK effector target binding (Kuertzinger et al 1981). CD18 molecules either alone or in conjunction with alpha chain mediates adhesion (Beatty et al 1981). In addition to these, a movel adhesion molecule ICAM-1, a ligand for LFA-1 may also participate in NK activity (Megoba et al 1988). (CD56 is a sialylated isoform of the neural cellular adhesion molecule N.CAM (Lanier et al 1989). Recent evidence suggest that CD56 plays a role in NK cell target interaction together with CD2 and leukocyte function antigen LFA-1 (Nitta et al 1989). CD16 is a receptor for antigen-antibody complexes and thus involved in ADCC (Ortaldo et al 1988). Binding of immune complexes to CD16 structures on NK cells leads to a slow increase of intracellular $Ca2^{+}$ (Windebank et al 1989). It has been shown that the CD3 zeta protein is expressed in NK cells and Co-precipitates with larger complex of unknown nature (Anderson et al 1989). The importance of this finding becomes apparent when we consider that in T cells

the CD3 complex is associated with the T cell receptor and zeta chain is responsible for tranduction of activation signal following antigen recognition (Baniyash et al 1988). NK cell and T cell functions are deficient in patients with adhesion complex defect syndrome. These individuals fail to express the beta chain (CD18) common to adhesion molecule LFA-1 (CD11a) and C3bi receptor (CD11b) (Springer et al 1987).

The most widely used targets for ADCC are either antibody-coated chicken RBC (Perlmann and Perlmann 1970) or antibody coated Chang liver cells (Kay et al 1977), human RBC (Talpaz et al 1982). Sheep RBC coated with TNP serum has been used for testing antibody dependent cell functions and Fc receptor activities of mouse peritoneal macrophage cell line IC 21 (Scrio et al 1979). Lymphoblastoid cell lines such as Daudi and Raji are also used (Bradely and Bonavida 1982).

MECHANISM OF NK CYTOTOXICITY

The NK lytic mechanism has been intensely investigated in a number of laboratories (Wright and Bonavida 1981, Saksela et al 1982, Dennert et al 1985, Henkart et al 1985, Targen and Deen 1985). A stimulus secretion model had first been proposed by Roder et al (1978), according to which NK cell recognizes and binds to the target, after which secretes cytotoxic mediators which lyse the target in a lymphocyts independent phase of reaction. Direct evidence for the presence of postulated cytotoxic mediators was consequently

reported (Wright and Bonvida 1982). It was found that murine or human NK effector cell populations cocultured with NK sensitive tumor cells release NKCF into the culture supernatant (Wright et al 1983).

A model proposed by Wright and Bonavida (1983b) postulated an interaction of the target cell with NK cell, the stimulation by target cell of the NK cell to release NKCF which bind the target cell subsequently lyse them by a mechanism that is not yet understood well. Recently Roozemond et al (1987) have also proposed a multiple stage model of NKCMC. According to this model, NKCMC is divided into 6 different stages:

1. The NK cell receptor recognizes glycoproein present on the target plasma membrane (Ortaldo et al 1983) and binds to it. The binding of effector to the target cell requires the presence of Mg^{2+} (Farram and Targan 1983).

2. The binding of NK cell with the target triggers certain intracellular process leading to activation of the NK cell for further events known as 'post binding events' in lytic process. These events are further divided into a Ca^{2+} dependent programming for lysis and Ca^{2+} independent killer cell independent lysis phase (Wright and Bonavida 1983a). The activation of NK cell occures via membrane structures distinct from phase involved in recognition and binding. Evidence for this activation step is derived from experiments that shown

IFN pre-treated YAC-1 target cell can form conjugates with effector cells, although they are unable to stimulate effector cells to release NKCF. This implies that effector target binding alone is not sufficient to induce release of NKCF. Not much is known about the nature of this second signal but NKCMC is inhibited by protease or phospholipase inhibitors (Queen et al 1982). Hence it is possible to speculate that this step may involve enzymatic activity (Wright and Bonavida 1983b). A requirement for proteolytic activity has also been shown by others (Hudig et al 1984, Carpen et al 1986).

3. The activated NK cell shows rearrangement of cytoplasmic granules. There is a re-orientation of the micro tubules organising centre and Golgi apparatus in the effector cell and this is found to be a pre-requisite for lysis of bound targets (Kupfer et al 1985). The cytoplasmic granules of NK cells have been studied extensively using a homogenous population of granules, and several peptides with potential involvement in cytolysis have been characterized (Podack et al 1985). These factors include, in addition to NKCF, a pore forming protein (PFP) or Perforin, serine esterase enzymes and proteoglycans (Tschopp and Conzelmann 1986, Liu et al 1987). Rabbit antibodies to purified preparations of cytoplasmic granules from LGL, inhibit not only the cytolytic activity of the granule preparation itself but NK activity of LGL also (Reynolds et al 1987). Perforins are proteins which can be activated by Ca^{2+} to polymerize on target cell and form

transmembrane pores akin to those generated by the ninth component of complement, C9. Cell death could ensue from exchange of solute through these pores. Proteoglycans bind to and inactivate Perforin inside the intact cell, thus preventing autolysis of NK cells (Tschopp and Conzelmann 1986).

4. The fourth step is the release of NKCF, many studies have been carried out to understand the mechanism which leads to the release of NKCF. According to farram and Targan (1983), specific target effector cell interation is required because only NK sensitive target cells (K-562, Molt.4, U937, YAC-1) can induce NKCF release. Conversely Wright and Bonavida (1984) reported that NK resistant cell lines could also stimulate release of NKCF which indicates that there is no specificity at the level of induction of NKCF release from human, rat or murine effector cells. Activation of PBL by IFN, which acts by enhancing the Ca^{2+} dependent programming for lysis phase, enhances NKCF generation (Farram and Targan 1983). NKCF is also released by stimulation of NK cells with Con A (Wright and Bonavida 1984) as well as phorol ester + ionophore suggesting a role for protein kinase C activity in the process (Graves et al 1980, Roozemond et al 1987). Recentoy a inositol phosphate induced enhancement of NK cell activity has also been reported (Baten et al 1989). It has been further reported that the rigidfication of effector cell membrane results in the suppression of the mechanism responsible for the release of NKCF (Roozemond et al 1987).

NK cells are also known to secrete IL-2, IL-1 and IFN which may be toxic to some target cells under certain circumstances (Wright and Bonavida 1983a, Oppenheim et al 1986).

5. In the fifth step, the NKCF released by the effector cells bind to the target cell, membrane, Evidence indicates that NKCF binding sites are not found in equal amounts on different types of tumor cells. Some NK resistant targets lack NKCF binding sites, while other NK resistant lines can still adsorb NKCF, although they are resistant to lysis (Wright and Bonavida 1983b, Wright and Bonavida 1984). They postulated that the NK specificity of target cells is determined by their sensitivity to lysis by NKCF. NKCF binding site is distinct from recognition and activation sites (Wright and Bonavida 1983b). Though much is not known about the nature of the target cell receptor for NKCF, there are indications that it is glycosilated (Herberman 1985). The proposition, that NKCF binds to carbohydrate determinants on the target cell memberane is supported by the observation that pretreatment of K-562 with Tunicamycin caused a decrease in their sensitivity to NKCF (Blanca et al 1984).

6. The final step of the actual lysis of target cell by the processed NKCF has been under intense investigation. The process of internalisation of NKCF is not clear, but target cell fluidity seems to play a role in it. It has been shown that rigidification of target cell membrane reduces the sensitivity of target cell to the lytic action of NKCF and interferon with the lytic process. Thus it appears that NKCF

internalisation mechanism is lipid dependent and suppressed by membrane rigidification. These studies have also revealed the lipophilic nature of NKCF (Roozemond et al 1987). It has been reported that polyperforin causes a channel or ring like hole to be introduced in the target cell membrane, which allows the passage of toxins into the target cell that kills it. A hypothetical scheme was suggested for lymphocyte mediated lysis, according to which target cell death ensues by pore formation after Perforin polymerisation, or nuclear disintegration by nuclease activation and DNA degradation or by both the above mentioned possibilities (Podack 1985). Studies have shown that during killing mediated by NK cells and by CTL, the DNA of the target undergoes fragmentation into repeat units of about 200 bp suggesting the involvement of an endonuclease activity that is triggered during cytolytic event (Russel 1983, Liu et al 1987).

Recently Liu et al (1987) have isolated a cytotoxin from murine CTL that seems to belong to a family of Tumor Necrosis Factor (TNF) related peptides which also include Lymphotoxin (LT) and NKCF (Gray et al 1984, Wright and Bonavida 1987). Preliminary studies have shown that different forms of this TNF like cytotoxin and DNA fragmentation activities are present in helper T cells, activated T cells and Mast cells. These molecules due to their slow time course of killing are termed 'Leukalexin' (from the Greek word alxis, meaning to protect). These TNF related cytotoxins could represent members of a family of closely related Leukalexins (Liu et al

1987). Leukoregulin, a 45-65 KD glycoprotein has also been reported to act as a mediator of the NK cytotoxic action. It induces profound changes in membrane premeability and cell surface composition of target cells (Ransom et al 1985). Thus, as the picture of mechanism of NKCMC becomes clearer, it is emerging that killing mediated by lymphocyte including NK cell is quiet complex. The process most probably involves multiple mediators and mechanisms that operate in concert or independently to bring about the death of target cell.

With such an efficient inherent apparatus for cytotoxicity the question arises how lymphocytes protect themselves from these lytic factors. A hypothesis has been forwarded recently by Young and Cohn (1988), according to which NK cell membrane incorporates a special protein named Protectin which is similar to Perforin. The close homology between the two substances promote a faulty polymerisation of perforin. The protectin rapidly combines with any perforin monomer that reaches the effector cell membrane, thereby preventing either insertion of Perforin monomer that would end in pore formation (Young and Cohn 1988, Young and Liu 1988).

CHARACTERISTICS OF NKCF

The NKCFs are glycoproteins in which disulphide binding appears to be essential for lytic activity. Murine NKCFs exhibit an apparent molecular weight of 20-40 KD. (Wright and Bonavida 1985). They are heat sensitive and are inactivated at 63^{o}C for 2 hrs. They are also inactivated at low pH (pH

2.0), by reduction and alkylation and by exposure to Trypsin. The NKCF may be closely related to cytolysin released by NK cell upon degranulation (Wright and Bonavida 1985).

It has been hypothesized that NKCF may act in synergy with a secreted protease in order to elicit maximal cell lysis, which is in consistance with reports that serine proteases are involved in late killer cell independent lysis (Targan and Dean 1985, Wright et al 1985). Lysis by NKCF is known to take several hours while NK cells mediate lysis within 1-4 hours. However partially purified NKCF could kill target cells pretreated with Trypsin with similar kinetics as NK cells. This observation also favours possible synergestic activity of NKCF and Trypsin proteases in vivo (Ortaldo 1985).

Antibody inhibition studies have shown that NKCF activity is mediated in part of TNF or an antigenically related molecule in addition to some other distinct factor. TNF also was found to be insufficient for mediating NK activity as was suggested by lack of consistant inhibition of NKCMC by anti TNF (Wright and Bonavida 1987). On the other hand, Degliantoni et al (1985) have reported complete inhibition of NKCF activity by anti TNF. Thus it is obvious that different forms of NKCFs seem to be existing and further biochemical purification and characterisation of these are necessary for a better understanding of NKCMC process.

REGULATION OF NK ACTIVITY

NK activity is regulated by a variety of factors of which IFN and IL-2 have been studied most, especially for activation of NK cells (Ortaldo and Herberman 1984, Welsh 1984). Other substance known to modulate NK activity are IL-1, suppressor cells and soluble factors like Prostaglandins and neuro-hormones like beta Endorphin (Herberman 1982, Mandler et al 1986). NK activity of mice or rats is depressed non-specifically by drugs such as Cyclophosphamide or more selectively by antibodies such as anti-asialo GM-1 (Kassai et al 1980, Habu et al 1981).

INTERFERONS

The IFNs are a group of glycoproteins with molecular weights ranging from 15 to 70 KD (Herberman and Callewaert 1985), first discovered by Isac and Lindenmann in 1957. The three classes of IFN (IFN alpha IFN beta and IFN gamma) represent immunologically distinct proteins that share a number of functions like ability to induce antiviral state, inhibition of tumor cell growth and modulation of immune response (Welsh 1984). The IFN can modulate cytotoxic responses of several cell types implicated in resistance to cancer like NKCMC, ADCC (Herberman et al 1979), monocyte and macrophage mediated cytotoxicity (Herberman et al 1982) and T cell-mediated cytotoxicity (Lindhal et al 1972). The mechanism of action of many other Biological Response Modifiers (BRM) such as OK432 (Christmas and Moor 1984) Coryne

bacterium parvum (Lichtenstein et al 1985) Staphylococcal protein A (Catalana et al 1981) several different murine viruses particularly Lymphocyte Choriomenigitis Virus (LCMV), Bacillus Calamette Guerrin (BCG) (Holden and Herberman 1977), MVE-2 a Pyran copolymer (Wiltrout et al 1984) Cis-diamino dichloro platinum (CDDP) (Lichtenstein and Pinde 1986) polyionosinic acid and polycytidilic acid, occures through the production of IFN, which augments NK activity. NK activity in both conventional and nude mice could be substantially augmented by inoculation of mice with tumor cells bearing relevant antigens (Herberman et al 1977, Wolfe et al 1976, 1977, Tracey et al 1977). The NK cells can produce all the 3 types of IFN when stimulated with lectins, viruses, mycoplasma or tumor cells (Djeu et al 1982). Paradoxically, treatment of target cells with IFN protects them from lysis by NK cells (Uchida et al 1985). This may be due to Interferon induced enhanced expression of class I or II MHC antigen on the target cells (Zoller et al 1988, Karre et al 1986).

It has been shown that IFN cause activation, proliferation (Bioron and Welsh 1982) as well as suppression (Shaw et al 1979) of NK Cells. IFN can activate NK cells by inducing pre-NK cells to develop into normally functioning mature NK cells that can recognize and bind susceptible target cell, by increasing the proportion of lytically active NK cells, by increasing the rate of lysis as well as by facilitating recycling of NK cells (Welsh 1984).

INTERLEUKIN-2

It is a glycoprotein of molecular weight 15.5 KD, which is produced by T lymphocytes upon activation by mitogens or antigens. IL-2 acts in an autocrine or paracrine manner to stimulate T cell growth, up-grades IL-2 receptor expression and enhance functions such as phagocytosis and cell killing. It can maintain long term growth of T cells in culture as well as mediate many immunologic events in vivo and in vitro and play an important role in the generation of immune response (Rosenberg et al 1984).

IL-2 can augment NK as well as ADCC activities (Henney et al 1981, Shaw et al 1985). Purified IL-2 can directly activate LGL to enhance NK activity in the absence of adherent cells (Mayasaka et al 1984). IL-2 induces the synthesis of interferon gamma by NK cells, which can also augment NK activity independently (Svednersky et al 1984, Rook et al 1985). Combined treatment of IL-2 and IFN has a synergestic effect on enhancement of NK cytotoxicity (Svedenersky et al 1984, Itoh et al 1986). IL-2 has also been shown to augment ADCC against carcinoma or melanoma cell lines (Herberman et al 1985, Shaw et al 1988). In many instances, the effects of IL-2 on tumor regression have been directly associated with increased Natural Killer activity (Lotze et al 1985, Hinuma et al 1986, Papa et al 1986). The generation of Lymphokine activated Killer (LAK) cells and their subsequent use in adoptive immunotherapy have shown positive results in a number of malignancies (Rosenberg et al 1985).

INTERLEUKIN-1

Interleukin-1 (IL-1) is a glycoprotein with molecular weight 17 KD and has been demonstrated to augment NK cytotoxicities due to which it has been suggested that it may play a crucial role in NKCMC (Herman et al 1985).

SUPPRESSOR CELLS

Monocytes and adherent cells are found to suppress NK activity in certain malignant conditions like high grade brain tumors and lung cancer (Penn et al 1984, Uchida et al 1984). Certain T lymphocytes can suppress NK activity in mice (Zoller and Wigzell 1982) and granulocytes are shown to suppress NK activity of normal human donors (Kay and Smith 1983). Among several thymocyte fractions seperated by velocity sedimentation, a relatively faster sedimenting fraction showed remarkable suppression of splenic NK cell activity against YAC-1 and RL 01 targets (Nair et al 1979).

PROSTAGLANDINS

Prostaglandins are natural aliphatic acids that can suppress both NK activity and ADCC (Herberman et al 1982). Prostaglandins, especially of the E series, produced by tumor cells themselves can also inhibit NK and ADCC activities (Brunda et al 1980, Koren 1981). Prostaglandins have also been reported to induce T suppressor subsets of cells or suppressor factors (Fischer et al 1985). It has been proposed that prostaglandins, particularly PEG-2 exert their inhibitory effects by suppressing the expression of transferrin receptors (Ibayashi et al 1987).

NEUROHORMONES

The neurohormones, beta Endorphin has pharmacologic action resembling opiate alkaloids, which are the major substances secreted by the brain during physiologic stress of the body (Guilleman 1977). The beta Endorophin and Enkephalins, have been shown to augment NK activity in vitro (Mandler 1986). Beta Endorphin can also enhance the production of IFN and IL-2 by LGL (Mandler 1986). Serotonin is another neurohormone which has been shown to augment NK cytotoxicity against K-562 and other sensitive targets. The effect was dose dependent and required the presence of accessory monocytes (Hellstrand and Hermodossen 1987). Transforming Growth Factor beta (TGF beta) a 25 KD homodimer has been reported to inhibit NK activity and also inhibits augmentation of NK activity in response to IFN but not to IL-2 (Esperik et al 1988, Rook 1986). These results favour the concept of regulation of the immune system by neurohormones. Corticosteroids have a negative effect on NK levels. Steroid hormone receptors have been shown on NK cells and these hormones may influence the NK levels in vivo.

CONCANAVALIN A (CON A)

Human PBL pre-cultured in media containing 60 ug/ml Con A displayed impaired NK and ADCC against both allogeneic and autologous targets. The downregulation of NK and ADCC could be attributed to the effect of endogeneous suppressor cells generated in culture (Nair et al 1981). Con A induced

impairment in ADCC of of SRBC in syngeneic DBA/2 mice has been reported (Wei et al 1983).

IMMUNE COMPLEXES

Effector cell activity may be inhibited by antigen--antibody complexes and aggregated immunoglobulin that occupies effector cell Fc receptor percluding contact with antibody coated target cell (Bancu et al 1988, Wilson and Coombo 1985). Augmented NK and ADCC in rats bearing Dimethylbenzanthracene induced primary mammary adenocarcinoma, following plasma adsorption over Staphylococcus aureus has been reported (Ray et al 1982, Ray et al 1984). Authors have attributed the enhanced killing potential of PBMNC to removal of humoral blocking factors, particulary the immune complexes from effector lymphocytes, enabling them to function against tumors. Adherent Peritoneal Exudate Cells (PEC) cells harvested from Balb/c mice after 4 days of ip injection of OK432, has been reported to exhibit potent cytotoxicity against mouse carcinoma cells in the presence of immune serum from mice which has survived challenge with MM2 carcinoma cells (Murayama and T. Sugiya 1984).

Treatment of mouse spleen cells with specific alloantibodies augment NK activity against Fc receptor positive target cells (Saxena et al 1981, Brunda et al 1981). A reverse ADCC mechanism has been proposed for this (Saxena et al 1982).

IMMUNOREGULATION BY NK CELLS

LGL can produce a number of different cytokines that can modulate immune responses (Reynolds and Ortaldo 1987). Incubation of purified LGL with PHA or Con A resulted in secretion of IL-2 (Domzig and Stadler 1982), IL-1 (Abo et al 1983), IFN Gamma, Colony Stimulating Factor (CSF) (Kasahara et al 1983), B Cell Growth Factor (BCGF) and B Cell Differentiation Factor (BCDF) (Herberman 1985). LGL also have been found to produce IFN gamma in response to IL-2 and IL-1 upon stimulation with tumor cells (Herman and Rabson 1986). A subset of LGL can act as antigen-presenting cells while some NK cells have been shown to interfere with accessory cell function and also negatively regulate B cell responses (Scala et al 1985, Arai et al 1983). Exposure to autologous B cell has been shown to induce NKCF production by Human LGL (Saranath et al 1986). Monocyte mediated augmentation of NK activity has also been reported (Bloom et al 1986).

IMMUNE SURVEILLANCE AND THE ROLE OF NK CELLS

The observations that nude or neonatally thymectomised mice have high levels of NK activity and a low incidence of tumors, and the susceptibility of tumor cells to attack by NK cells independent of the expression of tumor associated transplantation antigens suggest an important role for effector cells other than classical CTL in immune surveillance against tumor cells (Herberman et al 1981). There is considerable circumstantial evidence favouring a role for NK cells in immune surveillance.

1. High NK activity in nude neonatally thymectomised mice consistant with their low incidence of spontaneous mammary tumors or chemical carcinogen induced tumors.

2. Augmentation of NK activity by retinoic acid, a tumor preventive agent and inhibition of NK activity by tumor promotors.

3. High incidence of lymphoproliferative diseases in patients with Chediak Higashi Syndrome, who have a selective deficit in NK activity.

4. High incidence of lymphomas in a colony of beige mice, which also have selective deficit in NK activity.

5. Low NK activity is seen in patients with X-linked lymphoproliferative disease.

6. Low NK activity in kidney allograft recepients who have a high risk of development of lyphoproliferative and other tumors.

There are several reports regarding the antitumor efficacy of NK cells in vivo (Brood et al 1981, Gorelik et al 1982, Habu 1981, Henna 1980, Whiteside and Herberman 1989). In mice with reduced NK activity, incidence of tumor was enhanced when syngeneic, allogeneic or xenogeneic tumors were transplanted subcutaneously (Sajo et al 1984). NK activity and the number of pulmonary metastases were found to be

inversely correlated (Brunda et al 1983). Depressed NK activity was observed in human lung carcinoma, malignant melanoma (Sibbit et al 1984) adenocarcinoma of pancreas (Fuma et al 1984) head and neck cancer, breast cancer (Wanebo et al 1980), bladder cancer (Shapiro et al 1984), ovarian cancer (Allavena et al 1982) cervical cancer (Satam et al 1986) and hepatocellular carcinoma (Chaung et al 1990).

Low NK activity has been observed in many hematological malignacies like Hairy Cell Leukemia (Smith et al 1985) Chronic Myleoid Leukemia (Fujiyama et al 1987) Chronic Lymphocytic Leukemia (Pattangale 1982). Acute Myelogenous Leukemia (Nasrallah and Miale 1983) and Hodgkin's disease (Beranyi et al 1986). NK cell deficiency in patients with Chronic Myelogenous Leukemia has been attributed to defective IL-2 production by T helper cells and NK cells (Chang et al 1989). In patients with hepatocellular carcinoma the decreased NK cytotoxicity observed could be related to defect in IL-1 production (Herman et al 1985). Schwartz et al (1987) have suggested a role for NK activity in preventing tumor matastases. Antimetastatic effect of Swainsonine, an indolizidin alakloid, through augmentation of NK cell activity in Balb/c mice bearing B16-F10 melanoma has been reported (Humphries et al 1988). Above findings support the role of natural immunity against blood born metastases. In patients with Aids it was observed that the NK cells failed to release NKCF upon stimulation by target cells. The defective triggar for NKCF release could be upregulated by IL-2 suggests that

in Aids patients the defective IL-2 production could be responsible for failure of NK cells to release NKCF (Wright and Bonavida 1987). Functional deficiency of NK cells in mice with beige mutation and in humans (Chediak-Higashi Syndrome) is due to lack of formation of intracellular granules (which contain cytolytic enzymes) and is associated with increased incidence of tumors (Ortaldo et al 1988). The concept of therapeutic role of NK cells in cancer has been supported by the observation that activated NK or NK like cells are responsible for antitumor function in mice (Morale and Pang 1986).

Under physiologic conditions ADCC could play a role in allograft rejection, elimination of infectious agents including viruses and parasites, immune surveillance against neoplasia and autoimmune phenomena such as haemolytic diseases caused anti-erythrocyte antibody.

Significant depression in ADCC activity in primigravides compared to non-pregnant women has been reported. These findings support the hypothesis that maternal acceptance of fetal allograft is due to suppression of cell mediated immunity (Hill et al 1986).

The antinumor activity of the ADCC mechanism was demonstrated by observing tumor cell lysis when monoclonal antibodies were incubated with effector cells (splenocytes) from nude mice (Herlyn et al 1982). Established human melanoma tumors have been reported to be eradicated by

simultaneously injecting tumor bearing mice with monoclonal antibody and splenocytes (Shultz et al 1985). Spleen cells from C_3H (MTV+) female mice have been found to exert relatively high antibody dependent cellular cytotoxicity against chicken RBC and human B leukemia cell line, SB tumor targets (Nair et al 1980).

Macrophage line P-388/D has been reported to lyse antibody coated chick RBC (Koren et al 1978). The denser subpopulation of macrophage enriched cells from mouse peritoneal macrophage of IC-21 line has been reported to be more effective in mediating ADCC (Scrio et al 1979). Enhanced antibody dependent killing of autologous tumor cells (Chattopadhyay et al 1986) and phagocytosis of sensitized sheep RBC (Bhattacharya et al 1986) by tumor associated macrophage (TAM) has been demonstrated during the progressive growth of S-180 ascites tumor in Swiss mice.

High efficiency of 50-day-old rat spleen cells in mediating ADCC of rat erythrocytes parasitized by Plasmodium berghei in comparison with 30-day-old spleen cell has been reported (Orago and Soloman 1986).

Suppression of established myeloma (MOPC-104E) in mice on administration of 0.4ml high titred anti IgM serum ip or iv had been reported. Since the antiserum virtually had no toxicity in vitro, more plausible explanation for this type of suppression include some type of ADCC or opsonisation (Douglas et al 1977).

ADCC effector cell capacity was found to be higher in animals transplanted with immunogenic spontaneously regressing tumors (Moloney Sarcoma virus induced) than in mice bearing poorly immunogenic matastasing MS2 sarcoma (Mantovani et al 1977). Increased ADCC paralleling with tumor growth has been reported in Balb/c mice transplanted with syngeneic mammary tumors (Lopez 1977).

Administration of monoclonal antibody combined with LAK cells can mediate ADCC in vivo, a significant reduction in number of lung metastases compared to the treatment of mice with LAK cells alone has been reported (Munn and Cheung 1986, Eisenthal et al 1988, Berinstein et al 1987). Combination of antitumor monoclonal antibody capable of mediating ADCC with rIL-2 therapy could result in the generation of potent antitumor effects against LAK resistant tumors (Kawase et al 1988). Peritoneal exudate cells (PEC) from mouse or adherent cells of human perepheral blood leukocytes have been shown to lyse human tumor target coated with mouse monoclonal antibodies IgG3; IgG2a subclass recognising relevant tumor antigen (Hellstrom et al 1988).

T CELL SUBSETS

T lymphocyte function can be broadly divided into effector and regulatory. The regulatory functions involve T helper functions to help B lymphocytes to respond to thymic dependent antigens and antibody synthesis; T amplifier

functions which are capable of amplifying certain T cell mediated effector function and also suppressive function which suppress antibody synthesis and T cell effector function. The effector T cell functions include Delayed type Hypersensitivity, Cell Mediated Cytotoxicity and those involved in transplantation immunity including allograft rejection and Graft versus Host disease (GVH).

The adult murine thymus contains four subpopulations of thymocytes defined by T cell surface antigen CD4 ($L3T4^+$) and CD8 ($Lyt\text{-}2^+$) they are CD^+8^- and $CD4^-8^+$ (Single positive), $CD4^+$ $CD8^+$ (double positive), $CD4^-$ $CD8^-$ (double negative). The double positive makes up about 80% of the total thymocytes and most of these are thought to be terminal cells and undergo senescence. The thymus educated cells, thymocytes or T cells appear in peripheral blood as precursors and become activated only after specific stimulation with antigen.

HELPER/SUPPRESSOR T CELLS

The $Lyt\text{-}1^+23^-$ ($L3T4^+$) cells constitute approximately one third of the peripheral T cell pool. They are especially programmed to amplify the functional activity of other cells when activated by antigen associated with Ir region determinants. The amplifying effect of this T cell subset has been demonstrated for the production of antibody by B cell, for the differentiation of precursor to killer effector cells and for the activation of macrophages to participate in delayed hypersensitivity response (Huber et al 1976). In addition, this sub class is capable of inducing the developmet

of a subset of non-immune precursors of suppressor T cells to generate feed back inhibitory activity. $L3T4^+$ cells do not contribute themselves to CTL pool even though they account for the major proportion of the proliferative response in the mixed lymphocyte reaction. Many of the effect of helper inducer T cells are mediated by production of lymphokines. Lymphokines are potent pleotropic factors that control wide range of functions including activation, growth and differentiation of cells of immune system. Two T cell derived lymphokines are Interleukin-2 and Interleukin-4, functions as autocrine growth factors regulating the growth of T cells that produce them. More than one lymphokine may mediate same functions (Paul et al 1989).

The $Lyt\text{-}2^+$ ($CD8^+$) population comprises 5-10% of the peripheral T cells and include both cytotoxic suppressor cells. In contrast to $L3T4^+$ class, the $Lyt\text{-}2^+$ cells do not proliferate in response to I region difference in the MLR. Killer effector activity generated from either lymph node or spleen cells is mediated solely by $Lyt2^+$ cells (Mills et al 1983, Hurrel et al 1982) which also play a major supressive role after mitogen stimulation (Dye and North 1984). All pre-killer and killer activity seen after stimulation of lymph node cells with allogeneic lymphocytes and one half to three fourth of the splenic pre-killer activity are present in this subset.

Recently interest has focussed of a new subset of molecules best known in clinical studies by the nomenclature 4B4 ("helper inducers") and 2H4 ("suppressor inducers"). Recent progress indicates that these subsets: 1. are not distinct lineages but are different maturational stages, 2. can be more generally designated as naive T cells (encompassing the suppressor inducer subset) and memory T cells (encompassing the helper inducer subset (Sanders et al 1988, Takeuchi et al 1989), 3. differ in expression not only of the CD45R (2H4) and CDW29 (4B4) but also by at least five surface markers and 4. differ functionally in activation requirements and lymphokine secretion. Recently a novel monoclonal antibody which distinguishes between killer effector and suppressor effector cells in CD8 population based on an epitope on the LFA-1 antigen has been reported (Morimato et al 1987).

CYTOTOXIC T LYMPHOCYTES

CTL,defined by their capacity to kill specific targets cells in vitro have been shown to be an important effector cells in allograft rejection (Berk 1980, Cerottini and Brunner 1974), virus infection (Oldstone et al 1986, Zinkernagel et al 1986) and tumor rejection (Doherty et al 1984, Melief and Kast, 1990). The lytic activity of T lymphocyte was first described by Govaerts (1960), who demonstrated that thoracic duct cells from dogs which had rejected kidney allografts lysed donor kidney cells in vitro. CTL recognizes specific target cell

antigen displayed in close association with MHC class-1 molecules (Swain 1981, Swartz 1985, Salter et al 1989). The T cell receptor is crucial for the recognition, binding and activation of the CTL. T cell receptor (Ti) is disulphide linked heterodimer glycoproteins of 80-90 KDa with two subunits, Ti-Alpha chain (43-54 KDa) and Ti-Beta (38-49 KDa), closely associated with CD3 complex (Kronenberg et al 1986). Each chain has both constant and variable domains (Reinherz et al 1983, Kappler et al 1983a,b).

The Ti-Alpha and Beta chains have limited homology to immunoglobulin supergene family. Analogous to immunoglobulin heavy chain genes, Ti-Beta chains are assembled from recombinational events involving variable (V), diversity (D), joining (J) and constant (C) segments (Siu et al 1984). Ti-Alpha chains are similarly assembled but to date no D segments have been identified (Yoshikai et al 1985). The diverse antigen reactive repertoire of T cells (8×10^7) can be accounted for in part, from the joining of different V, J and D segments as well as combinatorial association between the Ti-Alpha and Beta chains (Hood et al 1985, Governman et al 1986).

Another T cell receptor Ti-Gamma-delta chain, ~55KDa and KDa glycoprotein (Saito et al 1984, Born et al 1987) has been reported to be expressed in T helper as well as on the surface of $T4^-T8^-$ thymocytes, felt to represent the most immature population of T cells (Bank et al 1986, Lanier and Weiss

1980). The Gamma-Delta positive cells mature in the fetal thymus prior to Alpha-Beta positive cells (Chain et al 1987a, Born et al 1986) and could be stimulated to produce IL-2 by Purified Protein Derivative (PPD) of Mybacterium tuberculosis (O'Brien et al 1989).

Peptides that are antigenic for T lymphocytes are ligands for receptors, the class I or II glycoprotein encoded by genes in MHC and the idiotypic Alpha-Beta chain T cell receptor. In such a trimolecular interaction the amino acid sequence of the peptide must specify the contact with both receptors, agretop residue bind to MHC receptor and epitope residue bind to the T cell antigen receptor. A synthetic delimitation of an antigenic site precisely identifies a predicted pentapeptide motif as the minimal antigenic determinant presented by class I MHC molecule recognized by a CTL clone (Reddehase et al 1989).

The interaction of the Alpha and Beta TCR with thymic MHC antigen is essential for the development of mature T cells (Scot et al 1989). The repertoire of receptors expressed by peripheral T cells is the result of two selective events during intrathymic development. Positive selection expands cells that are able to recognize foreign peptide presented by self MHC molecule (Murphy et al 1989). Negative selection involve clonal deletion or clonal abortion of auto-reactive T cells through an endogenous pathway of Apotosis, leading to self tolerance (Kappler et al 1987, Smith et al 1989). Induction of tolerance within the thymus depends on contact with dendritic cells (Van Boehmer and Schleiger 1984).

In addition to specificity for self + antigen many T cells have specificity for H-2 alloantigen. T cell clones with defined self + antigen specificity show non-random patterns of H-2 reactivity, which suggests that alloreactivity reflects cross reactive binding of epitopes shared between allo H-2 and self + antigen (Schwartz and Sredni 1982).

ROLE OF T3/Ti COMPLEX IN ACTIVATION

The T cell antigen receptor subserves two functions in antigen induced activation:

1. a recognitive function in which a specific antigen is recognized in the context of appropriate MHC molecules and

2. an effector function in which the recognitive event is transmitted across the plasma membrane to the interior of the cells, with resultant appearance of intracellular second messengers. Acessory molecules important for initial adhesion of the CTL with target cells are Lymphocytes Function Associated Antigen-1 (LFA-1) and Inter Cellular Adhesion Molecule (ICAM-1) CD2 and LFA-3. These molecules have been reported to enhance the avidity of interactions between T cells and antigen presenting cells and also possibly involve in recognition and signal transduction (Altmann et al 1989, Staunten et al 1989, Shaw et al 1986, Moingeon et al 1989).

$L3T4^+$ (CD4) and $Lyt\text{-}2^+$ (CD8) are the other accessory molecules which influence T cell specificity and/or triggering. The expression of these molecules are mutually

exclusive on the most peripheral T cell (Dialas et al 1983) and the type of T -accessory molecule displayed by T cells closely correlates the class of H-2 molecules that cell recognize: $L3T4^+$ T cells are restricted by H-2 class II and can transduce independent signal during T cell activation (Marth et al 1989) whereas $Lyt\text{-}2^+$ T cells are class I restricted and have been reported to involve in transmembrane signalling during T cell activation (Potter et al 1989). However, the issue of wheather T cell receptor (TCR) expresses (1) two binding sites, one for self H-2 and other for antigen (dual recognition) or (2) a single binding site specific for altered self determinants has still to be resolved.

Triggering of the T cell receptor (T3/Ti complex) in conjunction with class I MHC results in an increase in the concentration of cytoplasmic free calcium (Ca^{2+}), one of the intracellular events which is required for T cell activation. Other receptors appear to function as accessory molecules (TP-44, Ti, IL-1 receptor) which when stimulated are able to synergise with stimuli provided by the T3/Ti complex.

Studies have revealed that monoclonal antibody (moab) reactive with T3 could function as polyclonal agonist inducing resting human peripheral blood T cell within peripheral mono-nuclear cells to secrete the lymphokines IL-2 or IFN gamma (Chang et al 1982, Vanwauwe et al 1984) to express IL-2 receptors (Mauer et al 1984a, Schwab et al 1985). Analogous to antibody producing B cells CTL also show anamnastic response to subsequent antigen stimulation.

Regardless of the growth factors by which T cell proliferation is mediated, the induction of T cell proliferation by anti-T3 monoclonal antibody is dependent upon accessory cells (AC) (Chang et al 1982, Landegren et al 1984). Four ligands that bind to surface of the T cell can mimic the effect of AC in providing this second function: Moab reactive with Ti, T11, or TP44 as well as IL-1. Functions of AC can be provided by phorbol Myristate Acetate (PMA), a potent activator of Protein Kinase C (PKC) (Hara et al 1985, Weiss et al 1986a). Evidence supporting the role of the T3/Ti complex in PHA and Con A induced T cell activation is the observation that jurkat mutants which fail to express T3/Ti complex lose the capacity to produce IL-2 in response to either PHA or Con A (Weiss et al 1984b, Weiss and Stabo 1984).

The activation of T cells via stimulation of T11 (CD2) molecule has been termed the alternative pathway of human T cell activation to distinguish from the antigen dependent T3/Ti mediated pathway (Meuer et al 1984b, Meuer et al 1989). Recent studies have suggested that LFA-3 may represent the physiological ligand of T11 (Springer et al 1987). Other reports suggest that anti T3 and anti T11 moab can synergise in inducing proliferation of highly purified T cells (Young et al 1986).

The thy-1 molecule is 25-30 KD glycoprotein with two allelic forms expressed on mouse thymocytes, peripheral T cells, fibroblasts, epithelial cells (Reif and Allan 1984).

Studies have shown certain antibodies reactive with thy-1 could be mitogenic for murine T cells (Smith et al 1982) and also induce IL-2R, IL-2 or IFN gamma production (Gunter et al 1984, Macdonald et al 1985).

Several reports have implicated cell surface molecule TP44 of 80-90 KD disulphide linked homodimer expressed on the surface of all T4 and approximately 50% T8 human T cells (Hansen et al 1980, Yamada et al 1985), potentially playing an important role in T cell activation. Moab 9.3 identifies this molecule, can substitute for the second function provided by AC. A murine homologue of this molecule have recently been identified (Nagasawa et al 1986).

T1 (CD5, TP67, LEU 1)

The murine homologue of T1 is Lyt-1 (Ledbetter et al 1981) and anti T1 can augment the proliferative responses and IL-2 production by anti T3 stimulated T cells (Ledbetter et al 1985, Ceupper and Boroja 1986).

IL-1 RECEPTOR

Numerous studies in the past have demonstrated that Accessory Cell function can be in part, reconstituted by soluble factors in the supernatants of adherent cells (Mizel 1982). IL-1 can synergise with PHA or immobilised anti-T3 in the induction of IL-2 production or IL-2R expression (Williams et al 1988). Phorbol ester PMA can substitute soluble factors liberated by AC.

Synergy between Ca^{2+} ionophores and phorbol esters in T cell activation

Combination of Ca^{2+} ionophores and phorbol esters can mimic the effect of activation of antigen, but cannot byepass the requirement for the IL-2 mediated proliferative signal (Weiss et al 1984a, Truneh et al 1985). The activation signal delivered by Ca^{2+} ionophores is an increase in Ca^{2+} concentration. The only known phorbol ester receptor is Protein Kinase-C (PKC). The addition of PMA to intact T lymphocytes leads to the phosphorylation of IL-2 receptor, T200, the Transferrin receptor and T3 chain of T3/Ti complex as well as to the hyper phosphorylation of HLA Class I antigen (Shackelford and Trowbridge 1986, Samelson et al 1985).

The ability of Ca^{2+} ionophore and phorbol ester to deliver activation signals to T cell implies that T cell express cell surface receptors which regulate increases in Ca^{2+} concentration and activate PKC. Three seperate T cell surface structures which appear to signal by increasing Ca^{2+} concentration are T3/Ti, T11 and thy-1. Several lines of evidence suggest that an in increase in Ca^{2+} concentration serves as an intracellular signal for T3/Ti mediated activation (Weiss et al 1984b, Truneh et al 1985). Moab reactive with distinct sites of the T3/Ti complex can induce comparable increase in Ca^{2+} (Oettagen et al 1985, Lanier et al 1986). The conformational changes of T3/Ti may be induced by relevant ligand binding events and this results in receptor mediated signal transduction. Perturbation of T11 also increases Ca^{2+} in human thymocytes and NK cells that do not

express T3. clearly demonstrating the T11 mediated increase in Ca^{2+} does not require the cell surface expression of T3/Ti (June et al 1986, Alcover et al 1986).

Signalling pathway that have been implicated in T cell activation includes changes in nucleotides and opening of voltage gated K^{+} channels (Decoursey et al 1984). Mitogen derived intracellular signal other than Ca^{2+} and PKC, during T cell activation could be possible through the above mechanism. Many genes that are activated during the initial phase of T cell activation are the Oncogenes C-myc and C-Fos (Read et al 1986), the IL-2 receptor and a variety of lymphokines including IL-2 and IFN gamma.

Receptor mediated increases in (Ca^{2+}) and activation of PKC

A signal receptor mediated event, the hydrolysis of a membrane phospholipid, phosphatidylinisitol biphosphate (PIP2), can stimulate both intracellular pathways. The turnover of PIP2 generates two products with second messenger capabilities, Inositol 1, 4, 5 triphosphate (1,4,5 IP3), which mobilises intracellular Ca^{2+} and diacylglycerol (DG) which activates PKC (Berridge 1983, Berridge and Irvine 1984). In vitro DG greatly increases the affinity of PKC for phospholipid and Ca^{2+} allows PKC activation occur at Ca^{2+} concentration with in the intracellular range. Phorbol esters have effect on PKC similar to those of DG, activating PKC at Ca^{2+} concentration in the nanomolar range (Catagna et al 1982).

MECHANISM OF ACTION

CTLs recognise lysable targets by binding to tumor cell surface antigens and class-1 proteins of MHC complex (HLA in man/H-2 in mouse).

The prelytic adhesion of CTL to target cell is absolutely dependent on Mg^{2+} and occurs optimally under defined conditions of temperature and pH. T cell surface antigens T8, Leu3, T4, T11 and LFA-1 in man and Lyt-2, and L3T4 in mouse have been implicated in adhesion process. Non-specific conjugate formation involving these antigens preceds recognition of specific antigen and class specific antigens by T cell receptor (Spits et al 1986). In mice, T cell activation via the receptor results in early phosphorylation of a protein analogous to human T3 chain and triggering lytic machinery (granule exocytosis) of CTLs (Samelson et al 1985). The role of T3 in NK mediated cell cytotoxicity is unclear. It is now clear that binding and lysis (CTL-Target Cell) are two distinct cellular events.

In addition to CTL-TC plasma membrane inter digitations, granules, golgi stacks and actin accumulate in contact region of effector cells with in 15-30 minutes, after conjugate formation. The microtubule organising centre (MTOC) in effector cell is also polarised towards the area of cell contact (Geiger et al 1982). On continuous monitoring of CTL-TC interaction noted that on contact of CTL with TC through leading edge, nucleus immediately moves away from region of contact and the polarised granules fuse with plasma membrane of CTL in the vicinity of attachment area.

Activation of the membrane bound phospholipases A2 activity has been suggested in NK cell mediated lysis (Hoffman et al 1982). Proteolytic enzymes, localised on effector cell surface play a role in cytotoxic reactions mediated by activated peripheral blood lymphocytes (Gayzel et al 1975); CTLs (Redelman and Herdig 1983) and NK cells (Goldfarb 1985). NK cells have demonstrated the presence of a polypeptide of 50 KD (a serine protease) which appears only after conjugate formation (Petty et al, 1984). A parallel study with CTL demonstrated the presence of 28 KD polypeptide at a 300 fold higher concentration in CTL than in helper T cells, B cells and non cytotoxic T cells (Pasternak and Eisen 1985). The specific expression of serine esterases by CTL were further confirmed by isolation by CTL associated with DNA clones (Lobe et al 1986, Gershenfeld and Weismann 1986; Brunet et al 1986). The escretion of hydrolytic enzymes of lysosomal origin at CTL-TC contact site represents delivery of lethal hit (Zagury 1982). It has been shown that CTL killing depends on extracellular chloride (Cl^-).

The lethal hit initiates with progressive series of cytoplasmic convulsive movement in the TC followed by nuclear and plasma membrane blebbing (Zeiosis) with an increase in transmembrane fluxes and loss of cytoplasmic contents. Zeiosis is not seen in antibody-complement-mediated lysis (Sanderson and Thomas 1977). Another novel mechanism proposed is the "internal disintegration mechanism", an autocatalytic cascade within the TC which results in nuclear membrane damage

and DNA fragmentation. These findings were confirmed for cell killing mediated by mouse NK cells (Sears and Christiansen 1985) and alloreactive CTL (Liu et al 1989). This view is justified by the fact that in CTL mediated lysis ^{51}Cr release is preceded by release of nuclear label, suggesting early onset of DNA breakdown (Ucker et al 1987).

Several soluble cytotoxic mediators are involved in cell killing. Lymphotoxin has been found to be cytotoxic for a variety tc tumor cells and involved in CTL killing. CTL are capable of producing lymphotoxin (LT) on stimulation with mitogen (Schmid et al 1986). Tumor Necrosis Factor (TNF) and NK Cytotoxic Factor (NKCF) have been identified in CTLL granules (Young et al 1987). It has been demonstrated that LT containing supernatant derived from CTL lines mediate DNA fragmentation into repeat units of 200 base pairs. Leukoregulin, a lymphokine secreted by NK cells play a role in cytostatic function on a variety of tumor cell lines (Barnett and Evans, 1986).

The expression of cytolytic granule contents appears to be related to action of interleukines. A T cell hybrid (PC60) becomes cytolytic and acquired cytoplasmic granules following induction with a combination of IL-1 and IL-2 (Masson et al 1985).

Isolated granules are capable of mediating the production of tubular lesions on target membrane. Tubular lesions with an internal diameter 150-170 A^{o} are seen at temperature above

$30^{o}C$ and in the presence of Ca^{2+}. The putative Pore-Forming Protein (PFP) from lymphocyte granules involved in tubular lesions produced on TC membrane was named Cytolysin (Henkart et al 1984). Mouse CTL and NK cell pore formers have been named as Perforin 1 and 2 (Podack and Dennert 1983). Human LGL and NK cells were shown to contain Ca related polypeptides in their granules whose biochemical and functional properties were similar to PFP from murine CTL and NK cells (Liu et al 1986, Zalman et al 1986a).

The purified PFP has potent hemolytic activity; one nanogram completely lyses 10^{8} SRBCs. Ca^{2+} is required for this lytic expression. Pure PFP lyses a variety of tumor cells EL-4, J774 M/S 49-1 lymphoma cells, K-562, S-194, YAC-1 and 3T3 cells (Young et al 1986). Amount of protein required to lyse nucleated cells are higher than required for RBC. Pore formation by lymphocyte PFP involves a "barrel stave" model - monomers aggregate like barrel staves surrounding a nucleus pore and grow in diameter. The monomers span the membrane and through lateral movement in the bilayer oligomers so that hydrophobic side of molecule is exposed to lipid membrane moeities, while hydrophilic sides line up the pore interior. Membrane damage by colloid osmotic mechanism ensues. The CTL then releases itself from the target cells by an important but poorly understood process of dissociation and can move on to lyse another target cell (recycling).

Due to close proximity of CTL-TC the released PFP may bind efficiently to target bilayer. Any unbound PFP is inactivated by serum. The requirements of neutral pH and Ca^{2+} for expression of membranolytic activity ensures that PFP is packed in granules in an inactive form as cytoplasmic compartments are acidic and low in free Ca^{2+} may increase to favour degranulation. Non-requirement of Ca^{2+} ion in some cases suggests existence of more than one pathway of cell lysis by CTL (Goldstein 1987). Proteoglycans have been identified in mouse CTLL granules (Young et al 1987).

A general mechanism of killing by target membrane damage may be by pore formation. Pore forming proteins are a very interesting field of study and may be one of the mechanism of cell-mediated cytolysis.

Lymphokine Activated Killer Cells

Beginning in 1980 Reosenberg and colleagues (Rosenberg 1984) described a technique for generating Lymphokine Activated Killer (LAK) cells capable of lysing fresh tumor but not normal cells. The incubation of resting murine splenocytes or human peripheral blood lymphocytes with IL-2 for 3-4 days resulted in generation of cells that can lyse NK cell resistant tumor targets.

The characteristics of LAK cells have been extensively studied. These cells represent lytic population quite distinct from NK cells or cytolytic T lymphocytes and their

phenotypic surface markers are characteristics of non-MHC restricted killer cells. LAK cells can be either CD3 positive or negative are non-adherent and E-rosette negative (Roberts et al 1987) and bear NK like markers such as CD11 and NKH1 (Leu-19) (Lewis et al 1986) IL-2 is the sole signal required for the generation of LAK cells, as demonstrated by experiment using purified homogenous recombinant IL-2 (Rosenberg et al 1984).

Studies have demonstrated that the adoptive transfer of LAK cells in conjunction with IL-2 can mediate the regression of progressive leukemia in mice (Johnson et al 1989), established pulmonary and hepatic metastases in a variety of animal tumor models (Lafreneire et al 1985, Papa et al 1986, Lafreneire and Rosenberg 1985, Mule and Rosenberg 1989). IL-2 appeared to stimulate the in vivo expansion of LAK cells with maintenance of cellular functions (Ettinghausen et al 1985). With the advent of recombinant IL-2, studies with LAK cells alone or with recombinant IL-2 alone were attempted (Lotze et al 1985, Rosenberg 1984). No antitumor responses were seen in any of these early studies using activated killer cells alone. After these phase-I studies, a combination of LAK cells and rIL-2 was administered to patients with advanced cancer and regression of tumor was seen in some patients (Rosenberg et al 1985, Rosenberg et al 1987, Rosenberg 1989). West and co-workers (West et al 1987) reported that the toxicity resulting from continuous intravenous infusion of IL-2 was less than that seen with bolus administration. Many

of the side effects of IL-2 are probably attributable to lymphoid infiltrates in vital organ to a vascular permeability leak induced by IL-2 that leads to fluid retention and interstitial edema which can compromise organ function (Wagstaff et al 1989) and to the ability of IL-2 to lead to the secretion of other lymphokines such as gamma Interferon and TNF which have a range of physiological effects and toxicity of their own (Lotze et al 1985).

While earlier reports had described LAK progenitors as distinct from NK cells (Grim et al 1983, Merluzzi et al 1984, Andriole et al 1985, Ballas et al 1987), it has now been established that $CD3^-$ NK cell population is the chief progenitor of LAK activity (Itoh et al 1985, Ferrini et al 1987, Heo et al 1988, Young et al 1988). However non-NK cytotoxic cells like $CD3^+$, cytotoxic T cells with NK like activity may also contribute to over all LAK activity when unfractioned lymphocyte preparations are cultured with IL-2 (Tilden et al 1987, Lefor et al 1988).

Earlier studies had concluded that LAK cells were non-T/non-NK, based on antibody and complement depletion experiments with anti-NK cell antibody. Later studies performed using Moab to FCR on NK (Leu11a or CD16 marker) and positive selection with fluorescence activated cell sorter, determined that pre-LAK cells were $Leu11^+$ ($CD16^+$) granular lymphocytes with NK activity. Moreover LAK cells are neither

inhibited by cyclosporin A and nor do they express T cell differentiation marker (Grim et al 1985, Lanier et al 1987).

Lymphokines like IL-2 and IFN augment NK activity primarily by acting on mature fully differentiated cells. However there are certain factors like Hemopoietin-1 (H-1), Interleukin-3 (IL-3) and Interleukin-1 (IL-1) which are known to synergize with IL-2 and act at the level of induction of NK cells from less differentiated precursors (Jubinsky et al 1985, Migliorati et al 1987, Migliorati et al 1988). Neither IL-1a or IL-1b alone are able to induce NK cells from bone narrow precursor cells but both synergise with IL-2 to augment independent induction of NK from bone marrow presursors (Migliorati et al 1987, Migliorati et al 1988). Tumor Necrosis Factor also known to synergise with IL-2 to produce LAK cells (Ortaldo et al 1985). Interleukin-4 (IL-4), initially termed BSF-1, is known to act primarily on T cell like precursors of LAK cells than NK cells (Mule et al 1987, Peace et al 1988). rIL-2 stimulated non-adherent splenocytes have been reported to display enhanced motility than unstimulated (Ratner and Hapner 1988). Cytotoxic granules isolated from LAK cells have been shown to mediate Ca^{2+} dependent killing of tumor cell lines K-562, Raji and Daudi and the presence of P1 Perforin has been demonstrated in the granules using anti-mouse P1 and anti-human C9 in Western blot analysis (Lewrey et al 1988). It has been reported that T11, T8 or T3 molecules are not involved tumor cell lysis mediated by LAK effector T cells (Bagnasco et al 1987). IFN gamma has

been shown to reduce the sensitivity of tumor cells to lysis by NK cells and LAK cells. The finding that IFN pretreatment reduces the ability of sub-population to tumor cells to induce LAK effector cells to initiate lysis, supports the view that NK and LAK mediated lysis are closely related.

TUMOR INFILTRATING LYMPHOCYTES

More recent clinical studies have used a different type of activated killer cell called Tumor Infiltrating Lymphocytes (TIL) (Rosenberg et al 1986, Topalian et al 1987). Using IL-2 it is possible to expand the lymphocytes that infiltrate into stroma of solid tumors. In contrast to LAK cells, TIL are classic cytolytic T lymphocytes and show specific cytolytic activity against tumors from which they are derived (Rosenberg et al 1988). Immunobiological studies of the characteristics of the lymphocytes infiltrating human malignant melanoma have (Itoh et al 1988) revealed that majority of cytoxic T lymphocytes generated from melanoma biopsies after IL-2 activation were $CD3^+$, $CD8^+$ and $CD16^-$ autologous specific CTL which recognize as yet unidentified malanoma target antigens by a recognition mechanism which involves the T cell receptor.

In experimental animal tumor models, these TIL can be 50 to 100 times as potent as LAK cells in mediating the regression of micrometastases (Rosenberg et al 1986, Rosenberg 1990). The human TIL can exhibit specific MHC restricted lysis of tumor. Clinical trials with TIL are being pursued in the treatment of advanced cancer in humans and objective

remission in 11 of 20 patients with metastatic melanoma treated with TIL (Rosenberg et al 1988, Rosenberg 1990).

ADOPTIVE IMMUNOTHERAPY

This involves in vitro stimulation and development of cells that are cytotoxic to tumor cells, outside the organism. It is easier to control factors which can interveneon desired reaction in culture bottles. This form of immunotherapy is called adoptive immunotherapy. The transfer to the tumor bearing host of cells with antitumor activity, has substantial therapeutic attractiveness as an approach to treating human cancer (Rosenberg 1984, Rosenberg 1986, Rosenberg et al 1987, Rosenberg 1990). The major obstacle to the development of successful adoptive immunotherapies for treatment of cancer in humans has been the inability to develop immune cells with specific reactivity for human tumors that could be obtained in large enough numbers for transfer to tumor bearing patients. In weak syngeneic system, cytotoxicity mediated by antigen-specific CTL is often difficult to detect and transient. This may be due to low frequency of effector cells or the presence of factors interfering with lytic mechanism (Vasudevan et al 1977). The discovery of T cell growth factor or IL-2 (Morgan and Ruscetti 1976) has facilitated the propagation and cloaning of antigen-specific cytotoxic T lymphocytes. Macrophages and other antigen-presenting cells (APC) are also involved in the generation of CTL. Both mouse and human macrophages, upon appropriate stimulation, release

leukocyte activation factor (IL-1), which stimulates IL-2 producing T cells poised by contact with antigen to secrete IL-2. IL-2 stimulation capacities are neither antigen-specific nor MHC restricted. The primary action of IL-2 is its ability to stimulate the growth of activated CTL that bear IL-2 receptor. IL-2 has been successfully employed in recent years for long term culture of CTL lines, cloned, or uncloned, murine and human derived effector cells produced in MLTC or MLR culture of lymphocytes from tissues of normal, senstized or tumor bearing animals (Kedar and Weiss 1983). IL-2 or TCGF produced by murine T lymphocytes in response to Con A stimulation is a protein of Mol-Wt. 30,000 - 38,000 which promotes the development of functional CTL and continued growth of normal CTL (Gilles and Smith 1977). The mitogenic activity of IL-2 is generally assessed by its capacity to stimulate tritiated thymidine in microcultures of IL-2 dependent CTLL-2 cell line.

A vast variety of literature have recently emphasized the potential use of adoptive immunotherapy by Lymphokine Activated Killer or propagated CTL and proved their efficacy in controlling tumor growth in mice (Rosenberg 1984, Matia and Rosenberg 1986, Rosenberg 1989). The ability of IL-2 to stimulate antitumor activity of NK cells and cytotoxic T lymphocytes in vitro (Taniguchi et al 1983, Grim et al 1983, Domizg 1983, Rook et al 1983, Rosenberg 1985) coupled with successful IL-2 and cell therapy in several animal tumor models have been reported (Mazumdar and Rosenberg 1984, Lafreneire and Rosenberg 1985, Rosenberg et al 1988).

It has been demonstrated that adoptive transfer of specifically senstized T lymphocytes can efficiently mediate the regression of established local and metastatic MCA-105 tumors (Word et al 1988). Lyt-2^+ T cells have been shown to be solely responsible for regression.

The problem associated with lymphokine (Gamma IFN, IL-2 and TNF) therapy in cancer is that high toxic doses of lymphokine must be administered for any significant antitumor results. But recently it has been reported that these lymphokines when used in combination with each other at dosages well below the toxic level, synergise to produce enhanced antitumor effectiveness (Agah et al 1988, Yamasaki et al 1988, Mule and Rosenberg 1989). It has been suggested that IL-2 may provide growth of CTL population and Interferon can accelerate the recruitment of new effectors and activation of lytic process.

HUMORAL IMMUNITY:

This is antibody mediated complement dependent cytotoxicity and the mechanism does not rely on any cells as the effector arm. It was first introduced by Gorer and O'Gorman (1956) and has been used to detect a variety of antigen ranging from histocompatibility antigens to antigens associated with microorganisms. Cells originating from bone marrow which are not processed by the thymus but pass through the Bursa of Fabricius in the case of avians and Bursa equivalent lymphoid organs in the case of mammals (Probably

gut associated lymphoid follicles) are named as B cells. They form 20% peripheral blood circulation. They are seen in the germinal centres of lymphnodes. The immunocompetent B cells when they come into contact with an antigen transform first into blast cells and after divisional maturation into a clone differentiates into plasma cells which then starts to secrete the specific immunoglobulin into circulation. When the antibodies are attached to antigen present on the surface of the target cells the change in the tertiary structure of antibodies will attract and activate the complement components successively. The penultimate component of the complement will produce microscopic holes in the cell membrane. This leads to the osmotic entry of the water into the cell, thereby causing the target cell to swell at first and eventually to lyse.

Unlike the T cells, B cells do not form rosettes with unmodified erythrocytes from sheep, but they have receptor for Fc portion of the immunoglobulin and for activated 3rd component of the complement. Various class of immunoglobulin (Ig) may be expressed on the surface of the B lymphocytes. But any one cell generally expresses only one type of Ig, a notable exception is that IgM and IgD are both present on the surface of the same B cells. By studying the surface Ig on the cells in human embryos it has been found that IgM is the first immunoglobulin to appear on the surface of B lymphocytes. In embryos of 10 to 12 weeks, it is the only immunoglobulin at the surface of the cells. IgD next makes

its appearance followed by a switch of some cells to IgD and IgA. The surface of mice B cells will have the following receptors at different levels of differntiation of B cells. Surface immunoglobulin (SIg), receptor for the third component of complement (C3R), Immune Associated Antigen (Ia) and Plasma Cell Surface Antigen (PCSA). B cell precursor will be SIg negative, C3R negative, Ia negative and PCSA negative. During differentiation process, SIg becomes positive at first. In the next stage, the cell acquires Ia characters, and after the final maturation process the B cell in the peripheral blood becomes SIg, Ia and C3R positive and PCSA negative. When the B cell has finally transformed into plasma cell the surface characters are altered into SIg negative Ia negative C3R negative but PCSA positive. The surface characteristics are made use of in qualitative identification and quantitative isolation of B cells from mixed population. The first DNA re-arrangement occurs soon after a distinctive surface marker Ly-5 is expressed on large bone marrow lymphocytes which have basophilic cytoplasm with numerous polyribosomes. These cells may initiate synthesis of u heavy chains of IgM. 5×10^7 small lymphocytes are made daily in the bone marrow of mouse which migrate via circulation to the spleen. The macro-molecular antigens coded by Ly-5 gene are very immunogenic in rats. The Ly-5 molecules bear several unique epitopes which may be required by cells of humoral immune system. (Landreth et al 1981). Antibodies to Ly-5 inhibit antibody responses to particular antigens in vitro, may be by interfering with

macrophage B cell communication (Wortis et al 1982) and also depress T cell and NK cell-mediated cytotoxicity (Nieminen and Saksela 1986, Lefrancois and Bevan 1985), BP-I antigen is expressed in long term cultured lymphocytes and its density changes with age of culture (Witte et al 1986). The receptor for IL-2 is not restricted to T cells. It is also present on activated B cells. Large, cycling, pre-B cells considered to be in an activated stage are IL-2R positive (Lee 1987). A close functional relationship may exist between B cell Stimulating Factor, BSF-1, and murine Lyb-2 alloantigen (Yakura et al 1986). BSF-2 has been shown to induce final maturation of B cells to IgG secreting cells (Hirano et al 1986). Small amounts of LFA-2 are detectable on murine pre-B cell lines. LFA-1 is involved in leukocyte adhesion and function and may stabilise week recognition bonds between cells of the immune system (Somers et al 1986, Springer et al 1986).

Soluble mediators influence replication and differentiation of B lineage progenitors. The role of IL-3 in maintenance of B cell cultures is highly controversial. Hemopoietin-I (HP-1), a 17 KD molecule synergises with IL-3 and Macrophage growth factor (CSF-1) to influence multilineage progenitors (Stanely et al 1986). 4 distinct molecules were found to influence the final differentiation sequence of B cells. Two small 15 and 17 KD molecules from serum which augment functional maturation and surface IgM expression of normal B cell precursors (Jyonouchi et al 1983). IL-1 Alpha

and IL-1 Beta are influencing B lineage precursor cells (Lee 1987). B cell Growth Factor II (BCGF-II), like BSF-1 (IL-4), is a T cell derived substance which affects mature B cells (Dutton et al 1984).

The B cells form rosettes with sheep erythrocyte when coated with antisheep erythrocyte antisera plus complement. This is called erythrocyte + antibody + complement (EAC) rosette. B cells can be removed by passing through a column containing anti-immunoglobulin. Antibodies can cause target cell destruction by the following mechanisms; (a) Complement fixation (b) attracting K cells, Macophages or granulocytes mediating ADCC and (c) by opsonisation of the target cells and thereby making them more susceptible to macrophage activity, but it has been shown that leukemias are usually very sensitive to the cytolytic action of humoral iso-antibodies, while sarcomas are resistant (Moller and Moller 1962). Thus cells having higher concentration of surface iso-antigenic receptors are more sensitive to cytotoxic antibodies (Moller 1963). The antibodies are supposed to be very useful in preventing the blood born matastasis of cancer. The antibody and complement components are freely available in the blood and so the metastatic malignant cells could be rapidly lysed intravascularly. However the antibodies and complement components may not reach extravascular spaces in adequate quantities and so their cytolytic action will therefore be restricted in the case of solid tumors.

B cells share with other MHC class II expressing cells, the capacity to present antigen to T cells. specific B cells

take up the antigen via their immunoglobulin receptors and present the antigen to antigen specific helper T cells. Several aspects of the above mechanism still remain not clear, largely because the defects in the capacity of antigen presenting B cells to provide adequate stimulus to T cells (Chestnut and Grey 1986).

Of the 15 proteins in the complement (C) system five (C5, 56, C7, C8 and C9) are closely associated with the membrane attack complex (MAC) which causes lesion on the target membrane. The C5 generated in the vicinity of biological membrane combines with C6 to activate terminal lytic lesions. It is suggested that the hydrophobic regions insert into lipid bilayer to assemble the hydrophilic interior transmembrane channel. Polymerisation of C9 components can form lesions in the lipid membrane, but hemolysis is seen only when C9 is bound to C5b-8 complex (Young et al 1986). The minimial C9 required to form a functional lesion is one molecule. Oligomerisation of C9 allows formation of larger circular lesions.

The structural and functional similarities between complement and lymphocyte mediate killing suggest that structural effector proteins of these two systems are related (Lachman 1986, Young and Cohn 1986a). C9 has a number of bio-chemical and functional features which resemble Perforin/PFP. Rabbit polyclonal antisera against human C9 have been used in affinity purification of PFP localised in

human LGL and NK cells (Zalman et al 1986a). PFP and poly C9 form ion non-selective channels which remain permanently open, i.e., stable and voltage resistant channels are formed. C6 is a serine protease and its activity is linked with formation of C5b-C9 complex (Kolb et al 1982).

The observed immunological similarities between PFP and several C components of MAC reveal the possibility that lymphocyte PFP may be another member of the complement supergene family (Campell et al 1986). All these effector molecules may have emerged from the same ancestoral protein during evolution, but diverged and became specialised later to perform either cellular or humoral response.

In many tumors evidence of host humoral response especially to melanoma, neuroblastoma and osteogenic sarcomas have been reported (Ferrone et al 1977, Curry et al 1979, Hellstrom 1968).

The kinetics of antibody complement mediated cytolysis were studied in Babl/c mice bearing syngeneic spontaneous adenocarcinoma (ADK-IE) and peak activity has been observed at 10 days after tumor transplantation. (Landolf et al 1971). Three monoclonal antibodies, designated as H1, H2 and H3 raised against human null Acute Lymphoblastic Leukemia have shown to mediate complement dependent cytotoxicity against all human nucleated cells except for HLA lacking Burkitt's lymphoma cells & K-562 (Hartua et al 1984). Synergestic co-operation of four antitumor monoclonal antibodies has been

demonstrated in vitro complement dependent cytotoxicity assay (Maria et al 1984). Increased susceptibility of tumor cells to complement mediated lysis has been reported after treatment with lecithin. (Shlymour et al 1982).

In recent times monoclonal antibodies have found extensive application in biomedical research in the diagnosis and management of various malignant deseases. The application of monoclonal antibodies in understanding the biology and differentiation of benign and malignant breast lesions have been extensively reviewed by several workers (Arklie 1981, Colcher et al 1984). Monoclonal antibodies raised against mammary tumor antigens, cell membrane as well as human milk fat globule membrane representing functional cells of the gland have been used to detect differentiation antigens of mammary epithilium, there by identifying breast tumors. (Hilgers et al 1981, Menard et al 1983 and Hilkens et al 1984). Monoclonal antibodies 115D8 and E29 to epithelial antigens are extremely useful in the immunocytochemical diagnosis of all carcinomas, differentiating them from tumors of non-epithelial origin (Cordell et al 1985). A battery of monoclonal antibodies generated against mammary tumor antigens have found wide application and some are useful in identifying proliferating Cells at different phase of cell cycle and determining the mitotic activity (Kuenen-Boumeester et al 1988). Monoclonal antibodies raised against malignant gliomas have been shown to target specifically to tumor microvessels,

which is an interesting target for tumor therapy (Watkin et al 1989). In order to increase the cytotoxic effect of antibodies, they were combined with drugs which block the division of the cells (cytoreductive and antimitoic agents like Methorexate and Adriamycin), with radioactive substances, or with powerful toxins like toxin-A of Ricin. These remote controlled missiles present some advantages, but often suffer from great limitations. Antibodies coupled to Ricin, because of their great potential toxicity, have been limited to in vitro expulsions of bone marrow in human beings. Ritze and Colleagues (1982) have successfully used monoclonal antibody J5 to remove residual Acute Lymphoblastic Leukemia (ALL) Cells in vitro from autologous bone marrow, prior to re-infusion into patients primed with ablative chemotherapy and total radiation.

Another novel approach is the in vivo use of Streptavidin conjugated antibodies followed, after an appropriate period of time, by radioactive biotin either for imaging or for therapy. Biotin has extremely high affinity for Streptavidin, at the same time is small enough molecule that can diffuse rapidly through the most tissues in the body (Hanowitch et al 1987, Pagnelli et al 1988). A different rather interesting strategy has recently been proposed (Bagshaw et al 1989). In this system a prodrug is activiated at the tumor site by a non-mammalian enzyme conjugated to antibody. The prodrug is highly toxic, relatively stable and only gets activated by the target enzyme on tumor sites.

The use of 131 I labelled antibodies and metal chelated antibodies has been extensively studied by several workers using experimental lymphomas and other animal models for gamma camera imaging (Scheenberg et al 1982, Larson et al 1983).

The high ability of SWA-II murine monoclonal antibody to localise small cell cancer xenograft has been confirmed, by gamma scintigraphy. (Smith et al 1989). Extensive studies carried out on radioimmuno detection and localisation using Ga-67 incorporated with antibody to DLA antigen as liposomes revealed that anti-DLA antibody significantly improved the tumor take of Ga-67, thereby facilitating better visualisation of the tumor. Authors have attributed the enhanced tumor targetting to the specificity of anti-DLA antibody (Udayachander et al 1987). Imaging and scintiscan studies conducted using anti-DLA antibody and non-specific mouse immunoglobulin demonstrated that with anti-DLA antibody there was maximum uptake of Ga-67 by tumor providing the best images (Udayachander et al 1989).

The enhanced efficacy of specifically targetting antineoplastic drug, N-Acetyl-Melphalan monoclonal antibody conjugates against both subctuaneous and ascites murine thymomas have been reported (Smith et al 1988). Selective in vivo antitumor effects of monoclonal antibody with specificity against Ia bearig B cell lymphoma has been reported. Multiple doses of anti-Ia monoclonal antibody could produce regression in 50% of the animals bearing B cell lymphoma (Bridges et al

1987). Growth of Daunorubicin (DNR) resistant varient (HH 66 DR) of Alpha-Fetoprotein (AFP) producing rat ascites hepatoma (AH 66) has been found to be inhibited by specific antibody against highly purified rat AFP (Ohkawa et al 1989). Monoclonal antibody 3f 8E3 (IgG 3k) against human squamous cell carcinoma associated antigen, reacting with squamous cell carcinoma of oral cavity, larynx, oesophagus, lung and uterine cervix has been reported. (Tatake et al 1989). Organ specific monoclonal antibody G9 raised against colon carcinoma has been reported to exhibit high sensitivity and specificity in immuno assays (Gold et al 1989). It has been suggested that potential value of external irradiation to increase monoclonal antibody uptake by tumors is governed mainly by increased vascular permeability of the tumor soon after irradiation exposure (Kalofonos et al 1990).

A crucial and yet not unexpected problem that has been identified when using rodent monoclonal antibodies in humans is the sensitisation of the host to the administered xenogeneic protein (Courtney et al 1986, Schroff et al 1988). Immunisation of patients would cause immune complex disease and also abrogate any therapeutic effect due to rapid clearance of administered antibody. However there have been several approaches to overcome this problem. The first is to use existing rodent antibodies but to delay and reduce the intensity of human anti-mouse antibody responses by immuno suppressive agents such as Cyclophosphamide and steroids

(Begant 1989). The second approach is to try and induce specific unresponsiveness to rodent immunoglobulins by the use of immunosuppressive antibodies (Benjamin and Waldmann 1986) or by coupling substances such as PEG which apparently may convert xenogeneic monoclonal antibodies to specific tolorogens (Sehen 1989). A third way is to use human monoclonal antibodies produced from Epstein Barr Virus transformed human B lymphocytes or from human-mouse hybridomas (Sikora et al 1982). Human monoclonal antibodies to mammary carcinoma cells, have been generated using sensitized lymphocytes isolated from metastatic lymph nodes of patients resulting in human-human or human-mouse hybrids. These human monoclonal antibodies binding to malignant mammary epithetial cells may be used in radiolocalisation of tumors in vivo (Imam et al 1985). Recently a new technique has emerged exploiting the immunologic idiotype net work yielding high affinity and high specificity human monoclonal antibodies (Ritter et al 1987). Moreover by using recombinant DNA technology, it is now possible to engineer antibodies where only the antigen biding site is defined by mouse gene sequences and the rest of the molecule is human (Reichmann et al 1988). Anti-Carcino Embriyonic Antigen(CEA) recombinant mouse human chimaeric monoclonal antibody used in vivo diagnosis by immuno-scintigraphy of human colonic carcinoma has been reported to overcome some of the difficulties associated with repeated use of non-human Ig in human patients (Hardmann et al 1989).

Hybrid antibodies with dual specificities can be produced by the cell fusion of two immunoglobulin producing cell lines (Milstein and Gaello 1983, Clark and Waldman 1989). Of greater interest may be the ability of these bispecific antibodies to induce potent tumor killing by activiated T cells if these bispecific antibodies can crosslink T cells and antigens on the surface of tumor cells (Clark et al 1988, Clark and Waldmann 1989). A similar type of monoclonal antibody with dual specificity, which can trigger the lytic machinery of Gamma-Delta positive T cells in conjunction with recognition of ovarian carcinoma antigen has been reported (Ferrini et al 1989). A newly constructed CD4 immuno-adhesion molecule containing the GP 120 binding domain of the receptor for Human Immunodeficiency virus has been demonstrated to block HIV-I infection to T cells and monocytes probably through classical pathway of complement, or Fc receptor mediated phagocytosis or lysis by Killer cells (Capon et al 1989).

Levamisole:

Levamisole is the levorotatory isomer is tetramisole which is racemic 1, 2, 3, 5, 6, -- tetrahydro-6 phenyle--imidazole (2, 1, 6) - thiazole. It is also a deploarising neuromuscular blocking agent and inhibitor of cholinesterases and this property may be associated with pronounced anti-helmenthic activity.

Levamisole has attracted much attention because it appears to stimulate the immune responses. It has been reported to stimulate continuous delayed hyper sensitivity reaction in anergic elderly subjects (Verhaegen et al 1973) and patients with cancer and Hodgkins discease (Binominov and Ramot 1978). In patients and animals with hypofunctional cells Levamisole restores E rosette formation and boosts nucleeic acid and protein synthesis by T cells in response to antigenic stimulation. It stimulates differentiation of T helper, T suppressor/Cytotoxic cells and also stimulates lysosomal activity and plaque formation (Symoens and Rosenthal 1977). The drug can be immunosuppressive, as well as immunostimulatory depending on the dose and time when it is given in relation to antigenic challenge. Macrophages which are important in the afferent and efferent limb of immune response are probably involved in some of these actions of Levamisole. The precise mechanism of Levamisole's action on the immune response is not known but there are several reports regarding it's influences on T-lymphocytes and T-B lymphocytes interactions (Ramot and Biniminov, 1976) Renoux and Renoux (1974). An impressive body of evidence implicates influences exterted upon macrophage-monocyte population (Chattopadhyay et al 1986, Bhattacharya et al 1986).

Wanebo et al, 1978 studied the effect of Levamisol as a surgical adjuvant in the treatment of squamous cell carcinoma of the head and neck and a significantly decreased recurrence

rate was observed in Stage II patients with oral cancer treated with Levamisole. Chemoimmunotherapy with levamisole or with BCG plus Levamisole has been reported to induce prolonged remission of metastatic breast cancer patients (Hortobagi et al 1978).

Emblica Officianalis:

Emblica officianalis (Indian Gooseberry) phyllanthus emblica Linn (Euphorbiacea) is a small or medium sized deciduous tree often cultivated in garden. Amalaki an Ayurvedid drug prepared from the pericarp of dried fruits of plant is used in the treatment of ulcer and non-ulcer dyspepsia (Chawla et al1982) and gastro intestinal disorders (Malhotra 1984, Saxena and Jain 1982, Sethi et al 1982). It is a cardiotonic, cardiac refrigerant and employed in palpitation when it is due to anaemia (Tariq et al 1977, Shanmugasundaram et al 1983 , Thakur 1985). The drug has been reported to have pronounced antiviral properties (Antarkar et al 1980, Babbar 1982) and has been administered against acute viral hepatitis and other liver disorders (Singh et al 1983, Handa et al 1986, Suresh Kumar et al 1987). It is also used as aphrodisiac, antipyretic (Ahamed 1976, Ghosh 1976), anti-diabetic (Saley et al 1982, Upadhyay 1984) mild antibacterial agent (Rastogi et al 1982) and pronounced expectorant (Khorama et al 1960). There are several reports regarding the anti-coagulating property of the drug (Tawade, 1980, Rajyalaxmi 1982). Anti-cancer properties of the drug has been extensively

studied (Hartwell 1969, Nisteswar 1988). Emblica officianalis is one of the constituents of triphala which possesses antihelminthic and laxative properties.

The fruit is a rich source of ascorbic acid (Vit. C) (Roy et al 1987) and also contains phyllemblin, phyllemblic acid, ellagic acid, emblical phyllantidine, phyllantin acid and Tannin.

ASSESSMENT AND AUGMENTATION OF IMMUNOLOGICAL STATUS DURING TUMOR DEVELOPMENT

Submitted to the University of Calicut
for the Degree of

Doctor of Philosophy

In Immunology (Faculty of Medicine)

By

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1990

MATERIALS & METHODS

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MATERIALS

Animals and tumor model: Inbred Balb/c mice were maintained in the animal house and provided standard mice feed and water ad libitum. Dalton's Lymphoma ascites (a murine T cell lymphoma, Klein and Klein, 1951) obtained from Chittaranjan National Cancer Research Centre, Calcutta, India (Courtesy: Dr. U. Chattopadhyay and Dr. Roy Chaudhury) was maintained in ascites form by intraperitoneal passages. Balb/c mice, 6 to 8 weeks old, were given 10^7 Dalton's Lymphoma cells intraperitoneally. The tumor bearing mice were sacrified at different intervals of tumor growth. The investigations were conducted on spleen cells, peritoneal macrophages, Large Granular Lymphocytes (LGL) and serum separated from blood. Normal Balb/c mice, tumor bearing control and drug treated Balb/c mice were used for monitoring different immune parameters. (The details of these assays are given in the Methods Section).

The in vitro assays used to study the different immunological parameters are as follows:-

1. Short term ^{51}Cr release cytotoxicity assay for estimation of NK, ADCC, Macrophage mediated ADCC (MMADCC) and CTL activity.

2. Single cell cytotoxicity or conjugate assay for estimation of percentage active Killer cells.

3. Generation of NKCF by co-culture of effector (LGL) and target cells and estimation of the NKCF lytic potential.

4. Enumeration of T helper (T_H) and T suppressor (T_S) by immunorosetting technique using anti-ox IgM and anti-ox IgG sensitized ox RBC.

5. In vitro assessment of immunopotentiating action of drugs using jerne's plaque assay.

6. Antibody complement mediated cytotoxicity assay for the estimatimation of antibody response.

7. CTLL-2 proliferative assay of Interleukin-2 using tritiated thymidine.

Drug treatment schedule:

a) Levamisole: In one group of Balb/c mice, a single dose of 15 mg/kg body weight of Levamisole suspended in saline was given i.p. following tumor inoculation. In another group 1.5 mg/kg body weight of the drug was given i.p. and all parameters were studied.

b) Emblica officianalis: 20 mg/kg body weight of dried powder of the fruit suspended in distilled water was fed orally on every day following tumor inoculation, till the death of the animals and all immune parameters were studied.

Reagents:

1. Phosphate buffered saline (PBS) pH 7.2.
2. Reagents for cell culture:
   a) RPMI-1640 - Himedia Laboratories, Bombay.
   b) Fetal Calf serum (FCS) Sigma Chemicals, USA.
   c) Newborn calf serum (NBS), Hysel, India.
   d) L-Glutamine, Sodium Pyruvate, Mercaptoethanol, BDH, England.
   e) Antibiotics. Pencillin, Streptomycin, Gentamycin, Themis Chemicals, Bombay.
   f) Hepes (N-(2-Hydroxy ethyl) Piperazine-N E2-ethane sulphonic acid) p_H 6.8-8.2 Sigma Chemicals, USA.
3. Reagents for cytotoxicity assay:

a) Sodium chromate (^{51}Cr) obtained in the form of saline injections, Bhabha Atomic Research Centre, Bombay (specific activity/ml approximately 1 mCi, chromium content 10.50 ug/ml).

TARGET CELLS

Natural killer sensitive cell line K-562 and YAC-1 obtained from National Facility for Animal Tissue and Cell Culture (Govt. of India, Pune) was propagated as suspension cultures in RPMI-1640 plus 10% FCS.

Dalton's Lymphoma ascites tumor was maintained in ascites form by intraperitonial passages. Sarcoma-180 and Ehrlich's ascites cells were also maintained in ascites form in Balb/c mice.

Sheep RBC: Target Cells for ADCC was collected in Alsevier's solution, kept at $4^{o}C$ and used for not more than one week.

c) For single cell cytotoxicity assay on poly-L-lysine coated cover slips. Poly-L-Lysine (PLL): A stock solution of 0.2 mg/ml of Poly-L-Lysine (Sigma, USA) was made in PBS, pH 7.2, and stored at $4^{o}C$. It was diluted to a final concentratioon of 2 ug/ml and used in the conjugate assay. Trypan Blue 0.1% W/V solution (Fluka, Switzerland) was made in PBS and used for checking the viability of the cells.

d) For NKCF production and assay: RPMI-1640 containing final concentration of 1% Bovine Serum Albumin (Sigma) was used.

e) For antibody complement mediated cytotoxicity.

Antibody: On diferent days after tumor transplantation, the mice were sacrificed and blood collected by heart puncture, the seperated serum heat inactivated at $56^{o}C$ for half an hour and used.

Complement: For complement source young healthy rabbits were bled by ear venipuncture. Serum separated at $4^{o}C$ and stored in vials at $-100^{o}C$.

4. For surface phenotypic characterisation of effector CTLs. Anti-Lyt-2^{+} monoclonal antibody Sera-Lab, England.

5. **Reagents for Adoptive Immunotherapy:**

Cyclophosphamide (Endoxan-Asta; Khandelwal Laboratories Ltd., Bombay).

6. **Reagents for Production and Standardisation of Crude IL-2**

a) Reagents for production of rat growth factor.

RPMI-1640 containing 1% FCS, $5x10^{-5}$ M Mercaptoethanol, 100 units/ml penicillin, 100 ug/ml Streptomycin, 5 ug/ml Con A (Sigma Chemicals, USA).

b) Reagents for IL-2 assay:

Cell line CTLL-2 was grown in the presence of IL-2, 10-100 units/ml (or 1:10 dilution of rat spleen supernatant of 72 hrs in 5 ug/ml of Con A). RPMI-1640 containing 10% FCS $5x10^{-5}$ Mercaptoethanol, Pencillin 100 units/ml, streptomycin 100 ug/ml was used for culture.

IL-2 standard, Biological Response Modifiers Program (BRMP) National Cancer Institute, Maryland, USA interim reference reagent, (500 units/ml). ^{3}H-Thymidine (1mCi/ml) was obtained from Bhabha Atomic Research Centre, Bombay.

7. **Reagents for Cell Separation:**

a) **Percoll:** Sterilised percoll solution (Pharmacia, Sweden) was stored at 4^{o}C. Iso Osmotic Percoll (IOP) was prepared by mixing 90ml of Percoll and 10 ml of 10x Saline. The different

dilutions of Percoll for gradient were made with IOP and RPMI-1640.

b) **Nylon Wool:**

Sterilised Nylon Wool (Fenwall Laboratories, USA) was placed in 5ml syringe, washed twice with culture medium and kept at 37°C for 1 hr before use, immersed in culture medium.

c) **Tris-ammonium chloride buffer (pH 7.2)**

1. Ammonium Chloride 0.83 gm
2. Tris 2.06 gm
3. Distilled water upto 100 ml
4. pH adjusted to 7.2 - 7.4 with concentrated HCl.

d) Ethylene Diamine Tetra Acetic Acid (EDTA), BDH, England.

Alsevier's Solution (pH 6)

1.	Dextrose	2.05 gms.
2.	Sodium citrate	0.8 gms
3.	Sodium chloride	0.42 gms
4.	Citric acid	0.05 gms
5.	Distilled water upto	100 ml

Methods:

1. **Separation of Cells**

a) **Single cell suspension:**

Spleen from Balb/c mice was removed asceptically into a petri dish containing cold RPMI-1640 + 10% Newborn calf serum.

The spleen was gently teased with forceps to remove capsule and connective tissue and then very gently pressed through a sieve with plastic plunger of a disposable syringe in the presence of cold medium. The cell suspension was centrifuged at 400g for 5 minutes. The sediment resuspended and left standing at $4^{o}C$ for 45 minutes. The supernatent cells were taken, RBC lysed by treating with cold Tris-ammonium chloride buffer, pH 7.2 at $4^{o}C$ for 10 minutes. The cells were washed in medium containing 10% newborn calf serum and viability checked by trypan blue exclusion method.

b) **Removal of adherent cells:**

Adherent cells were depleted by incubating spleen cell suspension in a plastic petri dish at $37^{o}C$ for 1 hour. After incubation non-adherent cells were collected and washed in the medium.

c) **Removal of B cells:**

The method of Julius et al (1973) was followed. Non-adherent cells were layered on to a nylon wool immersed in culture medium in the 5ml Syringe. Incubation was done at $37^{o}C$ in a 5% CO_2 atmosphere. This process eliminates B cells and small amount of adherent cells remaining in the cell suspension. After incubation nylon wool was washed extensively with culture medium to collect the cells which were consisting mainly of T cells and LGL. They were further fractionated on Percoll discontinuous density gradient.

(d) **Isolation of LGL on discontinuous Percoll density gradient:**

(1) Preparation of gradient: The method of Timonen et al (1981) was followed. The IOP was mixed in different proportions with culture medium to obtain seven different concentrations of Percoll starting from 38.6% to 70.1%. Each fraction varied from the next by 2.5% increments. The refractive indices for 55% and 40% Percoll at 25°C were 1.3454 and 1.3432 respectively (Timonen et al 1981). The gradient was carefully layered in 15 ml test tubes under sterile conditions. The volume of each fraction was 1.5 ml, thus making the final volume of 10.5 ml for 7 fractions.

(2) Fractionation of cells by discontinuous Percoll density gradient centrifugation: After the careful layering of the gradient 2.5×10^7 nylon wool purified lymphocytes suspended in 1.5 ml of culture medium were placed on the top of the gradient. The tube was spun at 550g for 30 minutes at room temperature. Cells from 7 layers were then collected from the top with a pasteur pipette. The cells were washed once in medium containing 2% FCS, two more with normal saline in order to remove Percoll. The recovery of the cells was more than 75% and viability was 95% as judged by trypan blue exclusion.

(e) **Study of Morphology:** The cells from different Percoll fractions were suspended in medium RPMI-1640 at concentration of $2.5 \times 10^4/0.1$ ml and cytocentrifuged for 5 minutes at 125 g to uniform smear on glass slides. The cells were air dried

and fixed in Methanol for 15 minutes. They were stained with Giemsa for 20 minutes and washed with distilled water. The cells were then mounted in DPX (BDH) and the slides were scanned for the number of LGL under oil immersion lense of 100x magnification.

2. **Isolation of Peritoneal Tumor Associated Macrophages:**

The method described by Bhatacharya et al (1984) was used. The DLA tumor was grown in a group of 8-10 weeks old Balb/c mice by intraperitoneal inoculation of 10^7 tumor cells. For preparation of TAM, peritoneal cells were collected at different days of tumor growth from the DLA bearing mice and incubated in glass petri dishes at 37^oC for 45 minutes. The non-adherent cells were removed by repeated washing of the dishes. The adhered TAM were detached with 0.02% EDTA and were suspended in medium RPMI-1640 containing 20 mM Hepes and 10% FCS. These cells were morphologically identified as macrophages by staining with Giemsa, non specific esterase activity and Fc receptor positivity.

3. **In vitro generation of CTL:** Spleen cell suspension was prepared in RPMI-1640 containing 10% FCS and erythrocytes were lysed by treatment with Tris-ammonium chloride buffer (pH 7.2). After washing, viable cells were resuspended in complete medium composed of RPMI-1640, Pencillin (100 units/ml), Streptomycin (100 ugm/ml), 2 mM L-Glutamine, 1mM sodium-pyruvate, $5x10^{-5}$M Mercaptoethanol and 10% FCS. Mixed lymphocyte tumor cultures (MLTC) were set up in 15 ml culture vials, with $1x10^7$ responder spleen cells and $1x10^5$ Mitomycin-C

treated DLA cells in complete medium. After 4 days incubation at 37°C in a humid atmosphere of 5% CO_2 and air, cells from MLTC were harvested, washed, viability checked by trypan blue exclusion method and again resuspended to concentration of $1x10^6$ cells/ml and assayed for cytotoxicity against ^{51}Cr labelled DLA targets.

4. In vitro expansion of effector cells with human rIL-2:

(a) Expansion of CTL with human rIL-2: CTL generated after 4 days of MLTC were harvested, washed and again reuspended in complete medium containing 200 IU/ml of human recombinant IL-2. This was kindly donated by Dr. Jeffrey Rossio of National Institute of Health, USA. Cultures were further incubated at 37°C in CO_2 atmosphere for 7 days. After incubation cultures were harvested, washed 3 times in complete medium and assayed for cytotoxicity against ^{51}Cr labelled DLA targets.

(b) Activation of LGL with human rIL-2: LGL seperated by discontinuous density gradient of Percoll was incubated in medium in the presence of 200 units/ml of human recombinant IL-2 for 72 hrs. at 37°C in 5% CO_2 air and assessed for cytotoxicity against ^{51}Cr labelled K-562 targets.

5. ^{51} chromium release cytotoxicity assay:

A short term (4 hrs.) ^{51}Cr release assay (CRA) was performed for estimating the NK, ADCC, CTL and Macrophage mediated ADCC. (Perlmann and Perlmann 1970, Brunner et al 1976).

(a) Labelling of target cells: Approximately $1x10^6$ cells/ml

target cells (YAC-1, K-562, DLA, S-180, Ehrlich's ascites, or sheep RBC) were incubated with 100 uCi of ^{51}Cr in 200 uL of medium for 45 minutes at 37°C. The labelled cells were washed three times and used as target cells at concentration of 10^4 cells/0.1 ml medium/tube.

(b) Effector cells: Spleen cells, purified LGL, Tumor associated macrophages (TAM) or CTL were used as effectors cells.

(c) Assay of NK, ADCC, CTL and MMADCC. A short term ^{51}Chromium release assay was performed in RIA tubes. The labelled target cells were incubated with different concentrations of effector cells to obtain effector target ratios of 10:1, 30:1 and 100:1 in 1.1 ml medium. All tests were done in triplicates. Spontaneous release of ^{51}Cr was determined by incubating target cells with medium alone and total release by incubating in 1 N HCl and the tubes were incubated at 37°C for 4 hrs. Finally volume made upto 2 ml with medium, centrifuged and 1 ml of the supernatant counted in a gamma ray spectrometer. Percentage of specific cytolysis calculated as

$$\% \text{ specific lysis} = \frac{\text{Experimental release - spontaneous release}}{\text{Total release - spontaneous release}} \times 100$$

The stanldard error for triplicate assay was always less than 10%. Spontaneous release was less than 10% of the total release for 4 hrs. assay. Data were analysed with student's 't' test.

6. **Single cell conjugate assay:**

Poly-L-Lysine coated coverslips were made as previously described by vargas-Cortes et al (1983). 22x22 mm coverslips were thoroughly washed with 70% ethanol, placed on plastic petri dishes and covered with 0.5 ml of a solution of 2 ug/ml) Poly-L-Lysine hydrobromide (PLL) in PBS. The coverslips were incubated at room temperature for at least 45 minutes and were washed with PBS to discard unbound PLL. 100 ul of the effector cell suspension was mixed with an equal amount of target cells ($1x10^5$ cells), pelleted by low speed centrifugation in conical plastic tubes for 4 minutes. The cell pellet was incubated for 15 minutes at 37^oC in a 5% CO_2 atmosphere. After incubation, most of the supernatant was aspirated anmd pellet resuspended in serum free medium (RPMI-1640 0.5 ml/tube). The mixture was plated on the PLL coated coverslips and incubated at room temperature for 20 minutes. The coverslips were then transferred to 37^oC incubator for 4 hrs. Control coverslips containing only target cells were prepared in the same way. After incubation, control and experimental coverslips were washed by drop wise addition of medium. The medium was decanted, the coverslips were stained for 3 minutes with trypan blue (0.1% in PBS) and washed. They were fixed with 1% formaldehyde in PBS. After washing, the coverslips were inverted to microscope slides to be scored under the light microscope.

Calculations:

The number of target binding lymphocytes and their cytotoxicity was calculated as previously described by Ulberg and Jondal (1981). Spontaneously dead target cells were determined in control slides by scoring the percentage of dead cells assessed by trypan blue staining. The proportion of lymphocytes formig conjugates with target cells (TBC) was determined by counting the frequency of lymphocytes binding to K-562 target. At least 200 lymphocytes were counted. The percentage of target cell binding lymphocytes that are cytotoxic was determined by the relation A=B-(BxC) were B is the percentage of conjugates containing dead target cells (100 conjugates counted) and C is the fraction of spontaneously dead targets. The percentage of active killer cells present in the LGL population is calculated by the equation (AxD) = 100 where D is the percentage of total lymphocytes binding to target cells (TBC). V_{max} is the number of target cells killed when the assay system is saturated with target cells. It has been calculated by applying the Michaelis-Menten laws for enzyme kinetics to the effector-target immune reaction (Ullberg and Jondal 1981. According to Ulberg and Jondal, it is possible to get a rough estimation of V_{max} for effector cells by using a effector target ratio 5:1 ($5x10^4$ lymphocytes and 10^4 target cells in triplicates). When chromium release assay is done the V_{max} is calculated by using the formula, $V_{max} = 1x4x10^3 + 4.2x10^2$ x (percentage of ^{51}Cr release at 5:1 ratio) (Ulberg and Jondal 1981).

Estimation of maximal recycling capacity (MRC)

MRC was calculated by combining the data from the ^{51}Cr release assay and conjugate assay. Thus V_{max} value was divided by the absolute number of killer cell, ie. the percentage of active killer cells multiplied with the number of effector cells in the V_{max} ($5x10^4$). MRC is an estimation of the average number of target cells that an active NK cells can kill in 4 hrs under optimal condition.

7. **NKCF production and assay:**

(a) Production of cell free supernatents containing NKCF: The method of Wright and Bonavida (1985) was employed $5x10^4$ K-562 or YAC-1 cells were co-cultured in 2 ml RPMI-1640 containing 1% Bovine Serum Albumin at 37°C in 5% CO_2 for 48 hrs. After incubation, the cells were spun down, supernatant was filtered through a 0.45u filter and stored at -20°C until assay for cytotoxicity.

(b) Microsupernatant assay: Cell free supernatant were assayed for cytotoxicity as described by Wright and Bonavida (1983). 10^4 YAC-1 or K-562target cells in 50 uL culture medium were incubated with 150 uL of test supernatants in 90 well sterile micro titre plates (NUNC). Cultures were set in triplicates, control cultures contained 150 uL medium instead of test supernatent. After 48 hours incubation at 37°C in 5% CO_2 cell viability was determined by trypan blue exclusion.

Viability of YAC-1 or K-562 in control culture, was always greater than 90%. The percentage of cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\% \text{ viability of control} - \% \text{ vitability of test}}{\% \text{ viability of control}} \times 100$$

8. Preparation of Rabbit anti-sheep RBC antibody (Amboceptor)

Anti-SRBC antibody was prepared by immunizing young rabbits with 50×10^6 SRBC/ml of saline with complete Freund's Adjuvant at weekly intervals (intramuscular or subcutaneous). Bleedings were done at 2-3 weeks followig initial immunisation. Sera seperated and heat inactivated at 56°C for half an hour to eliminate intrinsic complement activity and used for the titration of minimum haemolytic dose.

Determination of minimum Haemolytic DOSE (MHD):

It was determined by incubating 2.5% washed SRBC with serial dilution of anti-SRBC antibody (Amboceptor) in saline (1:10 to 1:6000), 0.2 ml of the preparation was incubated with fresh rabbit serum (1:1) diluted at 37°C for 30 minutes. Then the tubes were centrifuged and optical density of the supernatant was measured in a spectrophotometer and percentage of lysis was calculated. The MHD was calculated as that dilution of Amboceptor demonstrating 50% lysis of the senisitized SRBC. SRBC sensitized with 2 MHD was used in further cytotoxic studies.

9. Enumeration of T cell subsets (T_H and T_S cells)

Anti-ox IgM and IgG were prepared by immunising rabbits with 10% Ox RBC suspension in saline with complete Freund's adjuvant at weekly intervals for 2 months. Earlier bleedings were done at 2-3 weeks and late bleeding at the end of 2 months following initial immunization. Sera seperated and heat inactivated at 56°C for half an hour. The crude early antiserum was purified for IgM on Sephadex G-200 and late anti serum on DEAE Column for IgG. The IgG and IgM fractions were evaluated in an agglutination assay. Sub haemagglutinating dose was used for sensitization of Ox RBC.

(a) T cells with IgM receptors (T_H)

Equal volumes of ($4x10^6$ cells/ml) nylon wool non-adherent spleen cell suspension and 1% anti-Ox IgM sensitized Ox RBC were mixed, centrifuged at 100 g for 5 minutes and incubated at 4°C for 1/2 an hr. The pellet after gentle resuspension was counted for the number of rosettes forming lymphocytes.

$$\% \ T_H \text{ cells} = \frac{\text{total number of rosettes}}{\text{total number of rosettes + free lymphocytes}} \times 100$$

(b) T cells with IgG receptor (T_S cells)

$4x10^6$ cells of nylon wool non-adherent spleen cell suspension was mixed with 1% anti-IgG sensitized Ox RBC and the number of rosettes counted as in the case of IgM rosettes.

10. **Antibody-complement mediated cytotoxicity:**

Mouse serum was heat inactivated at 56^{o}C for 30 minutes to eliminate intrinsic complement activity. Dalton's lymphoma ascites cells were washed three times in RPMI-1640 viability checked and finally suspended to a concentration of $1x10^{5}$ cells/ml in the medium. Antiserum was serially diluted in culture medium so as to get 1:1, 1:2 and 1:4 dilutions of antibody. 0.1 ml of the antibody was added to each tube containing $1x10^{4}$ Dalton's lymphoma ascites cells in 0.1 ml of RPMI+10% heat inactivated serum. 50 uL of 1:1 diluted fresh rabbit serum was added as complement source. Final volume in all tubes was adjusted to 2 ml. Controls were kept without antibody + complement, with antibody + without complement and with complement + without antibody. All the tests were done in triplicates. The tubes were incubated at 37^{o}C for 2 hrs and cytotoxicity was assessed by trypan blue dye exclusion method.

11. **Jerne's Plaque Forming Assay:**

Eight weeks old adult Balb/c mice were Intraperitoneally immunised with $2.5x10^{9}$ SRBC and on 2nd, 3rd, 4th and 5th day of immunisation, the spleens were removed and single cell suspensions were made in medium RPMI-1640. About 50 micro-litre of 7% SRBC was pipetted into tubes containing 0.5 ml of 0.5% agarose, in HBSS and 20-50 uL of spleen cell suspension ($8x10^{6}$ cells/ml) was added to the tubes. The tubes were kept

at 45°C in water bath. After adding the spleen cells, the contents of the tube was immediately poured into a grease free slide spread into an area of "1x2" and allowed to solidify. The slides were kept on the incubation rack and space between the slides and rack was filled with complement (Fresh rabbit serum 1:1 dilution) slides were incubated for 1 hr at 37°C. The number of plaques were counted in a colony counter and expressed as the number of plaques per million lymphocytes.

In order to study the immunopotentiating action of the drug appropriate dose of drug and $2.5x10^9$ SRBC/ml were given i.p. into a group of Balb/c mice. Spleen cells were tested for plaque forming activity on 2nd, 3rd, 4th and 5th day of immunisation and number of plaques formed per million lymphocytes were compared with control.

12. Depletion of the Lyt-2^+ antigen bearing cells using monoclonal antibody and complement:

About $5x10^6$ cells were incubated with appropriate dilution of anti Lyt-2^+ monoclonal antibody and fresh normal rabbit serum 1/10 dilution for 30 minutes at 37°C. The treated cells were washed twice and assessed for cytotoxicity.

13. Adoptive transfer of rIL-2 activated CTL:

a) Recipient Balb/c mice were inoculated s.c. in the hind leg with $1x10^6$ DLA cells on day 0. On day 7, when tumor size reaches appreciable diameter, the mice were injected 180 mg/kg weight of Cyclophosphamide i.p. and 1 hr later 10^7 activated

CTL were injected i.p. Tumor diameters were measured with a caliper in experimental animals and controls. Tumor volume calculated as 4/3 II x r_1^2x r_2^2, where r_1 and r_2 are radii of the opposite diameters.

b) Winn neutralisation assay: IL-2 activated CTL ($1x10^7$ cells) mixed with $1x10^6$ tumor cells were injected s.c. in the hind leg of syngeneic mice. Tumor diameters were measured with a caliper 3 weeks after inoculation of cell mixtures. The percentage increase in life span (ILS) was calculated as follows:

$$\% \text{ ILS} = \frac{T - C}{C} \times 100$$

T = Mean survival time of treated tumor bearing mice.
C = Mean survival time of control mice.

14. **Production and standardisation of Crude Interleukin-2:**

(a) Preparation of crude Interleukin-2 (IL-2): Rat spleen cells (10^6 spleenocytes/ml) were incubated in complete medium containing 5 microgram/ml Con A for 72 hrs at 37°C in 5% CO_2 atmosphere. After incubation, cell free supernatant was taken and filtered with 0.45 u and stored at -70°C.

(b) Standardisation of crude IL-2:

Method of Gillis et al (1978) was employed. Murine CTLL-2 cell line was used in the lymphoproliferative assay. The proliferation of IL-2 dependent cell line CTLL-2 was estimated by culturing the cell line for 24 hrs in 5% CO_2 atmosphere, in the presence of serial dilutions of standard IL-2 (6.25 units/ml to 100 units/ml). CTLL-2 cells were harvested on 3rd day after IL-2 feeding. Cells were washed

with RPMI-1640 twice and resuspended to concentration of 10^5 cells/ml. 0.1 ml of the cell suspension was added to microtiter plate wells (10^4 cells/well) which already contained 0.1 ml of standard IL-2 different dilutions (100, 50, 25, 12.5 units/ml) or different dilutions of unknown sample. Plates were incubated at 37°C at an atmosphere of 5% CO_2 in air for 24 hours. Proliferation of CTLL-2 was estimated by pulsing with ^3H thymidine after 24 hrs. Incubation was continued and after 8 hrs wells were harvested on to filter paper strips. Strips were completely air dried. Filter spots were placed in scintilation vials and scintillation fluid was added. Counts were taken in a beta ray scintillation counter (Rack Beta, LKB Wallac). A graph was plotted with reciprocal dilution versus probit each sample. The dilution of the standard which equalled 50% maximum response from graph was determined. This reciprocal dilution was measured as the standard unit. 50% maximum dilution for unknown sample was graphically determined.

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RESULTS

RESULTS

Immune Profiles During Tumor Development:

The immunological status in tumor bearing animals reflected in the NK, ADCC, MMADCC, ACC, T cell subsets and CTL activities were assessed on different days (from day 0 to death of animal) after tumor transplantion. NK cell activity in normal mouse (as noted on day 0) was found to be 1.4% ± 0.4%. The activity showed a gradual increase from day 3 onwards (2.1% ± 0.29) and reached maximum on 12th day of tumor inoculation (59.67 ± 0.415) (Table 1). Thereafter activity was found to decline and animals were dead by 20th day. Effector: target ratio of 100 : 1 was found to give maximum percent specific cytolysis as shown in the Fig. 1 and Table-1.

Antibody Dependent Cellular Cytotoxicity, assayed using anti-SRBC antibody coated SRBC target cells, at different attacker : target ratios displayed detectable activity on the day 0 (normal) (12.48 % ± 0.37) with a peak on fifth day (43% + 1.6) (Table 2). Thereafter the activity was found to decline abruptly. High amount of specific lysis was observed in the effector : target ratio of 100 : 1 (Fig. 2).

As shown in the Fig.1 and Fig.2 optimum cytolysis was obtained at an effector : target ratio of 100:1 in 4 hrs ^{51}Cr release assay. Subsequently all the assays were carried out at an E:T of 100:1.

Resident Macrophage (RM) and tumor associated macrophage (TAM) mediated ADCC were assessed against sensitized, ^{51}Cr labelled SRBC target. Appreciable amount of antibody mediated

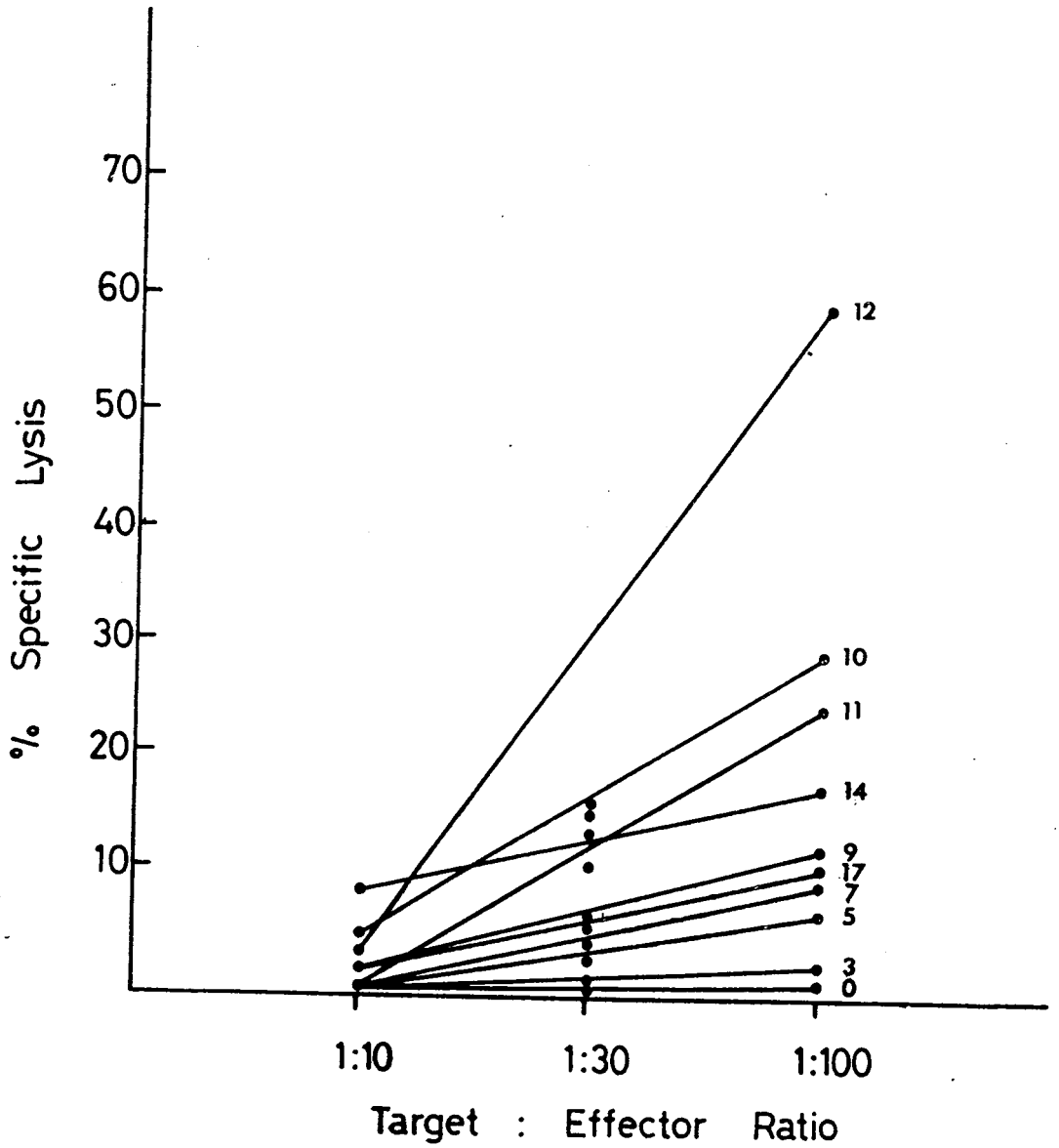


Fig. 1 - Natural killer cell activity against target K-562, assessed by ^{51}Cr release assay on different days after tumor inoculation at target:effector ratios of 1:10, 1:30 and 1:100. Numbers indicate days after tumor inoculation.

TABLE-1: Natural Killer Cell mediated cytolysis of ^{51}Cr labelled K-562 target on different days after DLA tumor inoculation.

No.	Days after tumor inoculation	No. of observations	Mean % cytotoxicity ± S.D. (Effector : Target Ratios)		
			10:1	30:1	100:1
1	0	3	0.9 ± 0.20	1.2 ± 0.51	1.4 ± 0.4
2	3	3	1.5 ± 0.60	1.8 ± 0.90	2.1 ± 0.29
3	5	3	2.1 ± 0.55	2.8 ± 0.25	7.8 ± 0.56
4	7	3	2.5 ± 0.65	5.2 ± 0.30	10.1 ± 0.24
5	9	3	3.6 ± 0.55	9.7 ± 1.60	12.59 ± 1.8
6	11	3	2.8 ± 0.60	3.3 ± 2.0	25.4 ± 0.73
7	12	3	4.2 ± 1.20	27.5 ± 1.20	59.67 ± 0.42
8	14	3	9.1 ± 1.10	14.5 ± 2.30	18.58 ± 0.63
9	17	3	2.4 ± 0.50	4.6 ± 1.02	7.85 ± 0.37

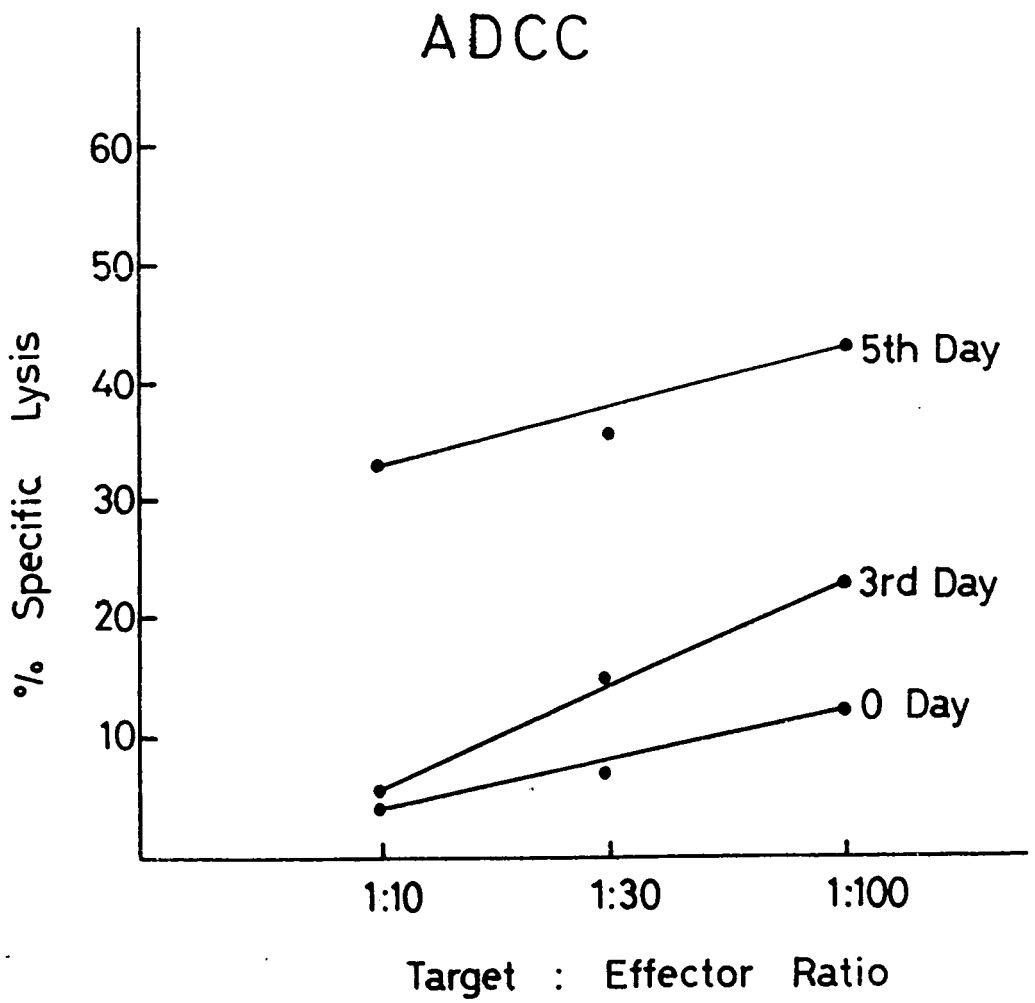


Fig. 2 - Antibody dependent cell mediated cytotoxicity against sensitized SRBC assessed by ^{51}Cr release assay on different days after tumor inoculation at effector:target ratios of 10:1, 30:1 and 100:1. Numbers indicate days after tumor inoculation.

TABLE-2: Antibody dependent cellular cytotoxicity of ^{51}Cr labelled sensitized SRBC on different days after DLA tumor inoculation.

No.	Days after tumor inoculation	No. of observations	Mean % Cytotoxicity ± S.D. (Effector : Target Ratios) 10:1	30:1	100:1
1	0	3	4.7 ± 0.83	7.4 ± 0.81	12.48 ± 0.37
2	3	3	6.6 ± 0.66	14.6 ± 1.0	22.6 ± 0.83
3	5	3	32.5 ± 1.75	37.6 ± 1.2	43.0 ± 1.6
4	7	3	N.D	N.D	N.D
5	9	3	N.D	N.D	N.D
6	11	3	N.D	N.D.	N.D.
7	13	3	N.D	N.D	N.D
8	15	3	N.D	N.D	N.D
9	17	3	N.D	N.D	N.D
10	19	3	N.D	N.D	N.D

N.D. = Not detectable

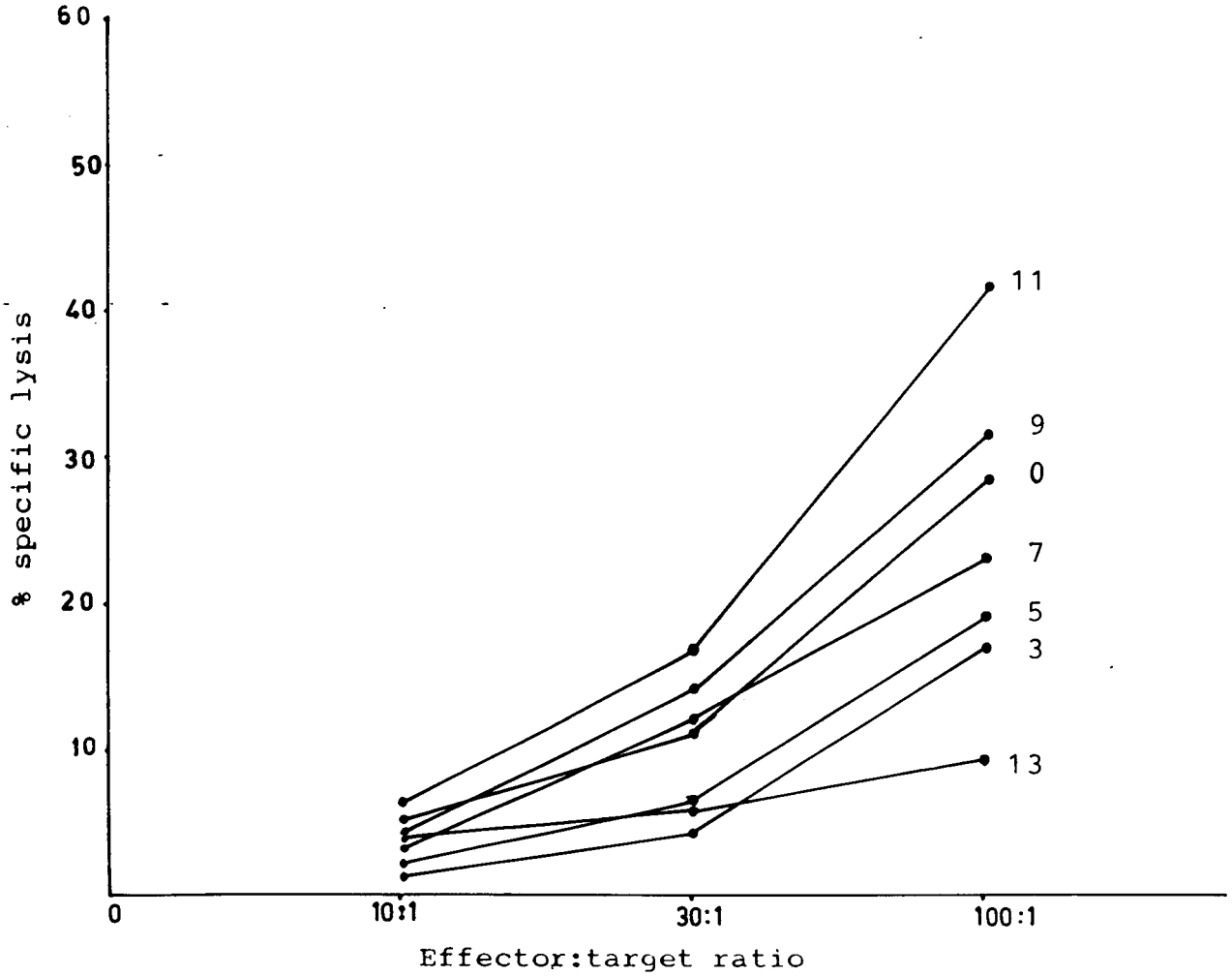


Fig. 3 - Macrophage mediated ADCC against sensitized SRBC assessed by ^{51}Cr release assay on different days after tumor inoculation at Effector:Target ratios of 10:1, 30:1 and 100:1. Numbers indicate days after tumor inoculation.

TABLE-3: Macrophage mediated ADCC of ^{51}Cr labelled sensitized at Effector : Target SRBC on different days after DLA tumor inoculation.

No.	Days after tumor inoculation	No. of observations	Mean % cytotoxicity ± S.D. Effector : Target Ratio		
			10:1	30:1	100:1
1	0	3	5.0 ± 1.32	10.8 ± 1.52	28.3 ± 5.3
2	3	3	2.1 ± 0.76	4.8 ± 0.80	17.5 ± 2.4
3	5	3	2.5 ± 1.0	6.4 ± 2.0	19.6 ± 2.5
4	7	3	3.5 ± 1.0	11.8 ± 1.52	23.3 ± 2.8
5	9	3	4.7 ± 1.4	14.2 ± 1.75	31.6 ± 3.2
6	11	3	5.9 ± 1.05	16.9 ± 3.0	42.3 ± 4.10
7	13	3	3.7 ± 1.58	5.8 ± 0.57	9.8 ± 1.96
8	15	3	N.D	N.D	N.D

N.D. = Not detectable

killing was displayed by RM (day 0) (28.3% ± 5.3). TAM mediated ADCC showed a gradual increase from day 3 onwards which peaked on day 11 (42.3% ± 4.10) (Table 3). Thereafter the activity was found to decline gradually to the basal level and no TAM mediated killing was detected on 15th day of tumor inoculation (Fig. 3).

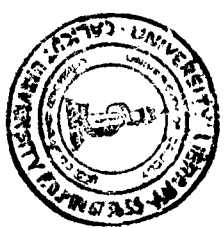
Cytotoxic T Lymphocytes generated in vitro using mixed lymphocyte tumor culture (MLTC) were tested for their cytoxic potential by ^{51}Cr release assay against radiolabelled DLA cells from day-1 to day-7 of incubation. In our studies peak CTL response was found on 4th day of co-culturing and negligible cytotoxicity was displayed by the spleen cells on the rest of the days. Hence this was used as a standard in further experiments. CTL activity estimated by release ^{51}Cr from labelled Dalton's Lymphoma Ascites tumor cells showed detectable activity on 10th day of immunisation (after 4 days of co-culture) (3.85% ± 0.15) and then the activity decreased gradually. Again high activity was observed in the effector : target ratio of 100:1. (Table-4).

Humoral immune response was demonstrated by using Dalton's Lymphoma Ascites tumor cells treated with anti-DLA mouse antibody and fresh rabbit complement. The activity was observed from the day 9 (10.5% ± 0.42) with a peak on the 13th day (43.3% ± 0.8) which gradually declined (Table 5). By the 19th day no activity was detectable (Fig. 4).

TABLE-4: CTL mediated lysis of ^{51}Cr labelled DLA tumor cells on different days after DLA tumor inoculation.

No.	Days after tumor inoculation	No. of observations	Mean % cytotoxicity ± S.D. (Effector : Target Ratio)		
			10:1	30:1	100:1
1	0	3	N.D	N.D	N.D
2	3	3	N.D	N.D	N.D
3	5	3	N.D	N.D	N.D
4	7	3	N.D	N.D	N.D
5	9	3	N.D	N.D	N.D
6	10	3	1.76 ± 0.72	2.76 ± 0.17	3.85 ± 0.153
7	12	3	1.3 ± 0.28	1.8 ± 0.20	2.23 ± 0.1203
8	14	3	N.D	N.D	N.D
9	17	3	N.D	N.D	N.D
10	19	3	N.D	N.D	N.D

N.D. = Not detectable



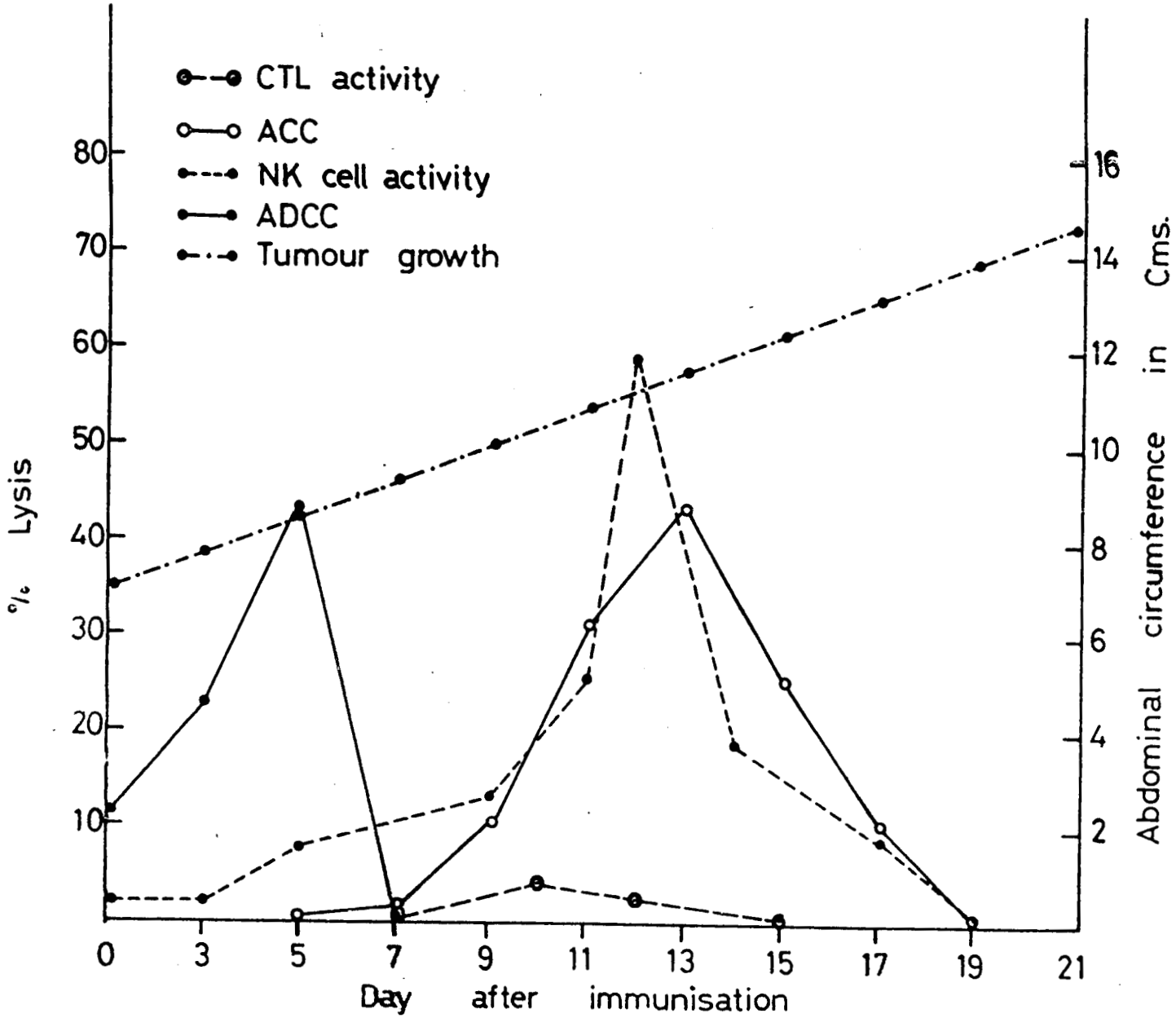


Fig. 4 - Profiles of natural killer cell activity, antibody dependent cell mediated cytotoxicity, CTL activity and antibody complement mediated cytotoxicity during tumor growth

TABLE-5: Antibody-complement mediated lysis of DLA tumor cells on different days after tumor inoculation assessed by trypan blue exclusion method.

No.	Days after tumor inoculation	No. of observations	Mean % cytotoxicity ± S.D.
1	0	3	N.D
2	3	3	N.D
3	5	3	N.D
4	7	3	2.1 ± 0.11
5	9	3	10.5 ± 0.41
6	11	3	31.5 ± 1.50
7	13	3	43.3 ± 0.80
8	15	3	25.1 ± 0.94
9	17	3	10.7 ± 1.10
10	19	3	N.D

N.D = Not Detectable

TABLE-6: Percentage of splenic T cells forming Ox IgM rosettes at different days after DLA tumor inoculation.

No.	Days after tumor inoculation	No. of observations	Mean % ± S.D
1	0	3	21.5 ± 2.0
2	3	3	32.0 ± 2.9
3	5	3	28.37 ± 1.17
4	7	3	31.1 ± 1.5
5	9	3	30.5 ± 0.85
6	11	3	31.85 ± 0.54
7	13	3	29.5 ± 0.41
8	15	3	24.5 + 0.83
9	17	3	26.0 ± 0.4
10	19	3	23.0 ± 0.4

TABLE-7: Percentage of splenic T cells forming ox IgG rosettes on different days after DLA tumor inoculation.

No.	Days after tumor inoculation	No. of observations	Mean % ± S.D.
1	0	3	24.7 ± 2.4
2	3	3	18.0 ± 0.83
3	5	3	23.5 ± 0.69
4	7	3	22.5 ± 0.42
5	9	3	22.4 ± 2.5
6	11	3	20.5 ± 0.63
7	13	3	26.7 ± 2.9
8	15	3	27.5 ± 1.6
9	17	3	26.5 ± 0.29
10	19	3	27.0 ± 0.41

The percentage of T_H cells in the spleen as assessed by immunorosetting with anti-ox IgM sensitised ox RBC increased gradually from day 0 to a peak on day 11 and then declined gradually to the basal level by 19th day of tumor inoculation (Table 6). The percent of T_S cells were found to be more or less consistent till 11th day and then increased gradually as the tumor growth progressed (Table 7).

Kinetics of NK cell activity:

When Percoll purified, LGL enriched fraction was used as effector cells against radiolabelled K-562 target in 4 hrs assay, an appreciable amount of cytolysis was displayed by normal LGL even at a low effector : target ratio of 5:1 (10.9% $\pm$ 1.27), which increased gradually to a peak on day 12 of post-tumor inoculation (20.5% $\pm$ 2.15). The activity was found to be reduced significantly on day 19 (2.26% $\pm$ 0.75). Pretreatment of effector cells on day 19 with 200 IU/ml of human rIL-2 or equivalent rat spleen supernatant in vitro, resulted in enhancement in killing potential of LGL (12.96 $\pm$ 2.74) (Table-8).

Single cell conjugate assay:

Among purified population of LGL 30-40% of LGL formed conjugate with K-562 targets indiscriminately, irrespective of the days after tumor inoculation. However percentage of conjugates containing dead targets increased gradually from

day 0 to day 12 of post tumor inoculation and was found to be significantly reduced on day 19 (10.4% ± 1.04) as compared to other days (Table 9). Percentage of active killer cells was found to be increased on day 7 (9.6%) and day 12 (10.16%) with respect to control (6.3%) (Table 9).

The estimated maximal recycling capacity of the effector cells however, did not show much variations when compared to normal control (Table 9).

NKCF Assay: Culture supernatant of effector cells with K-562 was tested for NKCF activity against K-562 in a micro-supernatant assay as described in the materials and methods section. Optimum activity was observed at an NKCF dilution of 1:3 in initial standardisation assays. (Fig. 5) Hence subsequent assays were carried out at 1:3 dilution of NKCF. Fig.6 shows the profiles of NKCF activity at different intervals of tumor development. Peak NKCF activity was observed on day 12 (40.3% ± 0.98) and on day 19 a significant fall (7.4% ± 1.4) ($P < 0.05$) in activity was noticed with respect to control (Fig.6, Table 10). Control cultures displayed more than 95% viability.

Incubation of day 19 effector cells with 200 IU/ml of human rIL-2 for 72 hrs at 37°C in 5% CO_2 atmosphere resulted in significant increase NKCMC (2.26 ± 0.75 to 12.95 ± 2.74) (Table 8) and in percentage active killer cells (2.58 to 7.2) (Table 9). Maximal recycling capacity of the effector cells,

TABLE-8: Natural killer cell (LGL) mediated cytolysis of radiolabelled K-562 target on different days after tumor inoculation at an Effector : target ratio 5:1 (values are mean ± SD from 3 observations in each group)

Days after tumor inoculation	% specific cytolysis
0 (normal)	10.9 ± 1.27
7	17.1 ± 2.40
12	20.53 ± 2.15*
19	2.26 ± 0.75**
19*	12.965 ± 2.73

* On day-19, LGL were presented with human rIL-2 as described in materials and methods and ^{51}Cr release assay was performed.

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

TABLE-9: V_{max} target binding cells (TBC), Fraction of dead conjugates and estimation of active NK cells and their recycling capacity with K-562 target cells. LGL isolated from the spleen cells of tumor bearing Balb/c mice at different intervals of tumor growth were used as effector cells (Values are mean ± SD from 3 observations in each group)

Days after tumor inoculation	V_{max}	TBC	Fraction of dead conjugates	Estimated No. of active killer cells	Estimated MRC
	x 10^3	%	%	%	
0 (normal)	5.97	37.83 ± 1.55	17.45 ± 1.78	6.3	1.89
7	8.48	36.33 ± 2.49	28.12 ± 2.29*	9.6	1.73
12	10.02	37.33 ± 4.11	29.2 ± 5.38	10.16	1.97
19	2.350	27.2 ± 4.9	10.4 ± 1.04*	2.58	1.82
19*	6.845	35.6 ± 2.7	21.3 ± 1.85	7.2	1.89

* On day-19 LGL were pre-treated with human rIL-2 as described in materials and methods and different parameters were studied.

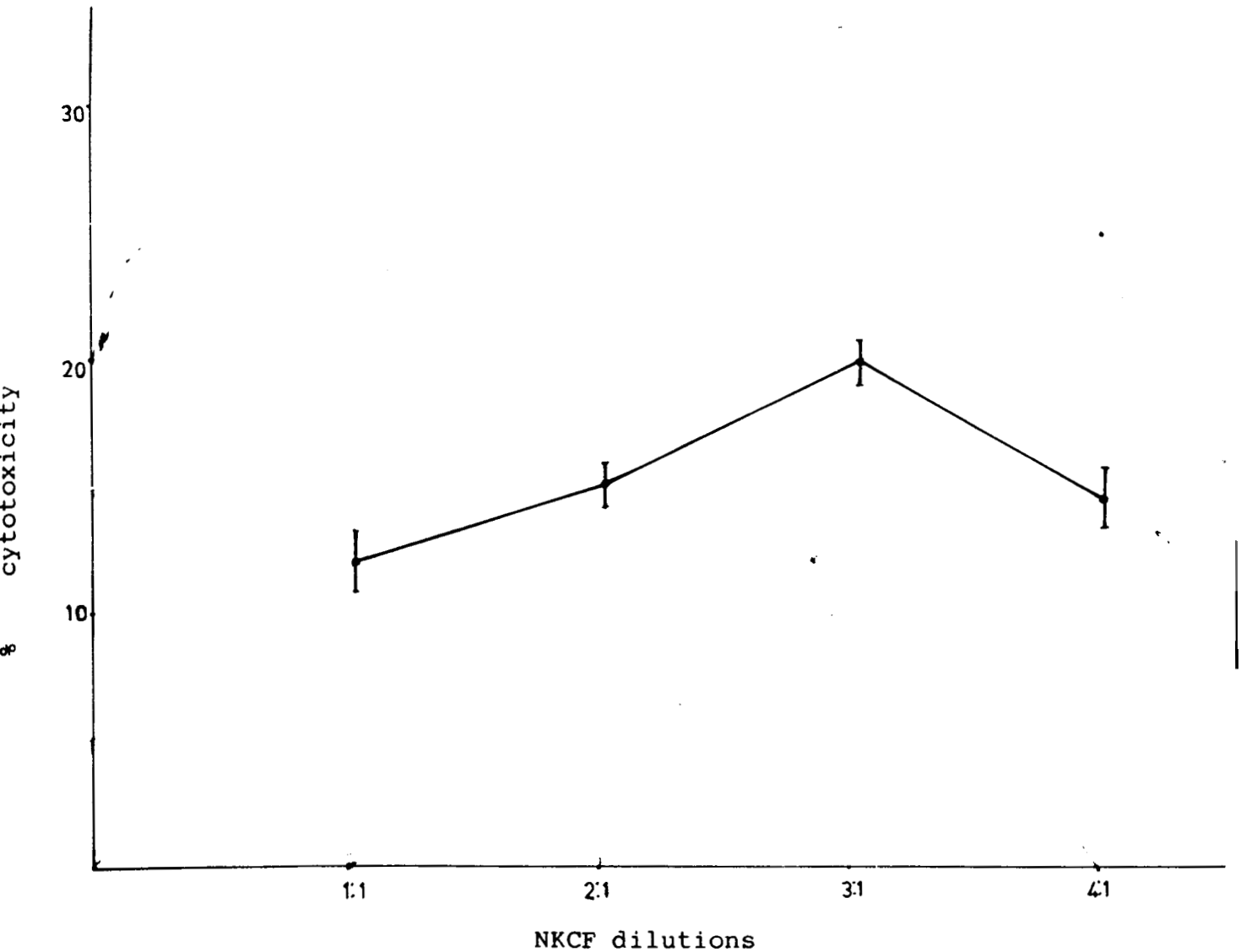


Fig. 5 - NKCF activity assessed against K-562 target cells in 48 hrs. microsupernatant assay at different dilutions of NKCF.

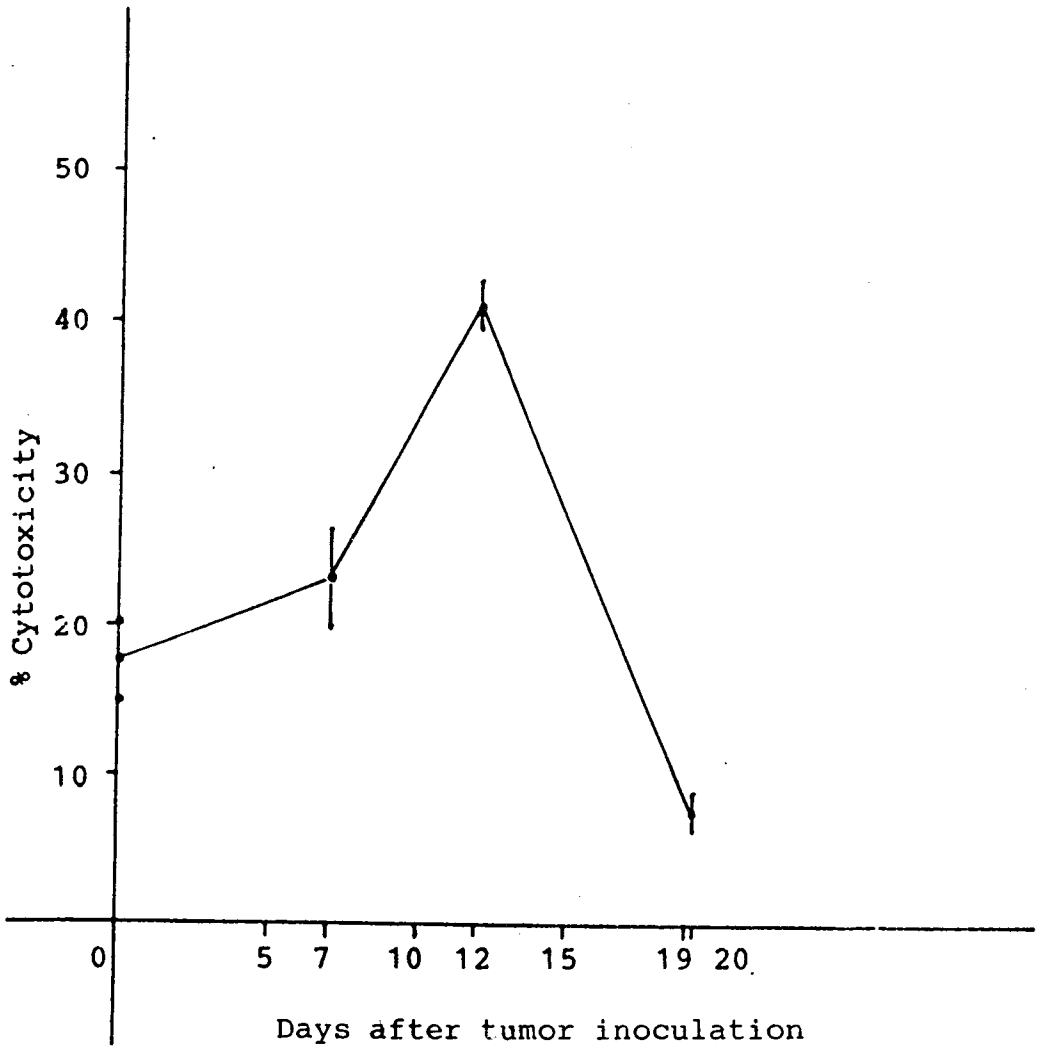


Fig. 6

Production of NKCF by LGL isolated from Spleen Cells of tumor bearing Balb/c mice at different intervals of tumor development assessed in 48 hrs. microsupernatant assay using K-562 target cells.

TABLE-10: Production of NKCF by LGL isolated from spleen cells of tumor bearing Balb/c mice at different intervals of tumor development assessed in 48 hrs. micro-supernatant assay using K-562 target cells. (Valuesare mean ± SD from 3 observations in each group)

Days after tumor inoculation	% cytotoxicity
0 (normal)	17.6 ± 2.6
7	23.6 ± 4.04
12	40.3 ± 0.98**
19	7.4 ± 1.4*
*19	18.4 ± 1.27

* On day-19 LGL were pretreated with human rIL-2 as described in materials and methods and NKCF activity was assessed.

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

however, did not show any variations (1.82 to 1.89) (Table 9). Moreover NKCF activity was found to be augmented after pretreatment of the effector cell with rIL-2 (Fig.6) (Table 10) (7.4 to 18.4) ($P < 0.001$).

In the above studies it was demonstrated that anti-tumor acivity of syngeneic murine CTL assessed in 4 hrs ^{51}Cr release assay was very low (3.85 $\pm$ 0.153) (Table 4). When these effector cells were further incubated with 200 IU/ml of human rIL-2 for 7 days, a significant enhancement in tumoricidal activity was observed against DLA target cells (27.86 $\pm$ 5.09), (E:T 30:1) (Table 11). Maximum amount of cytolysis was observed at an E:T ratio 100:1 (47 $\pm$ 2.20) (Fig. 7).

Adoptive Immunotherapy of transplanted solid tumors by IL-2 activated CTL:

Adoptive transfer of antitumor activity was performed as described in materials and methods. Balb/c mice were injected $1x10^6$ DLA cells subcutaneously and on day 7, when tumors became palpable, 10^7 activated CTL were injected intraperitoneally into a group of animals. Another group of animals were given 180 mg/kg body weight of Cyclophosphamide, 1 hr preceeding the injection of activated CTL. As shown in the Fig. 8, 10^7 CTL could suppress the tumor growth significantly in mice transplanted with $1x10^6$ DLA cells and treated with Cyclophosphamide prior to the adoptive transfer of activated

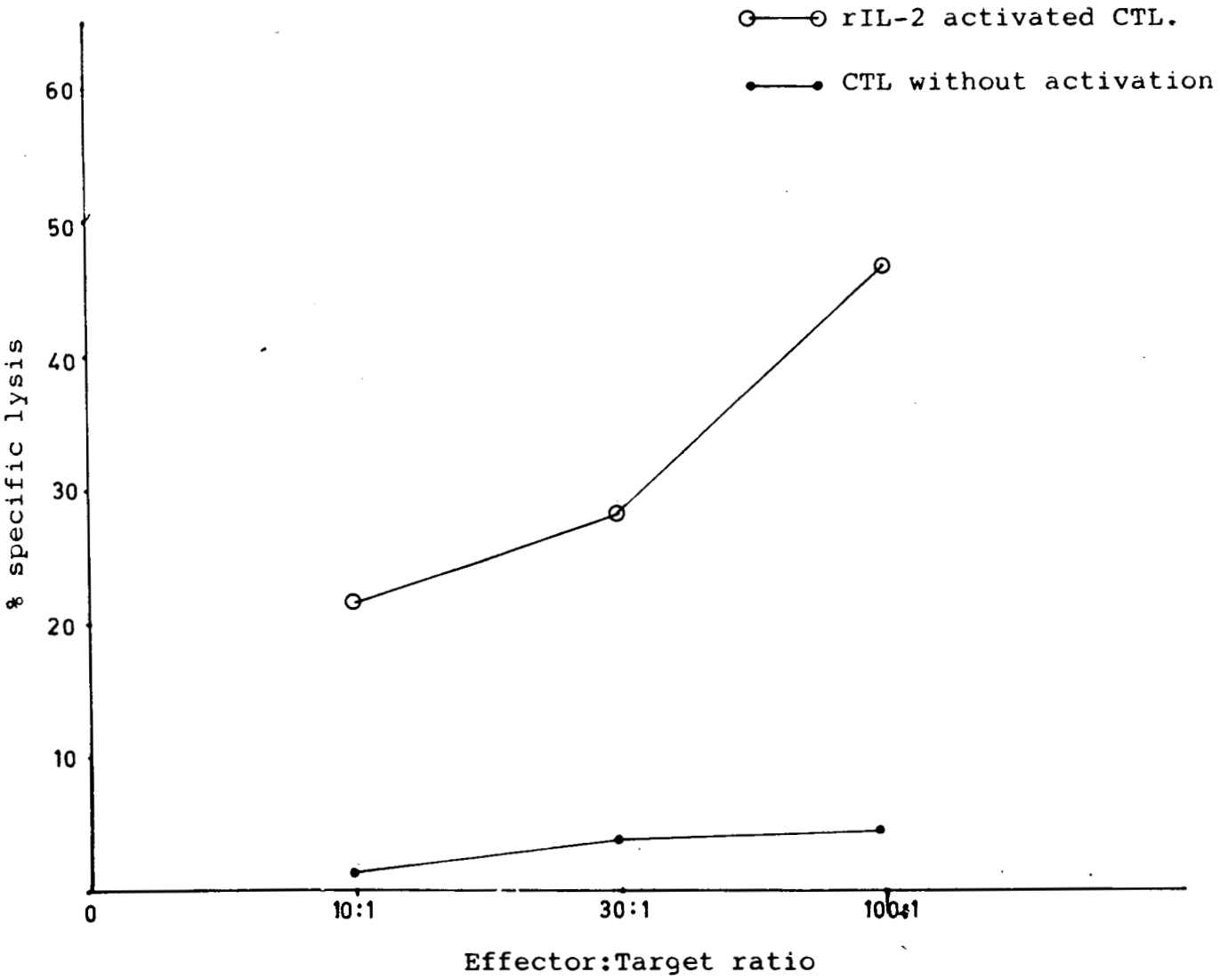


Fig. 7 - Cytotoxic T lymphocyte mediated lysis of ^{51}Cr labelled DLA target cells at different effector target ratios of 10:1, 30:1 and 100:1.

cells. Almost identical effect could be observed in mice treated with CTL alone.

Tumor growth was not suppressed in mice treated with Cyclophosphamide alone. All non-treated tumor bearing control mice died within 40 days after tumor inoculation, while 9% of the mice treated with Cyclophosphamide plus activated CTL were survived. Identical results were obtained in each of the three experiments repeated. The survived mice were shown to specifically reject DLA cells when they were reinoculated after 60 days.

Specificity of the Effector Cells:

Anti-tumor effectiveness of the activated CTL was tested against a spectrum of non-specific target cells including NK cell susceptible cell line YAC-1, Sarcoma-180 and Ehrlich's ascites tumor. As indicated in the table-11 negligible amount of tumoricidal activity was displayed by effector cells in 4 hrs ^{51}Cr release assay against these targets. Non-reactivity with the above targets showed the specific nature of the CTL.

Characterisation of Effector Cells:

Surface pehnotypic characterisation of the effector cell was performed as described in materials and methods. Effector cells after activation with rIL-2 were depleted of Lyt-2^+ lymphoid cells by treatment with anti-Lyt-2^+ monoclonal antibody and complement. As shown in

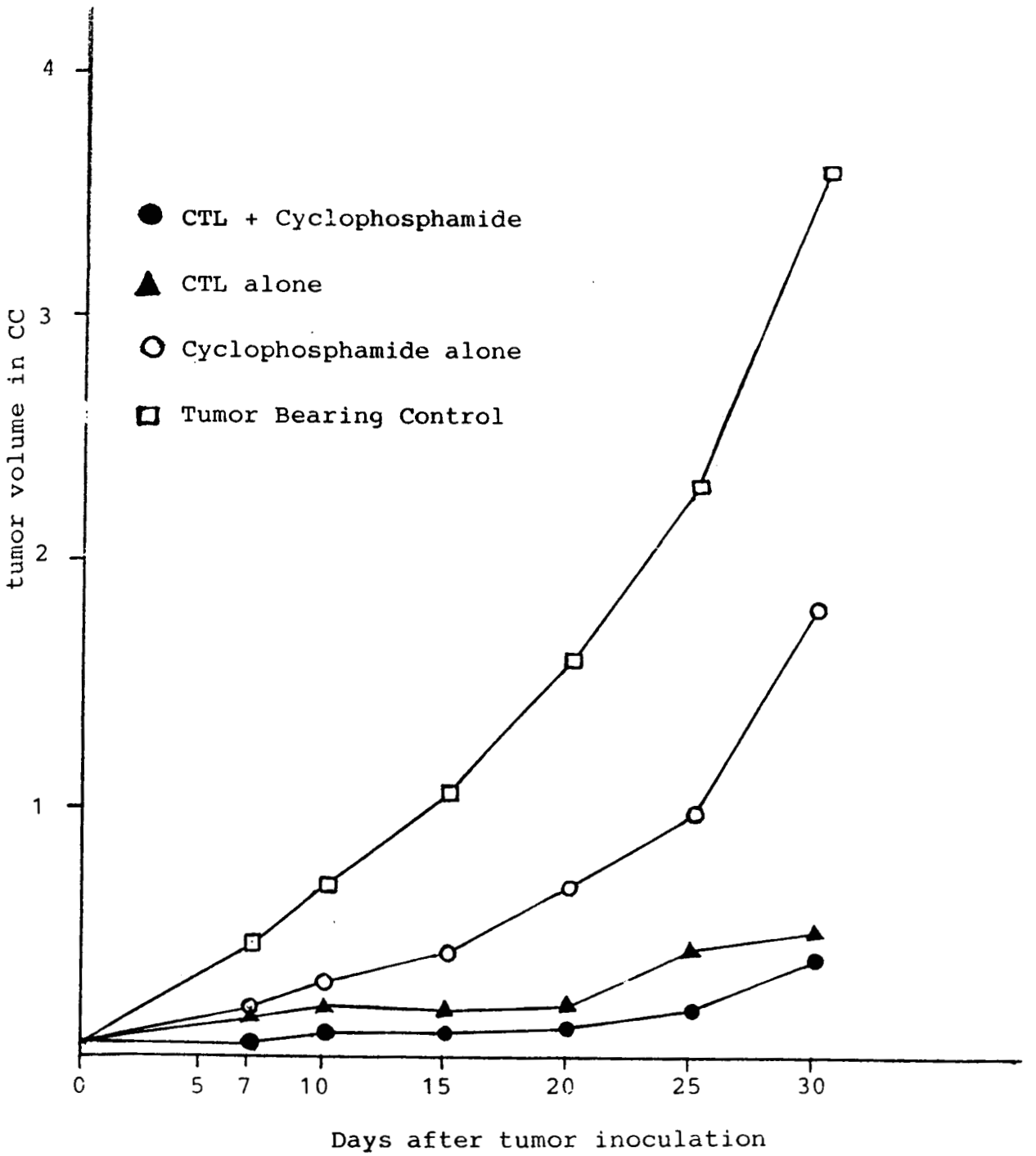


Fig. 8 - Adoptive Immunotherapy of DLA tumor with human rIL-2 activated CTL and Cyclophosphamide (180mg/kg)

TABLE 11: Tumor specificity of rIL-2 activated CTL against different tumor target cells.

	% specific lysis at E:T							
	D.L.A.		Ehrlich's ascites		Sarcoma 180		YAC-1	
	10:1	30:1	10:1	30:1	10:1	30:1	10:1	30:1
Without treatment	22.22 ± 2.4	27.86 ± 5.059	1.96 ± 0.40	3.8 ± 1.16	1.8 ± 0.66	4.3 ± 0.75	3.4 ± 0.75	4.70 ± 0.75
Treated with anti Lyt-2$^+$ antibody and complement	2.2 ± 0.61	4.3 ± 0.76	N.D	N.D	N.D	N.D	N.D	N.D

N.D : Not done

TABLE-12: Results of Winn type neutralization assay of two different syngeneic tumor cells with rIL-2 activated CTL.

Tumor	E:T[a]	% of inhibition of tumor take[b]	Tumor volume in cc[e] mean ± S.D.
DLA[c]	None	0 (0/6)[d]	2.52 ± 0.50
	10:1	100 (6/6)	0.45 ± 0.11**
S-180[c]	None	0 (0/6)	4.9 ± 0.28
	10:1	0 (0/6)	4.58 ± 0.43

a. Effector/target cell ratio

b. Results were evaluated 3 weeks after tumor transplantation.

c. 1×10^6 tumor cells/mouse s.c.

d. Numbers in parenthesis, free/total

e. Tumor volume in cc.

** Denotes highly significant ($P < 0.001$)

the table-11, treatment of the effector cells with anti-Lyt-2^+ antibody and complement totally abolished the anti-tumor activity of CTL against DLA cells in the in vitro killing assays.

Since the cytotoxic cells in vitro assays exerted their effect specifically on DLA target cells, the specificty was further tested by using Winn neutralisation assay as described in materials and methods. As the data in table-12 indicate the effector CTL could suppress only the growth of DLA tumor cells but not Sarcoma-180 in vivo, further confirming the specificity of the effector cells.

The Immunomodulatory effect of Drug Levamisole:

The modulatory effect of drug Levamisole on immune profiles of the tumor bearing animals as reflected in NK, ADCC, MMADCC, ACC and T cell subsets were assessed at various intervals of tumor growth after intraperitoneal administration of a single dose of (15/Kg body weight) Levamisole.

NK cell activity was significantly decreased in drug treated aniaml, compared to controls. A singificant reduction in the activity on day 5 ($P < 0.05$), day 7, 9, 11, 13, 15, ($P < 0.001$) was observed in drug treated aniamls compared to tumor bearing controls (Fig. 9) (Table-13).

ADCC showed significant enhancement on day 3 in drug treated animals with highly significant reduction on day

5 ($P < 0.001$), followed by a progressive increase ot a peak on day 11, thereafter a gradual decline and loss of activity after day 19. Augmentation of antibody mediated killing was significant on day 7, 9, 11, 13, 15 and 17 (Table-14, Fig.10).

An increase in T_H cell count was observed in Levamisole treated animals when compared to controls. The increase was significant on day 9, 15, 17 ($P < 0.05$) and 19 ($P < 0.001$) (Table 15). Suppressor T cells showed significant increase in day 3 in drug treated animals compared to controls (Table 16).

MMADCC showed a progressive enhancement from day 3 peaked on 11th and 13th day post tumor inoculation, thereafter a sudden decline in activity has been recorded in the levamisole treated animals. Augmented activity was significant on day 5, day 7 ($P < 0.05$) and day 11 ($P < 0.001$) (Fig.11 and Table-17).

Humoral response quantitated by antibody complement mediated lysis revealed considerable augmentation in antibody production following Levamisole administration. An early onset of humoral response was detected from day 5 onwards and sustained upto 15th day. Thereafter the activity was found to be decreased gradually to the basel level and was not detected on the 19th day. (Fig. 12). Enhancement in activity was highly significant on day 5, day 7 ($P < 0.001$), day 9, day 11, day 13 ($P < 0.05$) in treated mice with respect to tumor bearing control mice (Table-18).

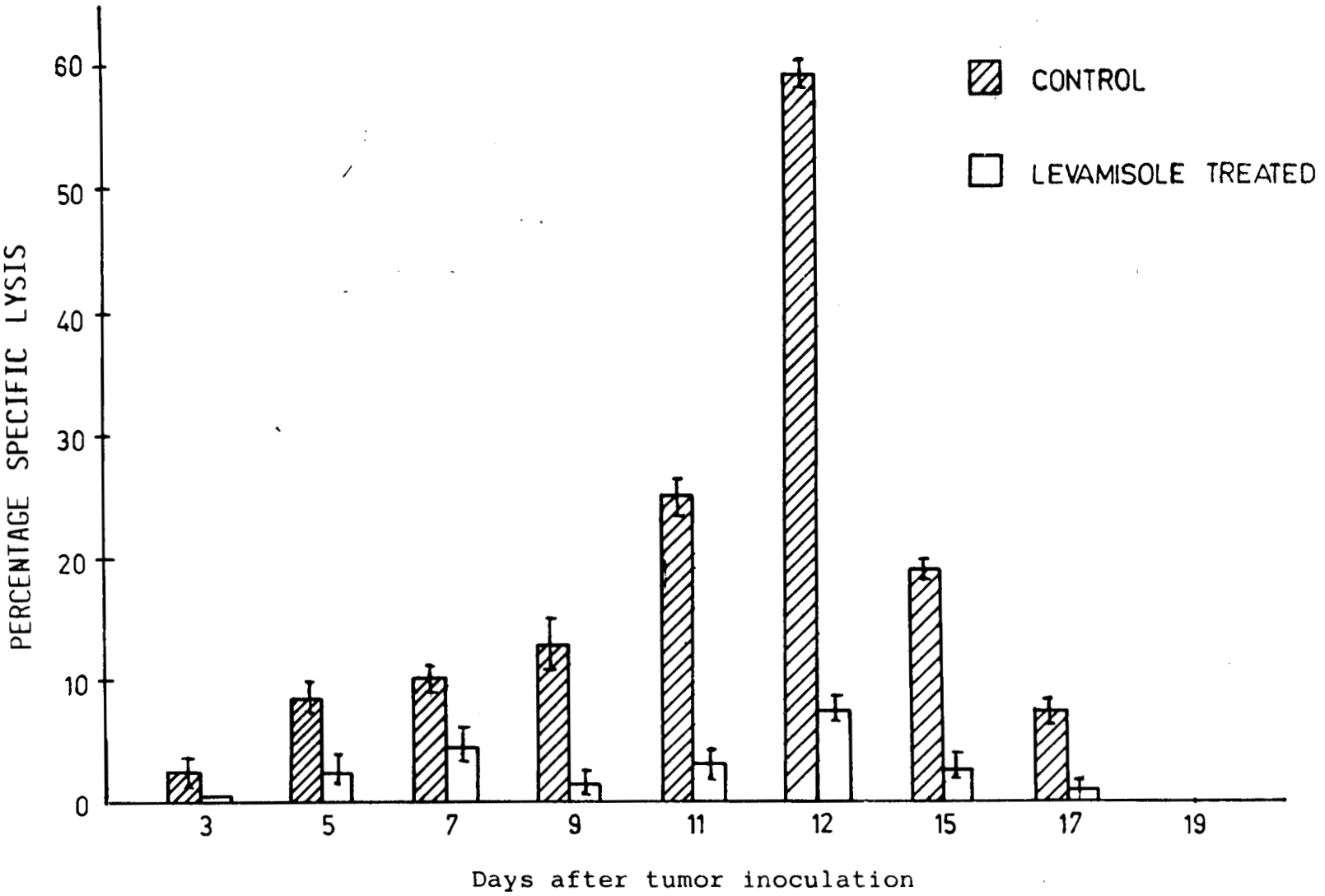


Fig. 9 - NK cell mediated cytotoxicity in tumor bearing Balb/c mice treated with 15 mg/kg weight of Levamisole at an effector:target ratio of 100:1

TABLE-13: NK cell activity against ^{51}Cr labelled K-562 at Effector : Target ratio 100:1

No.	Days after tumor inoculation	Control	Levamisole treated 15mg/kg body weight	Levamisole treated 1.5mg/kg body weight
		mean % specific lysis ± S.D.		
1	0	1.4 ± 0.4	N.D	N.D
2	3	2.1 ± 0.29	N.D	N.D
3	5	7.8 ± 0.56	2.05 ± 0.45*	10.2 ± 1.33*
4	7	10.1 ± 0.24	4.35 ± 0.54**	11.5 ± 1.0*
5	9	12.59 ± 1.8	1.3 ± 0.08**	12.5 ± 0.83**
6	11	25.4 ± 0.73	2.85 ± 0.53**	13.5 ± 1.1**
7	13	59.67 ± 0.51	7.75 ± 0.20**	15.0 ± 0.74**
8	15	18.58 ± 0.63	2.45 ± 0.45**	9.85 ± 1.03*
9	17	7.85 ± 0.37	N.D	4.9 ± 0.18
10	19	N.D	N.D	N.D

N.D. = Not detectable

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

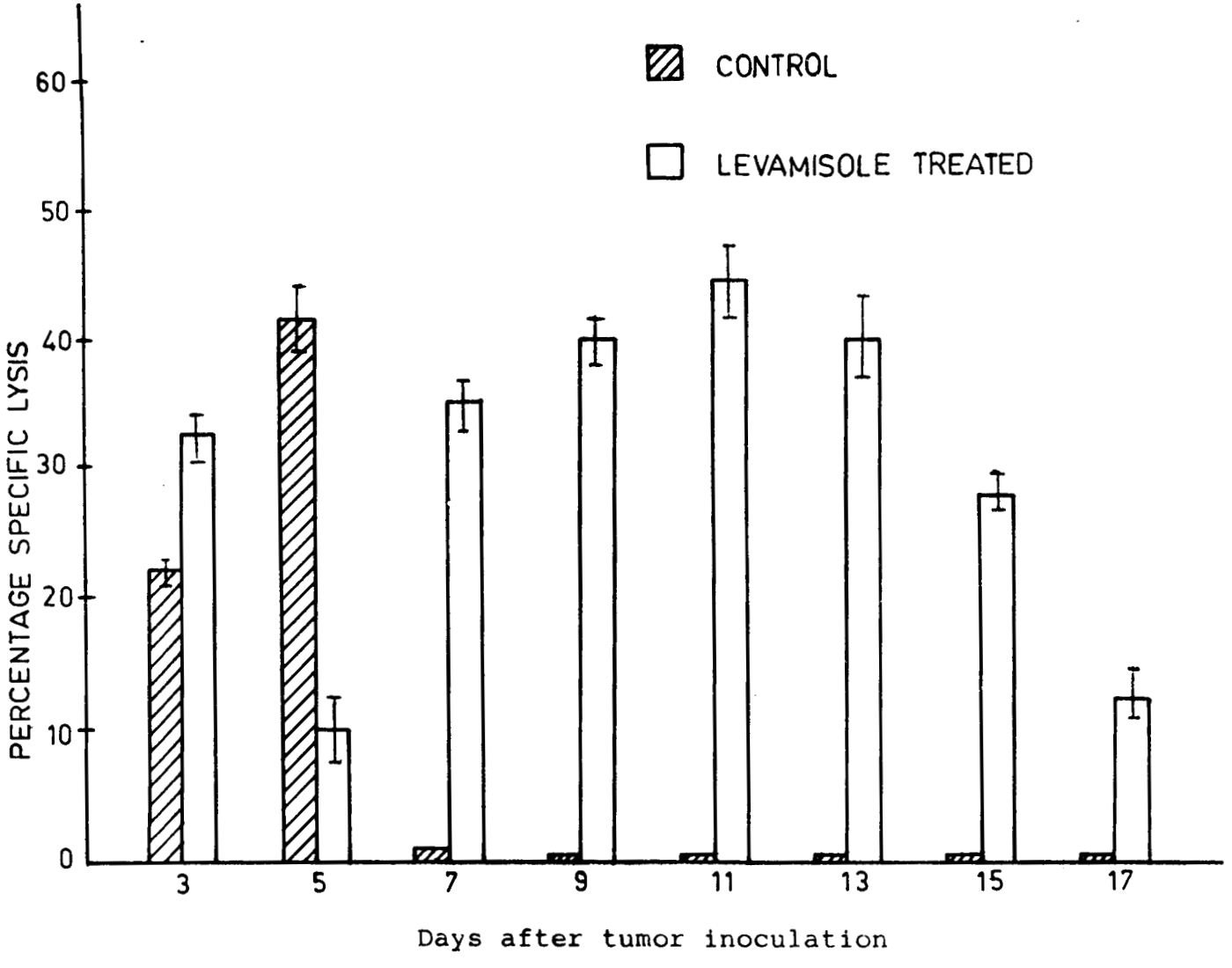


Fig. 10 - Antibody dependent cellular cytotoxicity in tumor bearing Balb/c mice treated with 15 mg/kg weight of Levamisole, at an effector:target ratio of 100:1

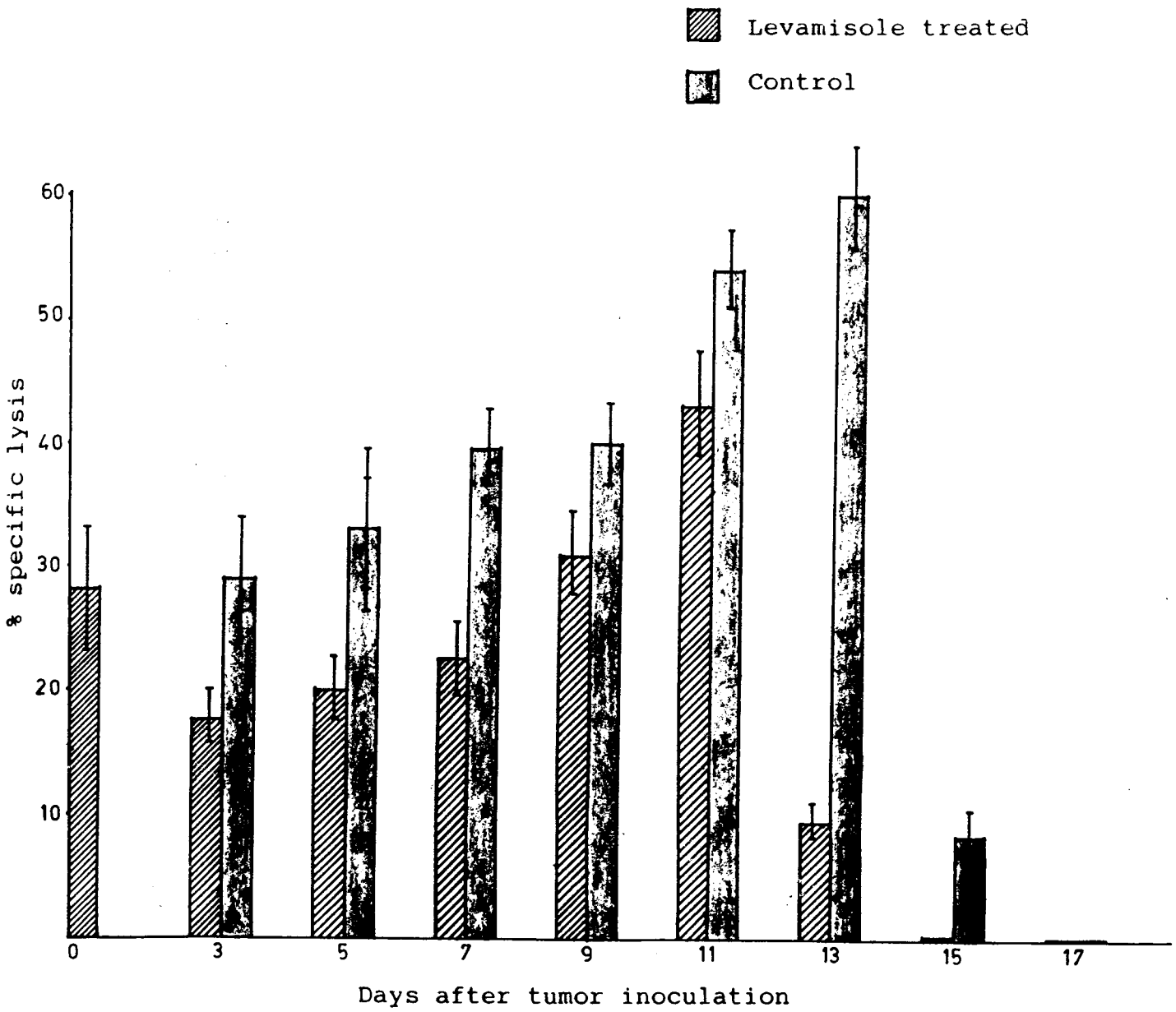


Fig. 11 - Microphage mediated ADCC in tumor bearing Balb/c mice treated with 15 mg/kg weight of Levamisole, at an effector:target ratio 100:1.

TABLE-14: ADCC against ^{51}Cr labelled, sensitized SRBC at Effector : Target ratio of 100:1

No.	Days after tumor inoculation	Control	Levamisole treated 15mg/kg body weight	Levamisole treated 1.5mg/kg body weight
		mean % specific lysis ± S.D.		
1	0	12.48 ± 0.37	12.48 ± 0.37	12.48 ± 0.37
2	3	22.6 ± 0.83	35.0 ± 1.6	22.16 ± 2.25
3	5	43.0 ± 1.6	10.0 ± 2.4**	5.5 ± 0.083*
4	7	N.D	35.0 ± 2.0*	8.95 ± 0.457**
5	9	N.D	40.0 ± 1.6**	12.16 ± 0.72**
6	11	N.D	43.7 ± 2.9**	13.0 ± 0.415*
7	13	N.D	41.0 ± 3.32**	10.5 ± 1.2*
8	15	N.D	27.5 ± 1.2**	4.9 ± 0.83*
9	17	N.D	12.5 ± 2.07**	N.D
10	19	N.D	N.D	N.D

N.D. = Not detectable

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

TABLE-15: Percentage of splenic T cells forming ox IgM rosettes in DLA tumor bearing Balb/c mice treated with Levamisole.

No.	Days after tumor inoculation	Control mean ± S.D.	Levamisole treated mean ± S.D.
1	0	21.5 ± 2.0	21.5 ± 2.0
2	3	32.0 ± 2.9	32.5 ± 4.15
3	5	28.37 ± 1.17	33.0 ± 1.24
4	7	31.1 ± 1.5	35.5 ± 0.7
5	9	30.5 ± 0.85	34.0 ± 0.73*
6	11	31.85 ± 0.540	37.75 ± 3.3
7	13	29.5 ± 0.415	32.75 ± 1.3
8	15	24.5 ± 0.83	31.5 ± 0.415*
9	17	26.0 ± 0.4	30.5 ± 0.623*
10	19	23.0 ± 0.4	30.25 ± 0.649**

Dose of Levamisole 15mg/kg body weight

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

TABLE-16: Percentage of splenic T cells forming ox IgG rosettes in DLA tumor bearing Balb/c mice treated with Levamisole.

No.	Days after tumor inoculation	Control mean ± S.D.	Levamisole treated mean ± S.D.
1	0	24.7 ± 2.4	24.7 ± 2.4
2	3	18.0 ± 0.83	26.0 ± 0.65*
3	5	23.5 ± 0.69	27.0 ± 1.5
4	7	22.5 ± 0.415	25.0 ± 0.41
5	9	22.4 ± 2.5	24.5 ± 0.62
6	11	20.5 ± 0.633	21.5 ± 0.561
7	13	26.7 ± 2.9	28.7 ± 0.20
8	15	27.5 ± 1.6	28.4 ± 0.75
9	17	26.5 ± 0.29	27.5 ± 0.48
10	19	27.0 ± 0.41	28.2 ± 2.5

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

Dose of Levamisole 15mg/kg body weight

TABLE-17: Macrophage mediated ADCC against ^{51}Cr labelled sensitised SRBC at Effector : Target ratio 100:1

No.	Days after tumor inoculation	Control (mean specific lysis ± S.D.)	Levamisole treated (mean specific lysis ± S.D.)
1	0	28.3 ± 5.3	28.3 ± 5.3
2	3	17.5 ± 2.4	28.0 ± 4.9
3	5	19.6 ± 2.5	32.8 ± 4.3*
4	7	23.3 ± 2.8	39.0 ± 2.9*
5	9	31.6 ± 3.2	40.5 ± 2.67
6	11	42.3 ± 4.10	54.3 ± 3.06
7	13	9.8 ± 1.96	60.5 ± 4.08**
8	15	N.D	8.3 ± 1.8

Dose of Levamisole 15mg/kg body weight

N.D. = Not detectable

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

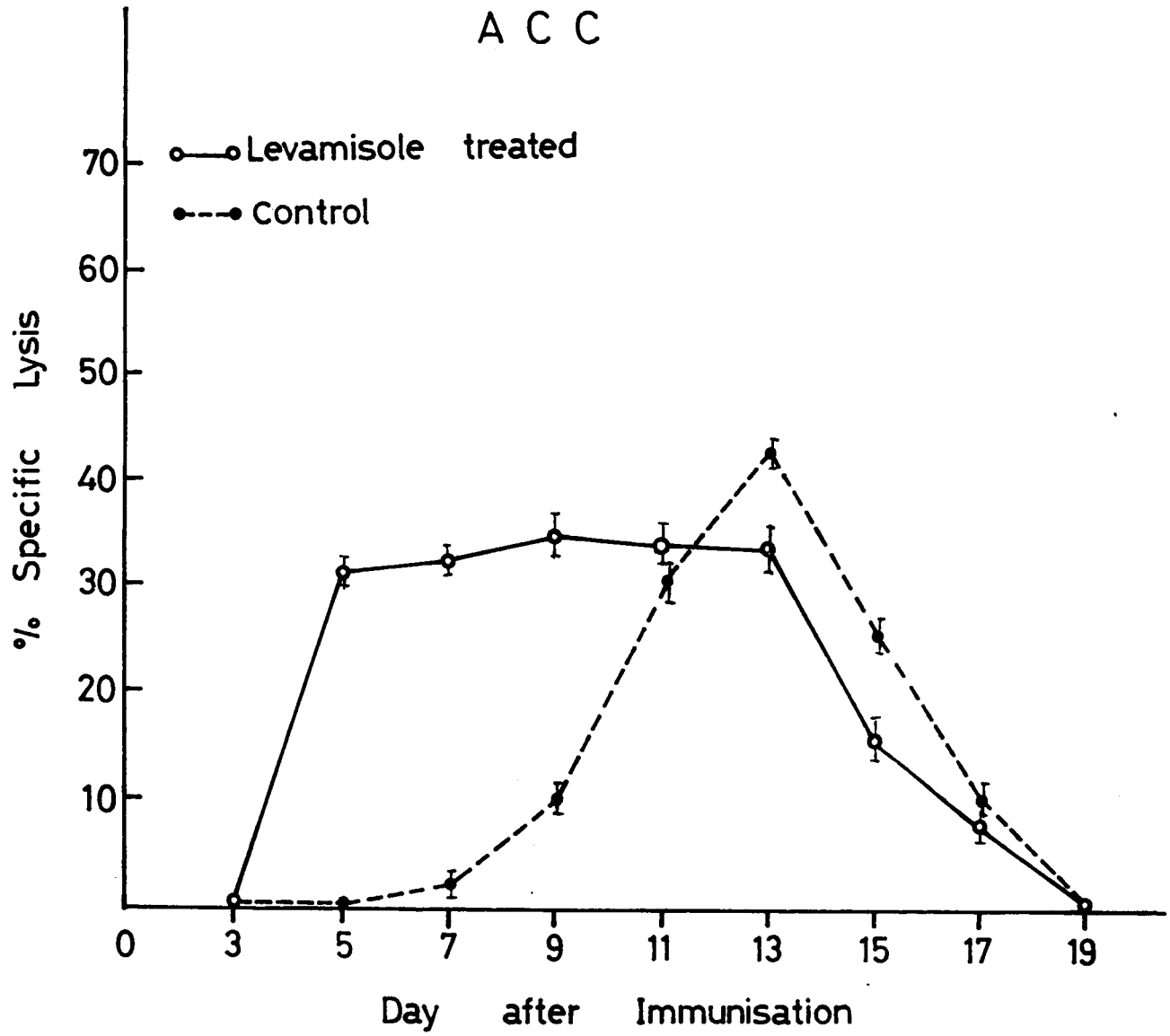


Fig. 12 - Antibody complement mediated cytotoxicity in DLA tumor bearing Balb/c mice treated with 15 mg/kg weight of Levamisole.

TABLE-18: Antibody complement mediated cytotoxicity against Dalton's lymphoma ascites cells.

No.	Days after tumor inoculation	Mean % lysis ± S.D. Control	Levamisole treated
1	0	N.D	N.D
2	3	N.D	N.D
3	5	N.D	30.5 ± 0.41**
4	7	2.1 ± 0.11	32.5 ± 0.41**
5	9	10.5 ± 0.41	34.9 ± 1.6
6	11	31.5 ± 1.5	34.2 ± 1.24*
7	13	43.3 ± 0.8	33.25 ± 1.4*
8	15	25.05 ± 0.94	15.0 ± 0.83
9	17	10.7 ± 1.1	7.5 ± 1.1
10	19	N.D	N.D

Dose of Levamisole - 15mg/kg body weight

N.D. Not detectable

*Denotes significant ($P < 0.05$)

**Denotes highly significant ($P < 0.001$)

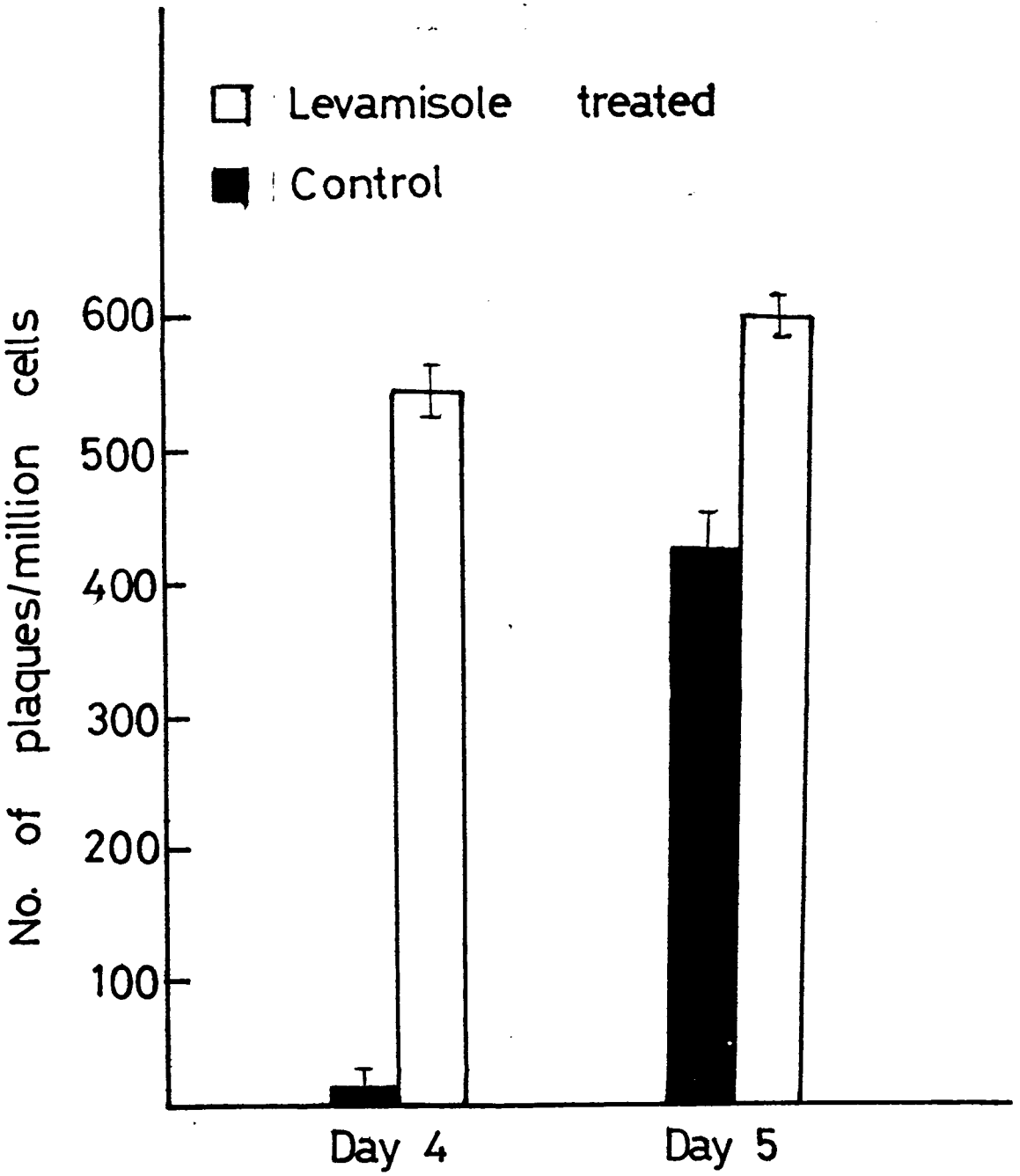


Fig. 13 - In vitro assessment of Immunomodulatory effect of drug Levamisole by using Jern's plaque assay.

TABLE-19 : Effect of Levamisole on life span of Dalton's Lymphoma Ascites tumor bearing mice.

	Dose in mg/kg body weight	Mean survival time (days)	% increase in life span
Control	No drug	20	--
Levamisole	15 mg	25	25%

Values are the mean of 6 animals per group repeated thrice.

In vitro assessment of Immunomodulatory action of Levamisole by Plaque forming assay showed significant enhancement in the number of plaque forming cells on the 4th and 5th day after immunisation when compared to control ($P < 0.001$) (Fig. 13).

All the above parameters of immunological response were studied following the administration of single dose of 1.5 mg/kg body weight of Levamisole. NK cell activity when compared to controls did not show significant difference on days 3 to 9 but the decrease was significant on day 11 to 19, whereas the overall activity was significantly increased on all days compared to 15 mg/kg body weight treated animals (Table-13). ADCC did not show any detectable activity on early days of tumor development (Days 0 and 3) with lower dose of the drug. An increase in activity was noted on days 7 to 17 when compared to control, though the activity was significantly lower than that of higher dose treated animals (Table-14).

Standardisation of IL-2:

In CTLL-2 proliferation assay of IL-2 50% maximum response was obtained at 45 units/ml of standard interleukin-2 (Interim reference reagent) whereas half maximum response was recorded at 1:6 dilution of rat spleen supernatent (Fig.14) (Table-20).

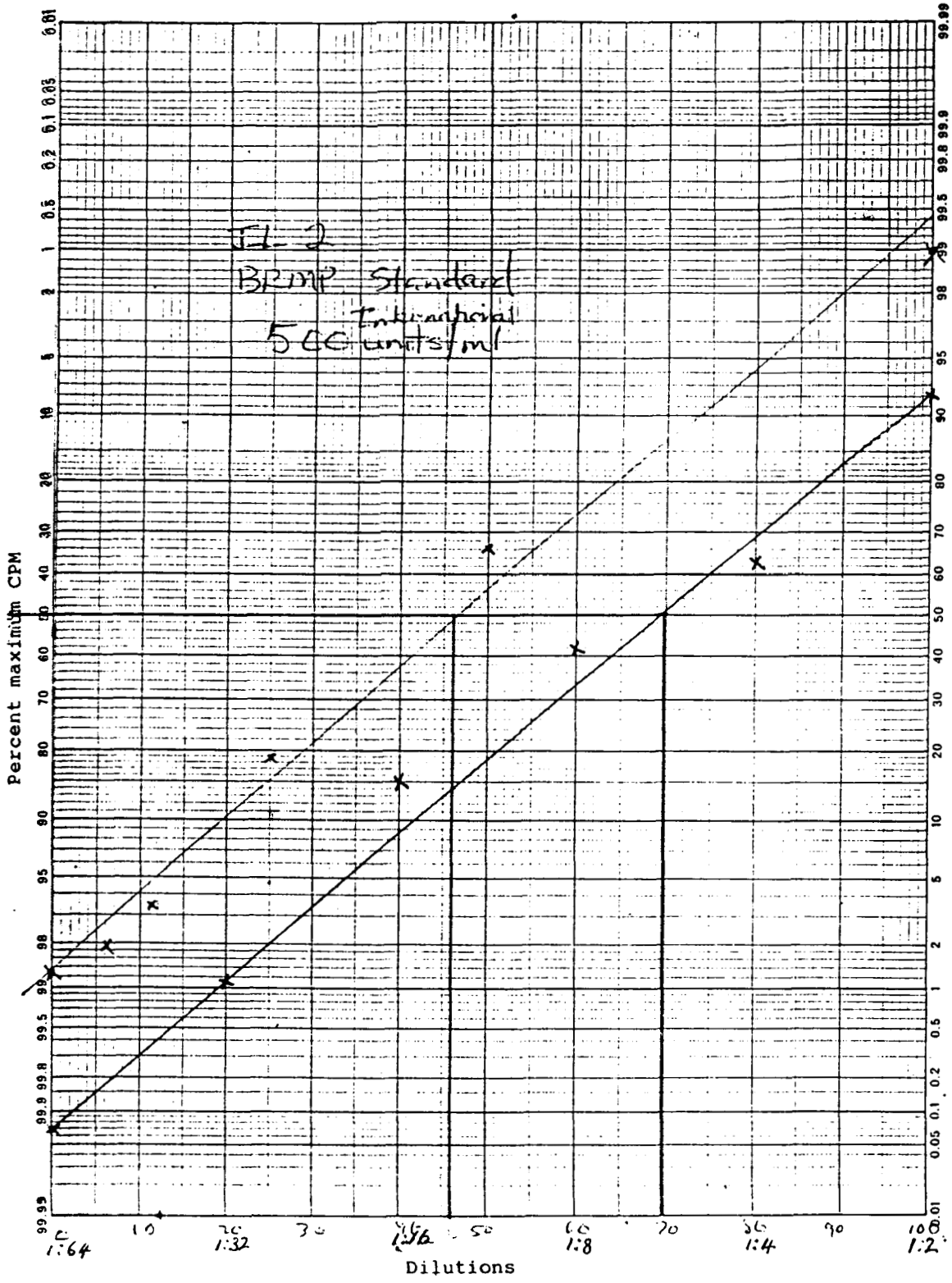


Fig. 14 - CTLL-2 proliferation assay of Interleukin-2

TABLE-20: Standardisation of IL-2 by using CTLL-2 proliferation assay.

	Dilutions	CPM	% Maximum
Standard	Control	579	1.3
	100 units	43430	99.0
	50 "	29371	67.0
	25 "	8326	19.0
	12.5 "	1564	3.6
	6.25	508	1.1
Unknown sample	1:2	40480	92.0
	1:4	28160	64.0
	1:8	18480	42.0
	1:16	6600	15.0
	1:32	484	1.1
	1:64	282 BG	---

Immunomodulatory Effect of Emblica officianalis:

The crude, dried powder of the fruit Emblica suspended in distilled water was fed at a dose of 20 mg/kg body weight orally, on every day, following tumor inoculation and all immune parameters were monitored at different intervals of tumor progression.

NK cell activity assessed against radiolabelled K-562 target cell, showed an exponential increase in activity from day 0 till day 11 of post tumor inoculation. Significant enhancement in NKCMC was documented on days 3, 5 ($P < 0.05$) 18 and 15 ($P < 0.001$) in drug treated animals with respect to tumor bearing controls (Table-21). Activity was found to peak on 12th day and thereafter a steady decline in activity has been recorded. NK cell mediated killing was not detectable on the 19th day of post tumor inoculation (Fig.15).

ADCC in Emblica treated animals, assessed against radiolabelled sensitized SRBC targets, revealed a steady increase in activity from day 3 onwards which peaked on day 11 and then an abrupt fall in activity was observed. Antibody dependant killing was undetectable on day 17 (Table-22). Enhanced cytotoxicity was significant on days 3, 7, 9, 11, 13 in drug treated animals when compared to control. However Effector cells from drug treated animals displayed reduced killing potential on day 5 when compared to tumor bearing control animals (Table-22 Fig.16).

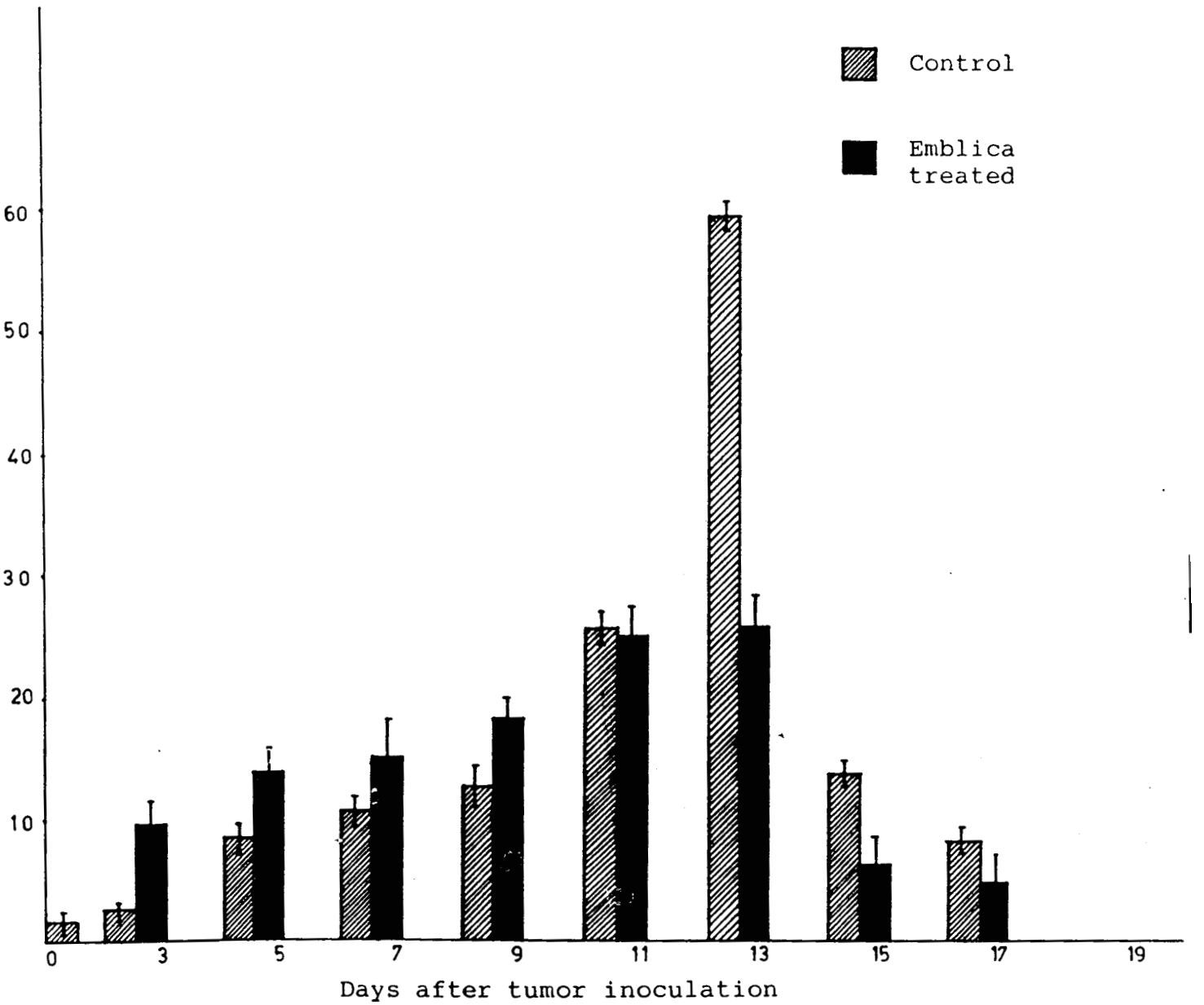


Fig. 15 - NK cell mediated cytotoxicity in tumor bearing Balb/c mice treated with 20 mg/kg weight of Emblica officianalis, at an effector:target ratio of 100:1

TABLE-21: NK cell activity in DLA tumor bearing Balb/c mice treated with Emblica officianalis (20mg/kg body weight), at an Effector : Target ratio 100:1

No.	Days after tumor inoculation	Mean % cytotoxicity ± S.D. Control	Emblica treated
1	0	1.4 ± 0.4	N.D.
2	3	2.1 ± 0.29	9.6 ± 2.10*
3	5	7.8 ± 0.56	12.6 ± 1.70*
4	7	10.1 ± 0.24	14.16 ± 2.71*
5	9	12.59 ± 1.8	17.66 ± 1.43*
6	11	25.4 ± 0.73	24.66 ± 2.88
7	13	59.67 ± 0.42	25.5 ± 2.24**
8	15	18.58 ± 0.63	6.0 ± 2.16**
9	17	7.85 ± 0.37	4.33 ± 1.24*
10	19	N.D.	N.D.

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

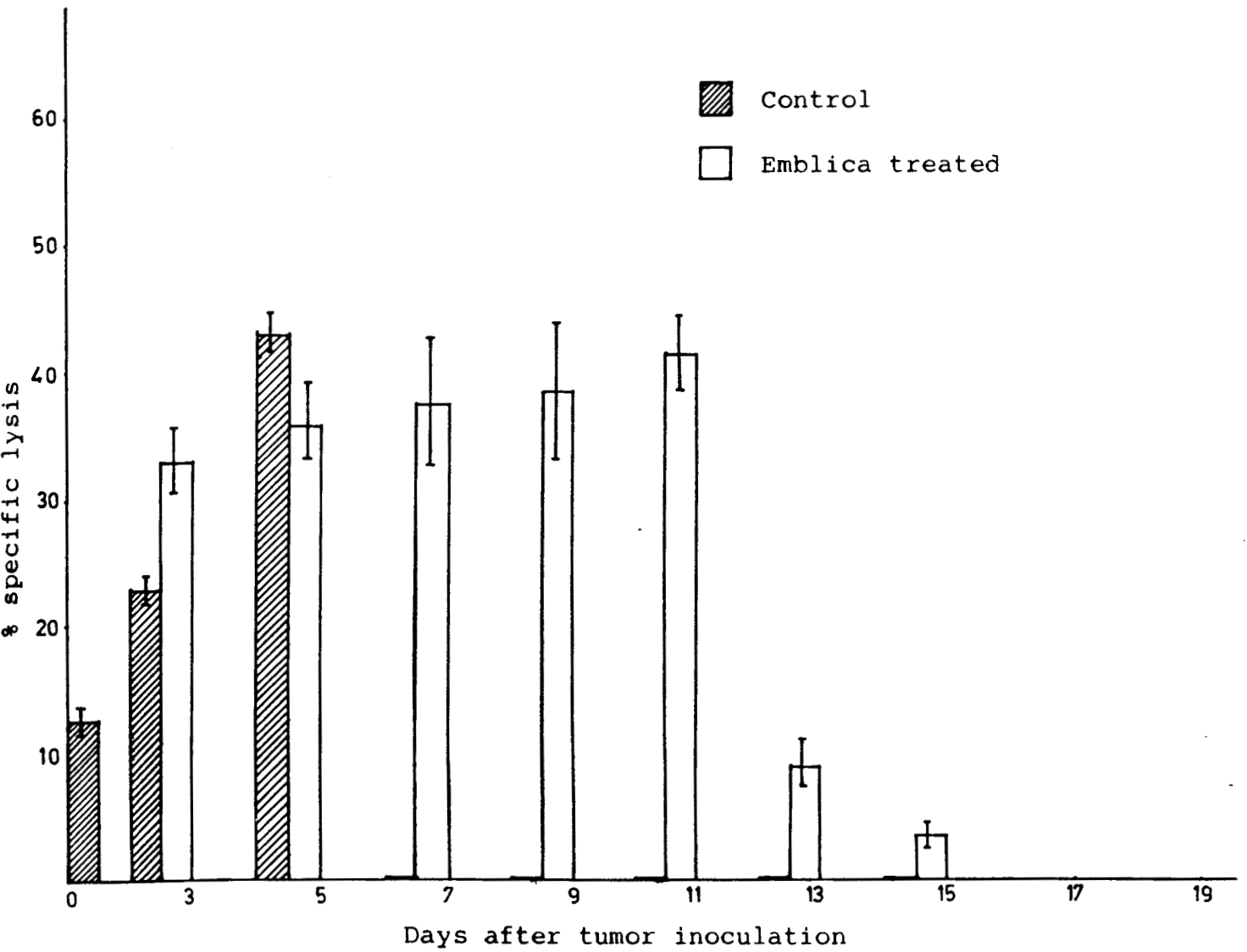


Fig. 16 - Antibody dependent cellular cytotoxicity in DLA tumor bearing Balb/c mice treated with 20 mg/kg weight of Emblica officianalis, at effector:target ratio of 100:1

TABLE-22: ADCC in DLA tumor bearing Balb/c mice treated with Emblica officianalis 20mg/kg body weight, at an Effector : Target ratio 100:1

No.	Days after tumor inoculation	Mean % Cytotoxicity ± S.D. Control	Emblica treated
1	0	12.48 ± 0.37	12.48 ± 0.37
2	3	22.6 ± 0.83	31.6 ± 2.6*
3	5	43.0 ± 1.6	35.83 ± 3.6
4	7	N.D	37.5 ± 4.50**
5	9	N.D	38.0 ± 5.35**
6	11	N.D	41.6 ± 2.50**
7	13	N.D	9.0 ± 1.08**
8	15	N.D	3.3 ± 0.89**
9	17	N.D	N.D

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

Macrophage (TAM) mediated ADCC assessed against sensitized SRBC in short term ^{51}Cr release assay did show a decrease in cytotoxic potential of the effector cells in Emblica treated animals with respect to control (Table-23) (Fig. 17).

Antibody Dependent Complement Mediated lysis in the Emblica treated animals was found to be profoundly enhanced as compared to controls. An early onset of antibody production from day 5 onwards which prolonged until 15th day of tumor inoculation was observed. The enhancement in antibody mediated lysis was significant on day 5, 7, 9 ($P < 0.001$) and on day 11 ($P < 0.05$) of post tumor inoculation. However on day 13 and 15 of post tumor inoculation drug treated animals showed a significant reduction in the activity and no activity was detected on day 17 (Table-24) (Fig. 18).

The percentage of splenic T_H was not found to be enhanced in Emblica treated animals as compared to control. A significant reduction was observed on day 9 ($P < 0.001$) and day 11 ($P < 0.05$) (Table-25). T_S cells did not show any significant change (Table-26).

In vitro assessment of immunomodulatory action of the drug Emblica by means of plaque forming assay revealed significant enhancement in the number of plaque forming cells on the 4th and 5th day after immunisation with respect to control ($P < 0.001$, $P < 0.05$ respectively) (Fig.19).

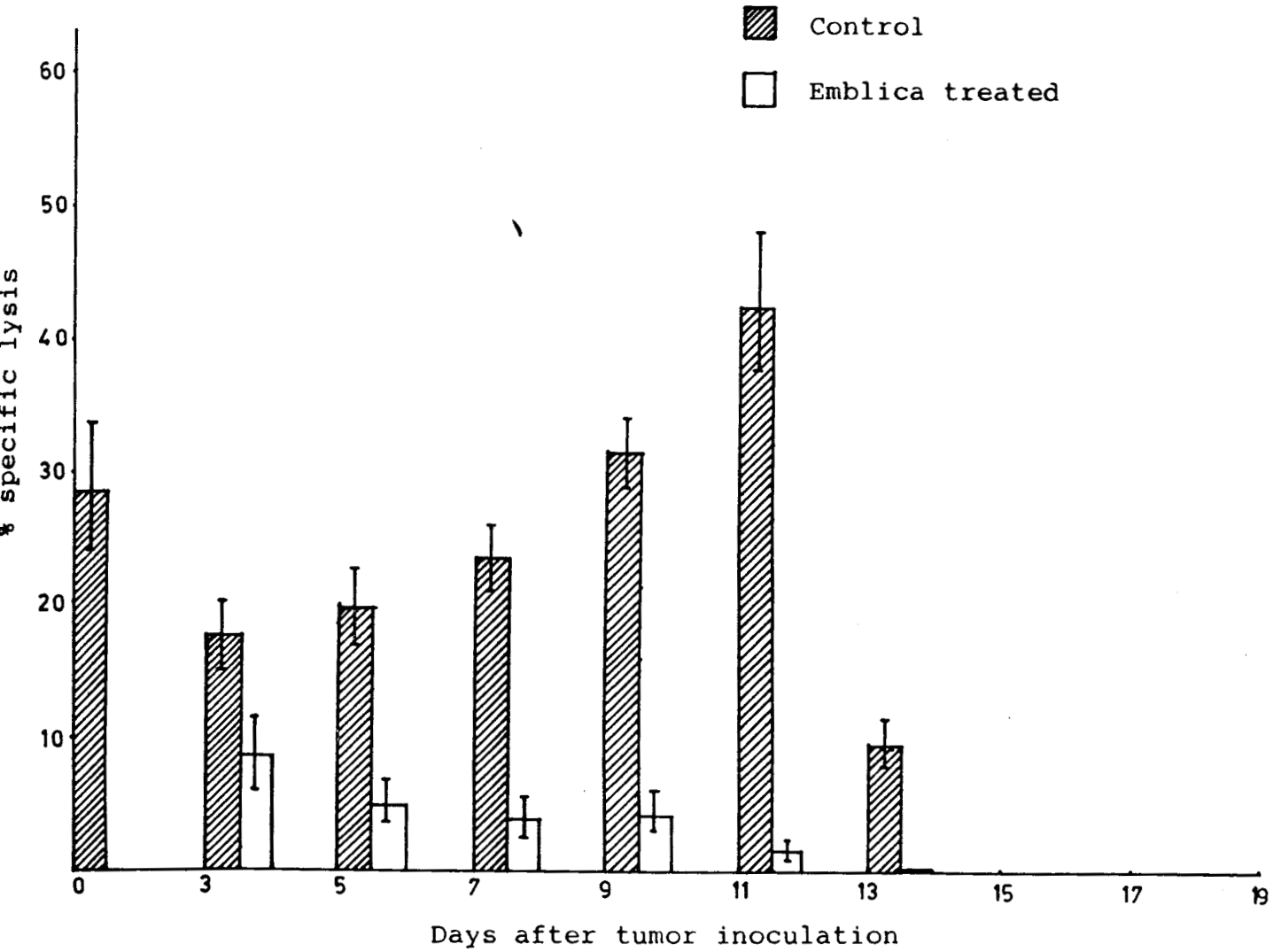


Fig. 17- Macrophage mediated ADCC in DLA tumor bearing Balb/c mice treated with 20 mg/kg weight of Emblica officianalis, at effector:target ratio of 100:1

TABLE-23: Macrophage mediated ADCC in DLA tumor bearing Balb/c mice treated with Emblica officianalis 20mg/kg body weight, at an Effector:Target ratio 100:1

No.	Days after tumor inoculation	% lysis ± S.D. Control	Emblica treated
1	0	28.3 ± 5.3	28.3 ± 5.3
2	3	17.5 ± 2.4	8.23 ± 2.8*
3	5	19.6 ± 2.5	4.21 ± 1.70**
4	7	23.3 ± 2.8	3.95 ± 1.49**
5	9	31.6 ± 3.2	1.26 ± 0.75**
6	11	42.3 ± 4.10	N.D
7	13	9.8 ± 1.96	N.D

N.D = Not detected.

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

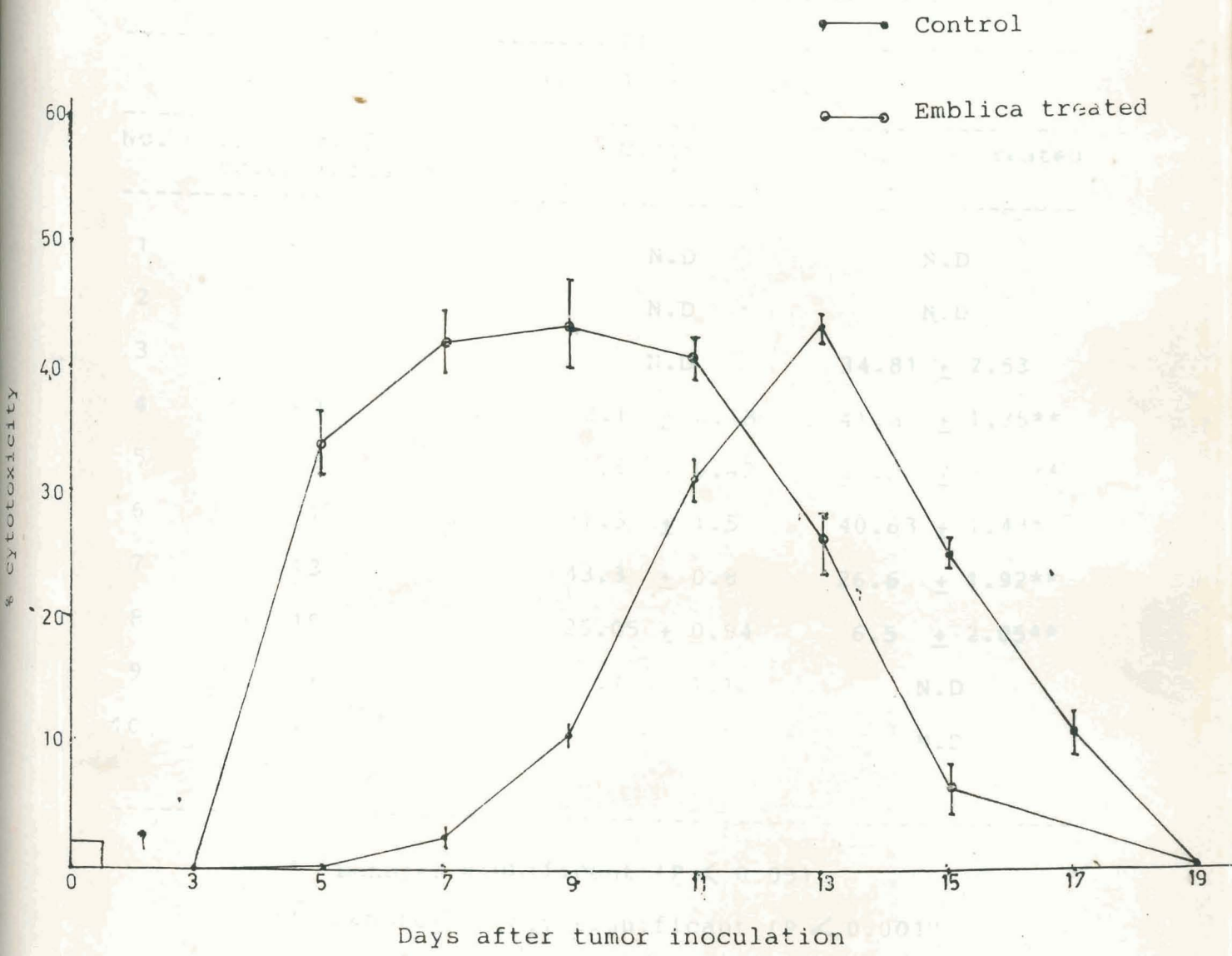


Fig. 18 - Antibody complement mediated cytotoxicity in DLA tumor bearing mice treated with 20 mg/kg weight of Emblica officianalis.

TABLE-24: Antibody complement mediated cytotoxicity in DLA tumor bearing Balb/c mice treated with Emblica officianalis 20mg/kg body weight.

No.	Days after tumor inoculation	Mean % cytotoxicity ± S.D. Control	Emblica treated
1	0	N.D	N.D
2	3	N.D	N.D
3	5	N.D	34.81 ± 2.53
4	7	2.1 ± 0.18	41.8 ± 1.35**
5	9	10.5 ± 0.42	43.2 ± 3.15**
6	11	31.5 ± 1.5	40.83 ± 1.43*
7	13	43.3 ± 0.8	26.6 ± 1.92**
8	15	25.05 ± 0.94	6.5 ± 2.05**
9	17	10.7 ± 1.1	N.D
10	19	N.D	N.D

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

TABLE-25: Percentage of splenic T cells forming ox IgM rosettes in mice treated with Emblica officianalis 20mg/kg body weight. Values are mean ± S.D. from 3 different observations in each group.

No.	Days after tumor inoculation	Control	Emblica treated
1	0	21.5 ± 2.0	21.5 ± 2.0
2	3	32.0 + 2.9	33.6 ± 2.5
3	5	28.37 ± 1.17	29.3 ± 3.68
4	7	31.1 ± 1.5	20.5 ± 2.8
5	9	30.5 ± 0.85	19.3 ± 2.05**
6	11	31.85 ± 0.54	23.6 ± 2.8*
7	13	29.5 ± 0.42	25.5 ± 2.05
8	15	24.5 ± 0.83	25.3 ± 3.39
9	17	26.0 ± 0.4	23.3 ± 1.78
10	19	23.0 ± 0.4	24.0 ± 1.63

* Denotes significant ($P < 0.05$)

** Denotes highly significant ($P < 0.001$)

TABLE-26: Percentage of splenic T cells forming ox IgG rosettes in mice treated with Emblica officianalis 20mg/kg body weight. Values are mean ± S.D. from 3 different observations in each group.

No.	Days after tumor inoculation	Control	Emblica treated
1	0	24.7 ± 2.4	24.7 ± 2.4
2	3	18.0 + 0.83	26.0 + 4.8
3	5	23.5 ± 0.69	18.6 ± 2.5
4	7	22.5 ± 0.45	19.0 ± 2.4
5	9	22.4 ± 2.5	22.0 ± 4.5
6	11	20.5 ± 0.633	28.5 ± 3.6
7	13	26.7 ± 2.9	21.0 ± 2.9
8	15	27.5 ± 1.6	26.6 ± 2.05
9	17	26.5 ± 0.29	27.3 ± 4.10
10	19	27.0 ± 0.41	28.16 ± 0.62

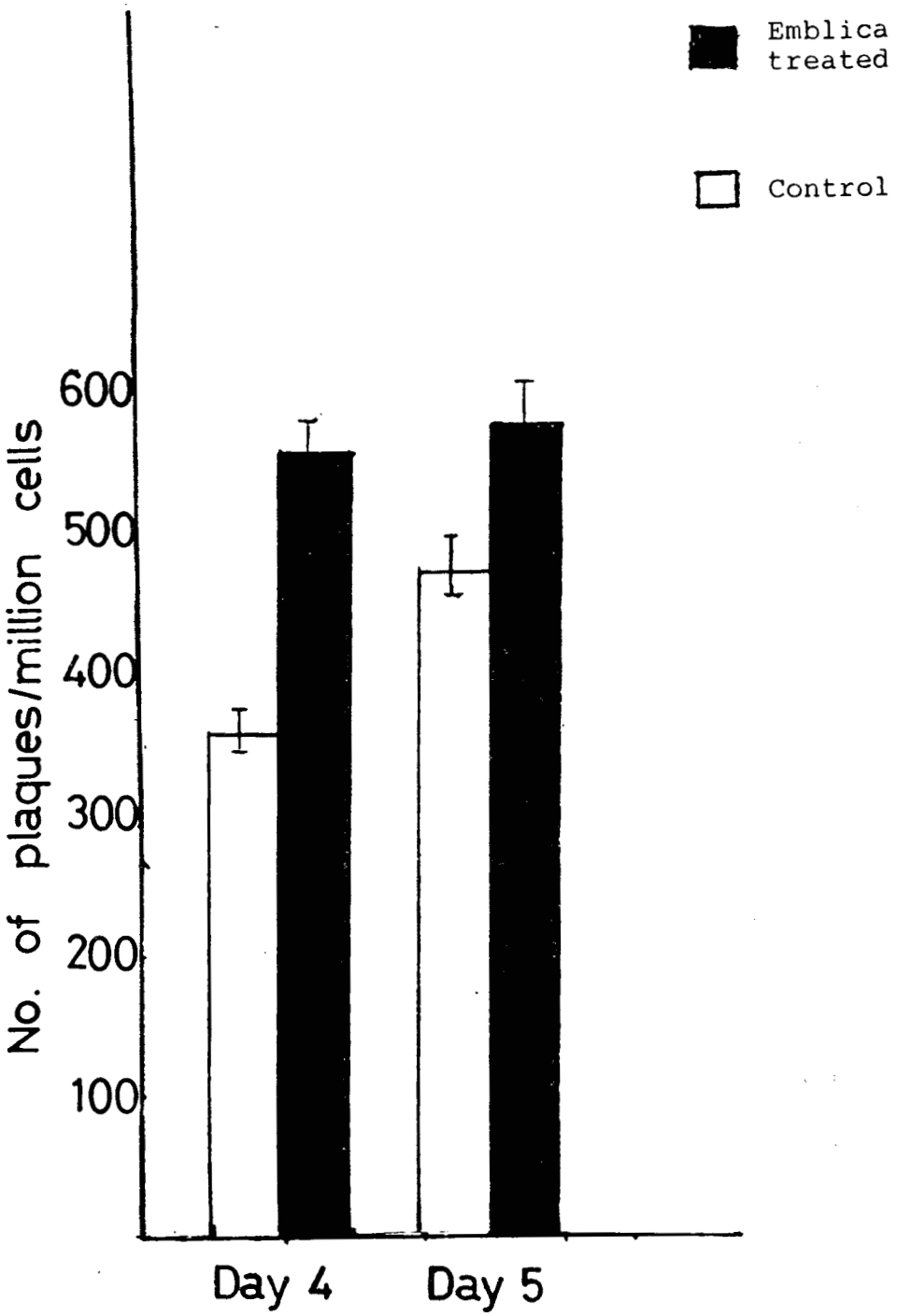


Fig. 19 - In vitro assessment of Immunomodulatory effect of drug Emblica officianalis by using Jern's plaque assay.

TABLE-27: Effect of Emblica officianalis on life span of DLA tumor bearing mice.

	Dose in mg/kg body weight	Mean survival time (days)	% increase in life span
Control	No drug	20	--
Emblica officianalis	20 mg	27	35%

Values are the mean of 6 animals per group repeated thrice.

An increased life span of 35% was observed in drug treated animals compared to control (Table-27).

ASSESSMENT AND AUGMENTATION OF IMMUNOLOGICAL STATUS DURING TUMOR DEVELOPMENT

Submitted to the University of Calicut
for the Degree of

Doctor of Philosophy

In Immunology (Faculty of Medicine)

By

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DISCUSSION

DISCUSSION

The growth of tumor is a multistep process characterised by progressively increased malignant potential of the tumor cell in the face of host resistance. Tumor specific or associated antigens generally expressed during malignant transformation of cells may elicit humoral and cell mediated immune responses in the host. Immune destruction of tumors is a complex process that involves a variety of effector mechanisms including cytotoxic T cells, macrophages, Natural Killer (NK) cells, K cells and humoral antibodies. One of the more contraversial issues regarding the progressive growth of immunogenic tumors in mice is the problem concerning the relative importance of inadequate host immunity and tumor heterogenity in the escape of tumor from immunological rejection. The very existance of antigenic tumors implies that neoplastic cells have escaped the immunological surveillance mechanism. Eventhough host immune system is mounting an antitumor immune response, often it seems inadequate, so as to cause relentless growth of tumor.

In this thesis, immunological profiles during the tumorogenesis of Dalton's lymphoma ascites tumor (a murine T cell lymphoma) as assessed by NK cells, T Cell, ADCC, MMADCC and ACC activities have been studied.

NK Cell activity is absent in new born spleen and matures to adult level in 4-8 weeks, followed by a period of decline

(Herberman et al 1975). In our studies, normal NK cell activity was found to be very low. In normal control mice NK cell activity against K-562 target cell has been reported to be very low (Lanza and Djeu 1982). Significant activity was detected on day 5 after tumor inoculation (7.8% ± 0.56). The activity increased gradually, peaked on day 12 (59.67% ± 0.42) and then declined to the basal level as the tumor growth progressed (Table-1). Augmentation of NK activity has been reported in mice inoculated with tumor cells bearing relevant antigen (Herberman et al 1977, Wolf et al 1976, 1977, Tracey et al 1977, Herberman 1982). Stutman in 1975 observed longer latent periods for the development of tumor induced by Moloney Sarcoma Virus in nude mice which expresses high levels of NK activity than normal. However since all the tumors that arise in nude mice grew progressively, NK cells did not appear sufficient to completely control the tumor growth. NK cells provide a transient suppression to the growth of tumor at a very early phase of tumor growth (Urban and Shreeber 1982, Sarin and Saxena 1989). However if the tumor byepasses rejection mechanism for some reason and reaching critical size NK cells would no longer be operative. Therefore NK cells may not provide absolute protection against tumor growth. It is possible that regression of tumor is mediated by combination of T cell and NK cell cytotoxic activities (Gerssen et al 1982). NK activity could no longer be detected after day 17, when tumor reaches maximum size. Progressive decline in NK activity as the tumor growth advanced, with little or no activity on day 19 has been observed during the tumorogenesis

of DLA. A similar NK cell kinetics have been reported in other tumor models (Dewey et al 1984). It has been reported that lower level of NK activity is detected at the site of progressive Moloney Sarcoma Virus induced tumor than at the site of regressing tumors (Gerssen et al 1981). The cytotoxic activity of NK cells has been shown to be inhibited by Fibrin Coagulation occured around tumor mass by murine plasma or serum (Gunji et al 1988). Monomeric IgG has been reported to negatively regulate NKCMC by inhibiting post binding events (mediated in part by cyclic AMP via Fc receptor on NK cells (Sulica et al 1982, Wilson and Coombo 1985, Bancu et al 1988). Increase in the titre of anti-DLA antibody demonstrated on day 13 tumor inoculation possibly could block NK cell mediated cytotoxicity. Sera from tumor bearing mice has been reported to inhibit NK cell activity and ADCC activities of murine spleen cells in in vitro cytotoxicity assays (Nair et a 1980). Brunda et al (1980) have demonstrated that splenocytes from mice bearing Moloney Virus induced sarcoma have severely depressed NK cell activity, which could be restored to normal level by administration of prostaglandin inhibitors. Prostaglandin PGE-2 produced by cells of Macrophage-monocyte series can negatively regulate NK cell mediated cytotoxicity (Koren et al 1981, Kennedy et al 1986).

Evaluation of ADCC by K cells in relation to tumor growth is a pre-requisite for understanding its contribution in host tumor interaction. In the present study detectable ADCC was observed on day 0, which progressed steadily to peak on day

5 post tumor inoculation. Thereafter abrupt fall of activity was recorded (Table-2). Increased ADCC activity paralleling with progressive growth of syngeneic mammary tumors in Balb/c mice has been reported (Lopez et al 1977). During the initial phase of tumor development, the tumor may release small amount of antigen which may lead to the production of antibody that in turn may bind to circulating antigen to form antigen-antibody complexes. The concentration of antibody produced initially may be small either because the TAA may be a weak immunogen or concentration may be very low, thus forming circulating immune complexes (CIC), which may remain in soluble form. These complexes could inhibit the lymphoid cells mediating ADCC by binding Fc receptors. Small concentrations of TAA observed in the sera of tumor bearing animals, during initial stage of tumor development may be responsible for blocking factors in plasma (Saha and Ray 1982, Ray Chaudhuri et al 1980). The sudden decline in ADCC observed after 5th day of DLA tumor transplantation could be attributed to plasma blocking factors; such as CIC and other soluble immunosuppressive factors generated during the early phase of tumorogenesis. Plasma blocking factors may either bind to Fc receptors on cytotoxic lymphoid cells thus abrogating their reactivity against target cells or act on suppressor cells by activating their suppressive effect (Ray Chaudhuri, 1980).

The major infiltrating components in primary and transplanted neoplasms have been identified as mononuclear phagocytes by surface markers and ultra structural studies.

Macrophage mediated ADCC (MMADCC) was assessed against ^{51}Cr labelled sensitized SRBC. Resident macrophages (RM) displayed considerable amount of antibody dependent killing (28.3% ± 6.3) as indicated in Fig. 3. Tumor Associated Macrophage mediated ADCC showed a gradual increase from day 3 onwards which peaked on day 11 post tumor inoculation (42.3% ± 4.10). The activity was found to decline after day 11 and no TAM mediated killing was detected on the 15th day of tumor inoculation. Higher erythrocyte-amboceptor (EA) binding by TAM as compared to that obtained by RM during the early days of tumor growth has been reported (Bhattacharya et al 1986). Binding of antibody coated SREC target constitutes the essential primary step in ADCC activity. The increased number of conjugates formed by TAM with sensitized targets indicates enhanced expression of Fc receptor function. Increase in the number of macrophage precurosrs in the bone marrow after 4 days of murine mammary tumor growth has been recorded (Baum et al 1972). With advancement of tumor growth, the ADCC mediated by TAM gradually declined to basal level at day 13 and at day 15 no activity could be detected. The observed impairment of cytolysis by TAM with advancement of tumor growth might be due to the release of inhibitory factors by growing tumors. A low density lipoprotein fraction of cell free ascites from syngeneic MM-46 tumor bearing mice was reported to inhibit ADCC in vitro (Yamazaki et al 1979). The inhibitory activity increased with the increase in tumor mass and the lipoprotein induced inhibition of ADCC was suggested

to be due to the functional depression of macrophages (Hattori et al 1980).

In our initial studies, peak CTL response was found on 4th day of co-culturing. Hence this was used as a standard in further experiments. CTL activity estimated by release of ^{51}Cr from labelled Dalton's Lymphoma ascites tumor cells showed detectable activity on the day 10 after immunisation (after 4 days of co-culture) and then activity gradually decreased (Table-4). Anti-tumor activity displayed by CTL in 4 hrs ^{51}Cr release assay has been found to be very low (3.85% $\pm$ 0.153). In weak syngeneic system, cytotoxicity mediated by CTL is transient and is often difficult to detect. This may be due to relatively low frequency of effector cells and/or the presence of factors interfering with lytic mechanism (Vasudevan et al 1974). It has been reported that anti-tumor activity of splenic CTL was not detectable by the in vitro cytotoxicity assay using 4 hrs ^{51}Cr release assay (Fuyama et al 1986). According to North and Bursuker (1984), T cells capable of causing regression of the recipient tumor were generated on about day 6 of tumor growth, reached peak number on day 9 and were then progressively lost until day 15 when their presence no longer could be detected. Both Meth-A and P815 Mastocytoma have the same kinetics (North and Dye 1985). Studies on P815 Mastocytoma bearing animals revealed that T cells were generated and lost in concert with the generation and loss of T cells capable of lysing P815 tumor cells in vitro. The progressive decay of concomitant immunity after

day 10 of tumor growth of DLA may be caused by the negative immunoregulatory function of suppressor T cells that are acquired progressively from day 9 onwards. Elevated level of T_s after day 11 tumor inoculation which sustained until day 19 has been documented. On day 12 of the growth of the DLA, tumor spleen contains a mixture of effector and suppressor cells such that neither type of cell is totally dominant. It has been demonstrated that an infusion of suppressor cells from donors with a 14-16 day Meth-A tumor can prevent concomitant immunity being generated (Bursuker and North 1985). The progressive blocking of further effector T cell production after day 12 of tumor growth results in declining of immunity. It has been reported that nine to fifteen days after tumor inoculation, tumor bearing spleen cells inhibited the growth of specific type of tumor cells suggesting the emergence of a specific cell-mediated immune response (Yamagishi et al 1983). Soluble form of B16 Melanoma tumor antigen suppressing immune response in C57 Bl/6 mice through inhibition of CTL activity has been reported (Takahash et al 1988). Isolation of low molecular weight inhibitor of lymphocyte proliferation from asictic fluid of P815 Mastocytoma cells in syngeneic DBA/2 mice has been reported (Corneleus and Norman 1988). Induction of specific T_s that block CTL generation has been suggested as the major route of escape of tumor cells from immune surveillance.

Humoral immune responses was demonstrated by using DLA tumor cells treated with antibody from tumor bearing mouse and

fresh rabbit complement. The activity was observed on day 9 (10.5% ± 0.415) with a peak on 13th day (43.3% + 0.8) which gradually declined and no activity was detectable by 19th day (Table 5). The kinetics of antibody dependent complement mediated cytotoxicity against spontaneous adenocarcinoma (ADK-It) were studied at various intervals of tumor growth and the peak has been observed at 10 days after tumor inoculation (Landolf et al 1977).

According to Saha and Ray (1982) and Ray Chaudhuri et al (1980), cytotoxic antitumor antibody activity increased directly with increase in size of the tumor. Antibody complement mediated cytotoxicity has been demonstrated in the serum of Balb/c mice bearing syngeneic fibrosarcoma (Menard et al 1977). The level of antitumor activity in serum increased following the incculation of tumor cells. The antitumor cytotoxicity revealed in vitro seemed to exert a specific in vivo protection as suggested by the indirect correlation found between the level of antitumor reactivity and growth of transplanted fibrosarcoma.

In the present study, a gradual decline in activity has been recorded after day 13. Humoral activity was not demonstrated after the 17th day of tumor inoculation. By this time, tumor reaches maximum size because of the accumulation of large amount of ascitic fluid in the peritoneal cavity. An exponential increase in the abdominal circumference with

progressive growth of the tumor has been noted. The impairment in the humoral response at the later stage could be explained by the presence of plasma blocking factors. The high concentration of TAA in the sera of large tumor bearing animals may induce the production of suppressor cells. Although antitumor antibodies are found at this stage, their production may be very soon limited because of the overall immunosuppressive actions on B cells by suppressor cells. Such antibodies may not gain access to the tumor cells because of the abundant quantity of TAA encountered. Large immune complexes are formed which may further stimulate activity of suppressor cells. The above hypothesis can be substantiated by the observed enhancement in the number of T_S with concomitent depression of T_H cells in tumor bearing mice from day 11 onwards (Table 6 and Table 7). There have been several reports on the role of suppressor cells in the development of tumors and also for promoting their growth (Uchida and Hoshino 1980, Giffored et al 1981, Mitzushima et al 1981, Tilken et al 1981). Complement mediated lysis of guinea pig ascitic Hepatoma tumor cells coated with specific rabbit antibody was inhibited by ascitic fluid or serum from tumor bearing animals (Bercizi et al 1977). Immunosuppressive factors has been also reported in Ehrlich's ascites fluid (Yamazaki et al 1973).

A crucial problem in tumor immunology is the depression of the immunological responses during progressive tumor growth. Mice bearing DLA tumor show appreciable specific and non-specific cellular and humoral immune responses after the

onset of tumor growth. But the antitumor activity steadily declined with progressive growth of rapidly dividing tumor cells and little or no tumoricidal activity was observed at the advanced stage of tumor development. Several reports have indicated that antigenic tumors can grow in vivo inspite of the fact that host may contain sensitized lymphocytes that have the ability to show antitumor cytotoxicity in vitro. These lymphocytes cannot deliver their cytotoxic effect against the tumor cells in vivo because of the immuno-suppressive factors (Ray 1982, 1983, Ray Chaudhuri et al 1980, Ray and Ray Chaudhuri 1983). Cell-mediated immunosuppression causing facilitation of tumor growth has been described as being mediated by specific T suppressor cells (Yamagishi 1983, Dye and North 1984). It has been reported that non-specific tumor facilitating cells are phagocytic adherent population, presumably macrophages. It has been further reported that tumor specific CMI was operative as long tumor diameter was 0.5 cm to 1 cm. With the increase in tumor size, the splenocytes showed tumor growth enhancement (Ray Chaudhuri et al 1980, Saha and Ray 1982). High concentrations of TAA in the sera of large tumor bearing mice may induce production of suppressor cells (Ray and Saha 1985). This may result in complete energy in large tumor bearing mice, allowing the tumor to grow uninterruptedly (Ray and saha 1985). Prostaglandin E2 produced by mononuclear suppressor cells has a major role in the regulation of both CMI and humoral response, T cell proliferation, lymphokine

production as well as macrophage and NK activity. Increased production of PGE by both naturally occuring and experimentally induced cancers has been reported (Roland 1980, Glaser 1980).

Several studies have suggested that circulating immune complexes (CIC) may favour tumor growth. An association between the concentration of CIC and poor prognosis is frequently observed in malignancy (Carpenter and Miescher 1983). Immune complexes can bind Fc receptors on target tumor cells and thus mask their antigenic sites or can react with lymphocytes abrogating their activity against tumor cells. Finally it can act on suppressor cells by activating their suppressive effect (Ray Chaudhuri et al 1980). Antibodies also had been reported to have the ability to facilitate tumor growth. It is not known what fundamental differences confer on them the ability to facilitate tumor growth in certain cases while destroy tumor targets in certain other situations. Extensive studies have been conducted by Ray et al (1984) on the role of immune complexes in cancer. It has been demonstrated that irrespective of the tumor model and tumor type, plasma adsorption can cause tumor regression, possibly through the elimination of blocking factors mainly CIC (Ray et al 1984, Ray and Bandyopadhyay 1983). The eclipse of concomitant immunity after the tumor reaches a critical size has been reported (North, 1984). Animals bearing tumor can posses macrophages capable of non-specifically suppressing T cell response in vitro (Mitzushima et al 1984).

North and Bursker (1984) reported that cells on passive transfer can inhibit the expression of passively transferred immunity against established tumor in TXB recipients were not acquired until about day 9 of tumor growth. Suppressor cells progressively increase in number after this time until about day 18 (North and Dye 1985).

The progressive decay of concomitant immunity after day 12 ot tumor growth of DLA in the present study may be caused by negative immunoregulatory function of suppressor T cells that are acquired progressively from day 11 onwards (Table-7). Bursuker and North (1985) demonstrated that an infusion of suppressor cells from donors with 14-16 day Meth-A tumor can prevent concomitant immunity being generated. Because of all available evidence is consistant with the interpretation that tumor induced suppressor T cells as well as plasma blocking factors such as circulating immune complexes and other soluble factors may act in concert in the case of DLA tumor to down-regulate already ongoing concomitant immune response. It is logical to Hypothesize that suppressor T cells are responsible for the escape of this tumor from immune destruction.

In the earlier part of the present study the kinetics of splenic NK cell mediated cytotoxicity during tumorogeneis of DLA tumor has been demonstrated. The subsequent investigations were intended to explain the observed NK cell profiles. NK cell target interaction is not subject to the

exquisite specificity shown in the case of lysis mediated by T cells and is not MHC restricted. For instance, rat NK and mouse NK cells are capable of lysing cells of the human Erythroleukemic line K-562 (Potter and Moore 1978, Brunda et al 1984, Lichenstein and Pinde, 1986). Two different methods of assessing NK cytotoxicity in vitro to dissect the relative importance of various steps involved in attaining the overall result have been used. The ^{51}Cr release assay has been combined with a recently established single cell conjugate assay using poly-L-Lysine coated cover slips to allow better quantitative analysis. With this double procedure, percentage of active killer cells, V_{max} and recycling capacity of the effector cells at different intervals of tumor progression have been estimated. LGL enriched population obtained by discontinuous density centrifugation of Percoll was used as effector cells, against K-562 target cells in both the assays. Enumeration of LGL forming conjugates with target cells provides a direct method for determining the number of cells capable of recognition and capable of potential lysis. Among purified population of LGL 30-40% of LGL formed conjugates with K-562 targets indiscriminately, irrespective of the days after tumor inoculation. It has been reported that all lymphocytes which bind to NK susceptible target cells are not capable of initiating cytotoxicity (Targan et al 1980, Bonavida et al 1984). Furthermore, it has been shown that normal cells, including adult fibroblasts may be bound by NK cells, which are resistant to lysis (Brooks et al 1984,

Trincheri et al 1981). These observations suggest that in addition to NK recognition structure expression, the induction of lysis is also influenced by inherent resistance mechanism which operates at post-recognitive stage of the lytic process. However, percentage of conjugates containing dead targets increased from day 0 to day 12 post tumor inoculation and was found to be reduced on the day 19, as compared to other days. Percentage of active killer cells was found to be increased on day 7 and day 12 with respect to control (Table-9). The estimated maximal recycling capacity of the effector cell however did not show any variation. Percentage of active killer cells, estimated at different phases of tumor progression correlates well with NK cell profiles studied using total spleen cells as well as purified LGL against K-562 target cells in 4 hrs ^{51}Cr release assay. Percoll density gradient enriched effector cells showed a similar lytic pattern against K-562 targets; even at a low effector : target ratio 5:1, in ^{51}Cr release cytotoxicity assay (Table-8). Previously it had been demonstrated that both NK and ADCC activities, spontaneous and IFN inducible, are confined to the LGL enriched fractions (Timonen et al 1981; Reynolds 1981). Approximately 20% of small conventional lymphocytes were reported to form conjugates with NK susceptible target cells, but are not associated with cytotoxic activity (Timonen et al 1981). This explains low cytotoxicity displayed by whole spleen cells at high effector : target ratio compared to LGL at low effector : target ratio.

Culture supernatant of effector cells and K-562 was tested for NKCF activity against K-562 target cells in a microsupernatant assay. Considerable level of NKCF activity was observed in the culture supernatent of LGL from day 0 and day 7. Peak NKCF activity was observed on day 12 and on day 19 a significant fall in activity was noticed with respect to the control. NKCF production by LGL has been found to be closely associated with lytic potential of the effector cell at different phases of tumor growth. Substantial levels of NKCF detectable in a 48 hrs ^{111}In release cytotoxicity assay of K-562 target cell has been reported in the supernatant of LGL co-cultured with K-562 target cells (Herberman et al 1984). An increase in mRNA for Interferon gamma and TNF alpha has been reported following exposure of NK cells to NK sensative cell line K-562 (Windebank et al 1988). Evidence has accumulated for the elaboration by NK cells of a NK cytotoxic factor or factors (NKCF) which interact with susceptible target cells and initiate their destruction (Wright and Bonavida 1981, 1982, Wright et al 1983, Farram and Targan 1983).

Since no impairement in conjugate formation was observed in the LGL of tumor bearing animals even at the advanced stage of tumor growth (day 19), when the NKCF production was significantly diminished, it is evident that post binding events in the cytotoxic process are mainly effected in the later stage. These include lymphocytes bound to dead targets,

the active killer cells, the killing potential and NKCF production. It has been proposed that initiation of lysis requires two, presumably independant sites on the target cell membrane, one of which serves as a binding site for NK cells and the other as receptor for NKCF (Wright and Bonavida 1983). It has been demonstrated that the decrease in NKCF release seen with Interferon pretreated stimulator cell cannot be accounted for by a decrease in the ability of effector cells to recognize and bind target cells (Wright and Bonavida 1983). Interferon pretreated YAC-1 cells exhibited a defect in their ability to stimulate release of NKCF from the effector cell. This defect is not due to a failure of NK cells to recognize and bind the targets, because Interferon pretreated YAC-1 cells form a normal number of conjugates with spleen cells. It has been suggested that the relative resistance of Interferon pretreated target cells to lysis in NKCMC could be attributed solely to a defect in their ability to stimulate release of NKCF from the effector cell (Wright and Bonavida 1983).

However, estimation of MRC showed minimal difference at different phases of tumor development. The observed reduction in the percentage active killer cells in conjugate assay and impairment in NKCMC in ^{51}Cr release cytotoxicity assay on day 19 could be attributed to a limited production or release of NKCF by LGL on day 19. Defective functioning of the lytic event which occur after effector cell target cell conjugate has been formed could be the basis for the decline in NKCMC

in animals at later stages of tumor growth. Depressed NK cell mediated cytotoxicity has been demonstrated in several tumor models at the later stages of tumor development (Brunda et al 1983, Sojo et al 1984).

Incubation of day 19 effector cells with 200 IU/ml of human rIL-2 or equivalent rat spleen growth factor for 72 hrs at 37^{o}C in 5% CO_2 resulted in significant increase in NKCMC (2.26% to 12.9%) (Table-8) and percentage active killer cells (2.58% to 7.2%) (Table-9). Similarly NKCF activity was found to be augmented after pretreatment of the effector cell with rIL-2 (7.4 to 18.4) (Table-10). The finding that the IL-2 induced augmentation of NKCMC in ^{51}Cr release assay correlates well with IL-2 induced augmentation of NKCF activity, supports the hypothesis that NKCF could be the lytic mediators in NKCMC. The ability of IL-2 to stimulate continuous activity of NK cells have been demonstrated in several animal tumor models (Hefeneider et al 1982, Pross et al 1982, Domzig et al 1983, Mazumdar and Rosenberg 1984). IL-2 can either directly activate LGL to enhance NK activity in the absence of adherent cells or induce the synthesis of Interferon gamma by NK cells which can also augment NK activity independently (Kashara et al 1983, Kawase et al 1983, Rooks et al 1985). Interferon or polycytidilic acid pre-treatment of effector cells has been reported to increase the number of active NK cell (Targan and Dorey, 1980), NKCF activity and NK activity in ^{51}Cr release assay. Interferon pretreated spleen cells produce approximately twice as much NKCF as untreated cells (Wright and Bonavida 1983). In conclusion, the IL-2 induced augmentation

of NK activity could be accounted for by an increase in synthesis or activation or release of NKCF.

In classical immunological teaching, cytotoxic T lymphocytes (CTL) would be considered as the main candidate for mediation of specific and potent resistance against tumor growth and progression (Herberman 1980). Earlier studies have demonstrated the low (% specific lysis) tumoricidal capacity of in vitro generated syngeneic murine CTL in ^{51}Cr release assay of tumor target cells (Vasudevan et al 1974, Fuyama et al 1986, Fearon et al 1988). It has been reported that in weak syngeneic system, cytotoxicity mediated by CTL is transient and often difficult to detect. This may be due to the relatively low frequency of effector cells and/or the presence of factors interfering with lytic mechanism (Vasudevan et al 1974, Fuyama et al 1986). One of the major hurdles confronted in earlier studies was the generation of large number of syngeneic CTL with antitumor activity. The discovery of T cell growth factor (TCGF) or IL-2 has facilitated the propagation and expansion of distinctive cell population (Smith et al 1979). IL-2 has been successfully employed in recent years for long term culture of CTL lines, and murine and human effector cells produced in MLTC or MLR culture of lymphocytes from tissue of normal, sensitized or tumor bearing animals (Kedar and Weiss 1983).

Therefore in the present study, attempts have been made to expand the in vitro generated CTL against DLA tumor, with

human recombinant IL-2 and test cytotoxic potential in vitro as well as in vivo against DLA tumor target cells. When the effector CTLs were further incubated with 200 IU/ml of rIL-2 for 7 days, a significant enhancement in tumoricidal activity against DLA tumor cell was observed in 4 hrs ^{51}Cr release assay (Table-11). The activated CTL also displayed growth inhibitory activity against DLA cells in vivo as shown in Winn neutralisation assay in Balb/c mice (Table-12). The ability of IL-2 to induce expansion and continuous growth of mature T cells in vitro (Smith 1984) suggested possible therapeutic application of IL-2 as a growth factor allowing amplification of useful specific T cell mediated immune response in vivo. IL-2 has been reported to stimulate antitumor activity of NK cells and cytotoxic T lymphocytes (CTL) in vitro (Domiz et al 1983, Grimm et al 1983, Rosenberg 1985), coupled with successful IL-2 adoptive cell therapy in several tumor models (Mazumdar and Rosenberg 1985, Lafriniere and Rosenberg 1985).

Extending these observations, it has been further shown that rIL-2 activated CTL were highly effective in suppressing solid tumor targets when adoptively transferred to tumor bearing syngeneic mice (Table-12). In addition, it is notable that surprisingly small number (10^7) of the cells, when transferred adoptively were sufficient to produce tumor regression in mice along with 180 mg/kg. wt. Cyclophosphamide i.p. given 1 hr before cell transfer. Almost a similar effect could be produced by the adoptive transfer of same number of

activated CTL alone (Fig. 8). In many adoptive tumor immunotherapy models, including virus induced tumors, the therapeutic action of $CD8^+$ ($Lyt\text{-}2^+$) CTLs in immuno competent animals is offset by "suppressor cells" that are usually $CD4^+$ (Greenberg et al 1984, Cheever et al 1986, North et al 1989). It has been reported that administration of suboptimal dose of Cyclophosphamide combined with adoptive cell transfer can significantly enhance the therapeutic efficacy of the effector cells (Glaser 1979, North and Dye 1982, North 1984). This may be due to the selective elimination of suppressor cells by Cyclophosphamide (Naito et al 1988).

Antitumor effectiveness of the activated CTL was tested against a spectrum of non-specific target including YAC-1 cells, Sarcoma-180 and Ehrlich's ascites tumor. Negligible amount of tumoricidal activity was displayed by the effector cells against these targets in 4 hrs ^{51}Cr release assay. Non-reactivity of the effector cells with non-specific targets revealed the specific nature of the effector cells. It may be noted that $CD8^+$ ($Lyt\text{-}2^+$) CTLs with therapeutic activity against virus induced tumors have been found to be directed against proteins encoded by the virus (Melief and Kast 1990). CTLs active against Moloney Sarcoma Virus induced tumors, for example, were found to be directed against membrane protein gP85 gag, which is related to the major internal viral protein (Vander Hoorn et al 1985). Tumor induced in rodents by the DNA tumor viruses, SV40, Polyoma viruses and human adeno-virus flourish in immunodeficient or immunosuppressed animals and

bear virus encoded tumor specific transplantation antigens (TSTA). These antigens have been shown to be the targets for T cell-mediated antitumor activity in immunocompetent aniamls (Sawada et al 1986, Bellgrau et al 1988). Specificity of the antitumor activity of CTL clone K4L has been demonstrated by in vitro cytotoxicity and tumor growth inhibition assays against murine leukemia L1210 (Rahman et al 1988). Cultured T cells (CTL/RFB) specifically cytotoxic to RFB-23 leukemia were derived by incubating spleen cells from animals immunised with RFB-23 in the presence of IL-2 and sensitizing antigen (Jhonson et al 1986).

Surface phenotypic characterisation of rIL-2 activated effector cells were performed by depletion of Lyt-2^+ lymphoid cells by treatment with anti Lyt-2^+ antibody and complement. Depletion of effector cells with Lyt-2^+ phenotype completely abrogated the anti-tumor activity of CTL against DLA cells in the in vitro killing assays (Table-11). Word et al (1988) have reported that adoptive transfer of specifically sensitized T lymphocytes can effectively mediate the regression of established local and metastatic tumors. Using monoclonal antibody, depletion of Lyt-2^+ but not L3T4$^+$ cells abolished the antitumor efficacy of CTL. Furthermore depletion of Ia$^+$ cells by monoclonal antibody treatment had no effect on secondary in vitro stimulated cells suggesting that tumor regression is solely mediated by Lyt-2^+ cells. Aurora and Hanna (1989) had studied the surface phenotype of

CTL generated against trinitrophenyl (TNP) modified syngeneic spleen cells and were found to be Lyt-2^+ cells with negligible reactivity with YAC-1 lymphoma cells. However, it has been reported that P815 specific syngeneic CTL (Lyt-2^+) could not be generated in the absence of L3T4^+ cells, whole function could be replaced with exogenous IL-2 (Bear et al 1988). Several investigators have shown that majority of the CTL, specific for syngeneic tumors were of Lyt-2^+ phenotype (Mills and North 1983, Greenberg 1986, Fearon et al 1990).

Since the cytotoxic cells in vitro assays exerted their effect specifically on DLA target cells, target cell specificity was further examined by the use of a Winn neutralisation assay. The effector CTL could suppress only the growth of DLA tumor cells but not S180 tumors, confirming the specificity of the effector cells (Table-11). 100% inhibition of DLA tumor growth was recorded in treated animals compared to controls. The above findings provide ample evidence for the antigen specific nature of the effector CTL. It had been demonstrated that injection of $25x10^5$ K4L CTL could almost completely inhibit the growth of 10^5 L1210 tumor cells in DBA/2 mice. But when the mice were challenged with L1210 and P388, all the mice died as a result of tumor growth suggesting that immunity did not attack on the third party byestander tumor cells (Rahman et al 1988).

Almost negligible degree of the tumor growth suppression was observed in the mice treated with Cyclophosphamide alone. All untreated control tumor bearing mice died within 40 days

of tumor inoculation, while 90% of the mice treated with CTL and Cyclophosphamide plus CTL surived. The surived mice were shown to specifically reject DLA cells when they were rechallenged after 60 days. Several workers have reported that after successful tumor therapy these CTL revert to memmory cells in lymphatic tissue where they can persist for long time (Cheever 1986, Klarnet et al 1987, Kast et al 1989). It is possible, however, to generate so called "helper independent" $CD8^+$ ($Lyt\text{-}2^+$) CTL clones that secrete larger amounts of IL-2, one such clone directed against Friend Murine Virus induced FBL-3 leukemia cells has been reported to eradicate disseminated leukemia without exogeneus IL-2 and provided specific immunologic memmory. These effector CTL when transferred adoptively retained for a long time in host and could reject the challenged tumor (Klarnet et al 1987). Many recent publications have emphasised the potential use of adoptive immunotherapy by lymphokine activated killer (LAK) or propogated CTL and proved their efficacy in controlling tumor growth in mice (Rosenberg 1984, Matia and Rosenberg 1986). Better efficacy of IL-2 expanded TIL in producing remission in mice bearing Mc-38 Colon Carcinoma simultaneously treated with Cyclophosphamide has been reported previously (Rosenberg et al 1986, Topalian etal 1989). In an animal model of weakly or nonimmunogenic methyl cholanthrene induced fibrosacromas, adoptively transferred TILs have been shown to be 10-50 fold more effective in vivo than LAK effectors (Rosenberg et al 1986, Rosenberg 1990). TIL are classic cytolytic T lymphocytes ($CD3^+$, $CD8^+$, $CD16^-$) and show specific

cytolytic activity against tumors from which they are derived (Rosenberg et al 1988, Itoh et al 1988). Adoptive therapy with cloned $CD8^+$ ($Lyt\text{-}2^+$) CTL now seems feasible in the case of virus induced tumors (Melief and Kast 1990). The mode of operation of these CTLs resembles that of $CD8^+$ cells, responsible for suppression of tumors bearing antigenic peptides with potent mutations (Boon et al 1989), allograft rejection (Rosenberg and Singer 1988) and viral immunity in general. These CTL were reported to be potent and highly specific in vivo (Byrne and Oldstone 1984, Zinkernagel et al 1985, Liehmann Grube et al 1988). The high efficacy of adoptively transferred cloned tumor specific CTL in eradicating large subcutaneous tumor masses (upto $10cm^3$) in tumor bearing T cell deficient nude mice has been demonstrated (Melief and Kast, 1990).

In summary, the present study demonstrated that permanent regressions could be induced in mice bearing lymphomas by combination therapy with IL-2 activated CTL and low doses of Cyclophosphamide. These studies provide basis for experimentally evaluating the use of CTL in the treatment of various haematopoietic malignancies.

Thus the profiles of immune responses during the development of DLA tumor have been studied. It has been further demonstrated that the kinetics of humoral and cell mediated immune responses show a definite pattern during the

development of the tumor. Further investigations were carried out on the immunomodulatory effect of the drug Levamisole on immune status of the tumor bearing mice as reflected in parameters such as NK, ADCC, MMADCC, ACC and T Cell subsets. The above parameters were studies at various intervals of tumor growth following intraperitoneal administration of single dose of Levamisole. (15mg/kg body weight). NK cell activity was significantly decreased in the drug treated animals compared to controls (Fig. 9). It has been reported that Levamisole at a concentration greater that 10^{-4}M inhibits NK cell activity. (Shaw and Dawson, 1982) Chattopadhyay et al (1986) have demonstrated the dose dependent contradictory effect of Levamisole. According to Renoux et al (1977) and Chirigos et al (1975) precursors of T lymphocytes were stimulated to differentiate and mature into T lymphocytes by Levamisole. Therefore with administration of Levamisole the transformation of pre-T cells into mature T cells must have taken place at a faster rate. It has been reported that there exists a feed back type of mechanism of inhibition between NK cells and T cells. Inverse relation between NK cells and T cells has been further confirmed by demonstrating increased number of NK cells in athymic nude mice (Urban and Shreeber 1982). Loor and Roelant (1979) have suggested that moust NK cells are pre T cells with low density of Theta antigen. Grossman and Herberman (1984) have put forward a hypothesis to explain NK cell genesis which says that differentiation of

NK and T cell is intimately related. The devergence of NK cell may occur at the stage of T cell receptor gene arrangement. They propose a partially adaptive rather than a rigidly programmed differentiation process of NK cells according to which the environment prevailing at the time of differentiation has a major influence on the direction in which the precursor cells develop. Recently Kalland (1987) has suggested that T and NK cells may share a common progenitor. The cell is propalled to T or NK cell development depending on the relative concentration of IL-3 and its receptor on progenitor.

Another possible mechanism by which NK cell suppression could be explained is through the inhibition of NK cell activity by Prostaglandins. Increased Prostaglandins production either by the tumor or by the host macrophages is important in inhibiting the immune response of the host (Ray and Ray Choudhuri 1981). Brunda et al (1980) have demonstrated that splenocytes from mice with Moloney Virus induced sarcomas had severely depressed NK cell activity which could be restored to normal by the administration of Prostaglandin inhibitors. Significant enhancement in Macrophage Mediated ADCC (MMADCC) from day 3 onwards till day 15 could be correlated to the impairement in NK cell activity observed in drug treated animals. It has been reported that tumor bearing mice that posses macrophages capable of inhibiting immune responses to certain antigens in vitro nevertheless are perfectly capable of mounting normal immune reponse to antigens in vivo (Forni et al 1982). Augmentation

of tumor associated Macrophage Mediated ADCC to autologous tumor cells and phagocytosis of antibody coated SRBC following in vitro treatment of the TAM with suitable doses of Levamisole has been reported (Chattopadhyay 1986, Battacharya 1986). Increase in the number of macrophage precursors in the bone marrow of C_3H mice bearing Mammary tumor transplants after 4 days of tumor growth was reported earlier (Baum and Fisher, 1972). ADCC showed a significant enhancement on Day 3 in drug treated animals with highly significant reduction followed by a progressive increase to peak on day 11, thereafter a gradual decline and the activity was completely lost after day 19. The above data revealed that Levamisole had differential effect on NK cell and ADCC acitivities depending on the functional status of the effector cells. A significant enhancement in ADCC by functionally depressed effector cells from day 7 onwards till day 17 was recorded in drug treated mice. Levamisole has been reported to restore impaired cellular immunity (Symoens, 1978, Gomi et al 1982).

The depression of ADCC on day 5 observed in the present study is difficult to explain. Liona et al (1974))) have reported that drugs such as Imidazole or Thiomidazole could activate Phospholipid Esterase with consequent reduction in intracellular cyclic AMP. Differential activation of enzyme by Levamisole, which is shown for changing intracellular cyclic AMP level (Symoens et al 1978) in cells with different functional status might explain varied effects of the drug. The modulation of target binding due to Levamisole associated

with changes in expression of surface receptors, presumably through alteration in the level of intracellular cyclic nucleotides has been reported (Rhodes 1975). Levamisole, when used for in vitro treatment, modulated the lysis of serum coated autologous tumor cells by tumor associated macrophages at early and late period of tumor growth in a dose dependent manner (chattopadhyay et al 1986). The mechanism of activity of Levamisole in either enhancing ADCC or suppressing NK cell activity could not be ascertained from present data. Changes in the level of intracellular cyclic necleotides due to this drug might be associated with observed modulation of ADCC.

Levels of T cell subsets were increased in Levamisole treated mice compared to control. Increased proportion of anti Ox IgM rosette forming splenic T cells were observed in drug treated mice. Remarkable increase in the level of T_H, starting from day 3 till day 19 was recorded (Table-15). Interestingly, humoral response demonstrated by complement mediated lysis correlates well with the above finding(Fig. 12, Table-18). T_S cells however did not show any significant enhancement except on day 3 (Table-16). Biniminov and Ramot (1975) reported that Levamisole in vitro significantly increased the proportion of E-rosette forming cells in peripheral blood from patients with Hodgkin's disease. Sampson and Lui (1976) hypothesized that the drug worked by selective impairment of suppressor cell function. However this view was contradicted later and it has been shown that

suppressor function was not impaired by the drug but was in fact potentiated (Sampson and Lui 1976). Thus it is evident that Levamisole is a nonspecific potentiator of T lymphocytes.

Complement mediated lysis in the drug treated mice were found to be augmented with an early onset of antibody production, which could be detected from day 5 onwards and remained without any peak till 15th day and then declined to the base line as the tumor growth progressed (Fig. 12). Levamisole may induce a prolonged and steady humoral response rather than a spiked response which is well correlated with the present findings. Invtiro assessment of the immunomodulatory action of the drug as evidenced by Jern's plague assay revealed maximum stimulation of plaque forming cells on the 4th day after immunisation in the drug treated animals. Levamisole has been reported to stimulate T_H, T_S/Cytotoxic cells, lysosomal activity and plque cell formation (Symoen and Rosenthal 1977, Gomi et al 1982, Gomi et al 1984). Increased life span of 25% was observed in drug treated animals as compared to control. From the above results, it is evident that Levamisole is a potent stimulator of humoral response in the murine model. Renoux et al (1978) have demonstrated enhancement of humoral response in mice treated with Levamisole.

The precise mechanism of action of Levamisole on the immunologic response is not known; but evidences point to influences on T lymphocytes and on T-B lymphocyte interactions

(Renoux and Renoux 1972, Cherigos et al 1973, Renoux and Renoux 1974, Chan and Simons 1975, Holden et al 1975, Mantovani and Sprefaeco 1975, Ramot and Biniminov 1976). An impressive body of evidence implicates influences exerted upon the macrophage monocyte population with macrophages in turn exerting a regulatory influence on lymphocytes (Schrieber et al 1975, Schmidt and Douglas 1976, Stecker et al 1976). Levamisole increases cyclic GMP level of lymphocytes and decrease the cyclic AMP level with a dose response to mitogen (Strom 1972, Holden et al 1975). Thus relationship similar to that of cholinergic agents that increase cyclic GMP levels, is associated with proliferation, lymphokine production and lymphocytotoxicity.

In summary, metabolic events in macrophages and lymphocytes suggest that cellular functions mediated by cyclic GMP and cyclic AMP account for the antianergic activity of Levamisole and that its activation of lymphocyte is regulated by macrophages.

Several indigenous drugs, either single or in combinations used in Ayurvedic medicines for improving vitality and to aid rejuvenation may also be useful as adjuvants in Cancer therapy. Ayurvedic drugs are less toxic compared to conventional chemotherapeutic drugs currently used in Cancer therapy. Emblica officinalis (Indian Goose berry);

is a good source of vitamin-C and is one of the major constituents in many of the Ayurvedic preparations. Eventhough the anticancer properties of the drug has been extensively reviewed (Hartwell 1969, Nisteswar, 1989) the immunological aspects still remain elusive. Therefore an attempt has been made to elucidate the modulatory effect of the drug Emblica Officinalis on immune profiles of the tumor bearing Balb/c mice.

20 mg/kg body weight of dried powder of the fruit (pericarp) was administered orally every day following tumor inoculation. NK cell activity assessed against ^{51}Cr labelled K-562 target cells showed an exponential increase in activity from day 0 till day 11 post tumor inoculation. A significant enhancement in NKCMC was documented on day 3, 5, 7 and 9 in drug treated animals with respect to tumor bearing control (Table-21). The activity was found to peak on the 12th day and thereafter a steady decline in activity has been recorded.

ADCC in Emblica treated mice showed a steady increase in activity from day 3 onwards which peaked on day 11 and then an abrupt fall in activity was observed. Antibody dependent killing was not detectable on day 17 (Table-22). Enhanced cytotoxicity was significant on day 3, 7, 9, 11, 13 in drug treated animals when compared to control. However it is very difficult to explain why effector cells from drug treated animals displayed reduced killing potential on day 5 with respect to tumor bearing control aniamls. There are several

reports regarding the immunostimulatory action of ascorbic acid, through its influence on various parameters of immunocompetence, particularly in subjects in immuno-suppressive drugs and in other risk groups (Siegel 1974, Siegel 1975, Dahl and Degree 1976, Anderson et al 1981). Thurman and Goldstein (1979) observed depressed immunological response in guinea pigs depleted of ascorbic acid. Siegel and Mortan (1977) have reported a three fold increase in Interferon level in mouse cell culture following Poly-I/Poly-C addition to mouse cell culture containing ascorbic acid. Also Interferon levels were reported to be increased in mice provided with 250 mg % ascorbic acid in drinking water and exposed to Murine Leukemia Virus in vivo (Anderson and Theron 1979). Patients with Chediak-Higashi syndrome suplemented with Vitamin-C markedly increased the percentage toxicity of killer cell function, over a five day supplementation period (Brin 1981). Brin and Katz (1981) further reported that injection of ascorbic acid in patients with Chediak-Higashi syndrome caused normalisation of depressed Natural Killer function. The Emblica has been reported to have pronounced antiviral properties (Antarkar et al 1989, Babbar et al 1982) and has been administered against acute viral Hepatitis and other liver disorders (Singh et al 1983, Handa et al 1986, Suresh Kumar et al 1981). The antiviral activity detected in plants appears to be carried out by protein molecules biologically resembling Interferon

but different from that of chick Interferon (Babbar et al 1982). Plant extract has been reported to induce antiviral activity in Ranikhet disease virus (RDV) in chorioallantoic membrane (CAM) cultures. Septilin, a crude mixture of Oleogum resins and extract of Phyllanthus emblica, Tinospora Cardifolia etc. have been shown to enhance Interferon production in the presence of Interferon inducer 6-MFA in mice challenged with Semiliki Forest Encephalitis Virus. Significant increase in the mean survival time of test mice challenged with Semiliki Forest Encephalitis Virus has been reported (Gupta et al 1985). From the above findings it is evident that pronounced antiviral activity of the drug may be due to Interferon or Interferon like substance. It has been reported that this antiviral substance is a protein and sensitive to trypsin treatment (Babbar et al 1982). Antioxidants such as Vitamins C and E, and corticosteroids/-non-steroidal anti-inflamatory agents which inhibit Prostaglandin Synthesis have been reported to be effective anti promotors in the mouse skin model (Slaga 1983, Fischer et al 1986). The augmentation of NK cell activity and ADCC in tumor bearing mice treated with Emblica officianalis could be attributed to Interferon or Interferon like substance, which has been reported to be induced by the drug. Therefore it is quiet logical to hypothesize that the drug can enhance NK cell activity and ADCC in vivo through induction of Interferon and Interferon like substances. However further work is required to prove which type of Interferon is induced by the drug.

Tumor associated macrophage mediated ADCC assessed against sensitized SRBC in ^{51}Cr release assay did not show any enhancement in the cytotoxic potential of the effector cells in drug treated mice with respect to control. The above findings contradict with the observed potentiation of Natural cytotoxicity in drug treated animals (Table-23).

Antibody dependent complement mediated lysis in the drug treated animals were found to be profoundly enhanced as compared to control. An early onset of antibody production from day 5 onwards which prolonged until 15th day of tumor inoculation was observed. The enhancement in antibody mediated lysis was significant on day, 5, 7, 9 ($P < 0.001$) and day 11 ($P < 0.05$) post tumor inoculation (Table-24). In vitro assessment of immunostimulatory action of the drug by Jern's plaque assay revealed significant enhancement in the number of plaque forming cells on the 4th and 5th day after immunisation with respect to control ($P < 0.001$, $P < 0.05$) (Fig. 19). Increased life span of 35% was observed in drug treated animals compared to control. It has been reported that increased serum levels of IgA, IgM and C_3 component of complement in individuals consuming 1 gm Vitamin-C per day for 75 days (Brin et al 1981). It has been reported that in human subjects depleted of Vitamin-C under controlled conditions, reduced humoral immunity was observed when the earliest signs of scurvy appeared (Brin 1981). The mechanism of action of

the drug on humoral arm of immunity cannot be ascertained from the limited data. However further work has to be done to explain the mechanism responsible for augmentation of humoral response.

Ascorbate has been shown to increase intracellular cyclic GMP in a variety of tissues (Goldberg and Haddox 1977, Anderson et al 1981) Which could be directly related to lymphocyte activation, potentiation of NK, ADCC and humoral response. The proportion of splenic T_H in drug treated mice did not shown any significant change except a reduction on day 11 ($P < 0.001$) (Table-25).

Emblica officianalis should therefore be considered as an immunopharmacological agent in prophylaxis.

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Submitted to the University of Calicut
for the Degree of

Doctor of Philosophy

In Immunology (Faculty of Medicine)

By

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AMALA CANCER RESEARCH CENTRE,
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1990

1850

SUMMARY & CONCLUSION

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One of the most critical questions in Cancer immunology is why the immune system fails to eliminate tumors arising de novo. The immune profiles during the tumorogenesis of Dalton's lymphoma ascites tumor as reflected in NK cells, T cells, ADCC and antibody complement mediated lysis have been studied in Balb/c mice. Spleen cells from tumor bearing mice displayed significant NK cell mediated cytolysis on day 5 after tumor inoculation which increased gradually to peak on day 12 and then declined to the basal level as the tumor growth progressed. Appreciable amount of antibody dependent killing was detected in normal mice, which progressed steadily to peak on day 5 post tumor inoculation and thereafter abrupt fall in activity was recorded. Resident macrophages showed considerable antibody dependent killing of sensitized SRBC. Tumor Associated Macrophage mediated ADCC showed a gradual increase from day 3 onwards and peak activity was found on day 11 past tumor inoculation. Detectable CTL mediated lysis was observed on the day 10 after immunisation (after 4 days of co-culture). Humoral response demonstrated by antibody complement mediated lysis of DLA tumor cells revealed detectable activity on day 9 with a peak on 13th day which gradually declined and lost. The proportion T_H cells in the

tumor bearing mice increased gradually to peak on day 11 while T_S cell were found to be more or less consistant till 11th day and then increased gradually as the tumor growth advanced.

The above studies have demonstrated that antitumor immune response in DLA tumor bearing mice show appreciable specific and non-specific, cellular and humoral immune responses after the onset of tumor growth. But the antitumor activity steadily declined with progressive growth of rapidly dividing tumor cells. The progressive decay of immunity after day 12 of tumor growth of DLA, demonstrated in the above study could be attributed to negative immunoregulatory function of suppressor T cells as well as plasma blocking factors such as circulating immune complexes. All available evidences from these studies lend support for the above hypothesis. Hence it is possible that the immune system fails to eliminate tumors not because neoantigens are absent, but rather because the response to these neoantigens is inadequate.

Further investigations were done using NK cell enriched fraction of cells obtained by density gradient centrifugation of percoll. Percentage of the active killer cells, V_{max} and recycling capacity of the effector cells at different intervals of tumor progression has been estimated by combining single cell conjugate assay and ^{51}Cr release assay. The percentage of active killer cells were found to be increased on the day 7 and the day 12, followed by a significant reduction on day 19 with respect to control. The estimated

maximal recycling capacity of the effector cells, however did not show much variations when compared to normal control.

Culture supernatent of LGL with K-562 was tested for NKCF activity at different intervals of tumor progression in a micro supernatent assay. Peak NKCF activity was observed on day 12, while on day 19 a significant fall in activity was noticed with respect to control.

Incubation of day 19 LGL with human rIL-2 for 72 hrs at $37^{o}C$ in 5% CO_2 resulted in significant increase in NKCMC, percentage active killer cells and NKCF activity. Maximal recycling capacity of LGL, however did not show any variation. The observed reduction in the percentage active killer cells in conjugate assay and impairment in NKCMC in ^{51}Cr release assay could be attributed a limited production or release of NKCF by LGL on day 19, which could be compensated by treatment of the LGL with IL-2. The finding that IL-2 induced augmentation of NKCMC in ^{51}Cr release assay correlates well with IL-2 induced augmentation of NKCF activity supports the hypothesis that NKCF could be the lytic mediators in NKCMC.

In the earlier studies, CTL have been shown to elicit low tumoricidal activity even after a secondary in vitro stimulation. When the effector CTLs were further incubated with 200 IU/ml of rIL-2 for 7 days, a significant enhancement in the killing potential against DLA tumor, was observed in in vitro assays. Extending, these observations, it has been

In the subsequent studies the modulatory effect of drug Levamisole on immune profiles of the tumor bearing mice were assessed after i.p. administration of a single dose of Levamisole (15mg/kg body weight or ⅛ 5mg/kg body weight). NK cell activity was totally abrogated in Levamisole treated mice when compared to control, while ADCC showed significant enhancement from day 3 to day 11 post tumor inoculation and declined thereafter. Significant increase in T_H cell count was observed in drug treated animals on day 9, 15, 17 and 19. Suppressor T cells showed significant increase on day 3 in Levamisole treated animals compared to control. Humoral response assessed by complement mediated lysis revealed an early onset of humoral response in Levamisole treated mice, which sustained upto 15th day of tumor inoculation, in vitro assessment of immunomodulatory action of the drug by Jern's plaque assay showed significant enhancement in the number of plaque forming cells on the 4th day after immunisation when compared to control. An increase in life span of 25% was recorded in treated animals. From the above results it is evident that Levamisole is a potent stimulator of macrophages, T and B lymphocytes. It could be possible that macrophages in turn exert a negative regulatory effect on lymphocytes and NK cells.

Finally the immunomodulatory effect of the drug Emblica officianalis on immune status of the tumor bearing Balb/c mice was investigated. Enhancement in NK cell and ADCC activities

further shown that rIL-2 activated CTL were highly effective in suppressing solid tumor targets when adoptively transferred to tumor bearing syngeneic mice. Surprisingly small number (10^7) of the cells, when transferred adoptively were sufficient to produce tumor regression in mice. Non-reactivity of the effector cells with non-specific targets such as YAC-1, S-180 and Ehrlich's ascites revealed the specific nature of the effector cells. Depletion of the effector cells with Lyt-2^+ phenotype completely abrogated the antitumor activity of CTL in in vitro killing assays. Target cell specificity was further examined by the use of a winn neutralisation assay and it has been shown that the effector CTLs could suppress only the growth of DLA tumor cells but not S-180, confirming the specificity of the effector cells. 100% inhibition of DLA tumor growth was recorded in treated animals compared to controls. The survived mice were shown to specifically reject DLA cells when they were rechallenged after 60 days.

In summary the present study has demonstrated permanent regressions could be induced in mice bearing lymphomas through adoptive transfer of IL-2 activated specific CTL, which are predominantly of Lyt-2^+ phenotype and revert to memory cells and persist for long time in the host. These studies outline a noel strategy for experimentally evaluating the use of CTL in the treatment of various haematopoetic malignancies.

was recorded in drug treated animals compared to control. Tumor associated macrophages mediated ADCC did not show any enhancement in treated mice with respect to control. Profound enhancement in humoral response has been demonstrated in Emblica treated mice. Increased life span of 35% was observed in drug treated animals. Augmentation of NK cell activity and ADCC in mice treated with Emblica could be accounted by interferon or interferon like substance which has been reported to be induced by the drug. Also ascorbate has been shown to increase intracellular cyclic GMP in a variety of tissues, which could be directly related to lymphocyte activation, potentiation of NKCMC, ADCC and humoral response. Emblica officianalis should therefore be considered as an immuno-pharmacological agent in prophylaxis.

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