INDUCTION OF NON-MHC RESTRICTED KILLER CELLS: DIFFERENTIAL INDUCTION OF EFFECTOR POPULATIONS BY TUMOUR CELL LINES

BY

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Men can do nothing without the make-believe of a beginning. Even Science, the strict measurer, is obliged to start with a make-believe unit, and must fix on a point in the stars' unceasing journey when his sidereal clock shall pretend that time is at Nought. His less accurate grandmother Poetry has always been understood to start in the middle; but on reflection it appears that her proceeding is not very different from his; since Science, too, reckons backward as well as forwards, divides his unit into billions, and with his clock-finger at Nought really sets off into the middle of things.

George Eliot from *Daniel Deronda*.

ABSTRACT

The nonadaptive immune response characterized by non-MHC restricted cytotoxic effectors appears to play a significant role in host cellular immunity against both infectious diseases and tumours. It is possible that cytotoxic responsiveness of these effectors to "altered" tumour cells also implies a capacity to induce the effector population. A systematic examination of different tumour cell lines did demonstrate a differential ability of tumour cell lines to induce effectors both NK cells and $\gamma\delta$ T cells. The properties and characteristics which made tumour cell lines into effective inducers were examined as well as the nature of the effector populations.

Lymphoblastoid B cell lines (LBL) were the most effective inducers of non-MHC restricted killer cell activity as they induced enhanced levels of cytotoxic activity and stimulated proliferative responses in the responder population. Different LBL alone or in conjunction with IL-2 were able to stimulate non-MHC restricted cytotoxic activity in NK cells, $\gamma\delta$ and $\alpha\beta$ T cells. The phenotype(s) which was induced was dependent on the specific LBL used in the induction system as well as the presence of IL-2.

The presence of Epstein Barr virus (EBV) infection was found to significantly enhance LBL cytotoxic and proliferation inductive capacity as well as the porportion of CD16+ cells. Studies using EBV^+ and EBV^- LBL suggested that at least two parameters were involved in the EBV^+ LBL induction process, the presence of a

stimulating antigen on the LBL which specifically stimulates CD16+ cells and a second element which results in the induction of IL-2. Neither parameter was sufficient alone.

Consistent with the hypothesis that a LBL cell surface molecule was involved in the induction was the observations that cellular contact was found to be essential. As well antibodies to 3 classes of adhesion molecules (CD2, CD18, & CD29) were found to inhibit LBL induction of non-MHC restricted killer cell activity.

Two LBL, RPMI 8226 and Daudi were found to be potent inducers of Vγ9 expressing T cells. This inductive capacity was not a general property of LBL nor did it relate to the presence of EBV nor to the tumour type of the B cell line. RPMI 8226 induced a population of γδ T cells which were heterogeneous in terms of their cell surface markers, patterns of proliferation and cytotoxic responses. A member of the groEL HSP family (HSP 58) has been suggested as the inducing molecule in Daudi cells. Although anti-HSP 58 was inhibitory to γδ T cell induction by RPMI 8226, Daudi and mycobacterial products evidence is presented which suggests this may not be a specific effect.

Collectively, the results suggest that some LBL cell surface stimulus can induce an activation and expansion of non-MHC restricted killer cells. In the present studies the expansion of CD16+ and γδ TCR+ effectors were examined. This inductive ability of LBL appears to relate in part to viral infection and in part to the phenotypic properties of the inducer. The nature of the stimulus is still unclear at this time but these results do suggest

that there is a clear distinction between target susceptibility and inductive capacity.

Papers associated with this research project.

1. Selin, L.K., Stewart, S., Shen, C., Mao, H.Q. & Wilkins, J.A. (1992) Reactivity of γδ T cells induced by the tumour cell line RPMI 8226: functional heterogeneity of clonal populations and role of GroEL heat shock proteins. *Scand. J. Immunol.* 36, 213

2. Selin, L.K., Stewart, S. & Wilkins, J. A. Induction of non-MHC restricted killer cells: Differential induction of effector populations. (submitted for publication)

3. Selin, L.K., Stewart, S. & Wilkins, J.A. Preferential induction of CD16+ natural killer cells by lymphoblastoid cell lines: role of Epstein-Bar virus (EBV) and cell surface molecules. (manuscript in preparation)

4. Wilkins, J.A., Selin, L., Stewart, S., Sivananthan, K. & Stupak, D. (1992) The interactions of γδ T cells with extracellular matrix: receptor expression and utilization patterns. *Scand. J. Immunol.*, 36, 213

5. Freedman, M.S., Ruijs, T.C.G., Selin, L.K. & Antel, J.P. (1991) Peripheral blood γ T cells lyse fresh human brain derived oligodendrocytes. *Ann. Neurology*, 30, 794

CONTENTS

GENERAL INTRODUCTION

In 1960 it was observed that a kidney transplant in dogs resulted in the development of lymphocytes cytotoxic *in vitro* for donor kidney cells (Govaerts, 1960). This simple observation began the extensive study of cytotoxic lymphocytes which has grown to include various cellular reactions of adaptive immunity, directed against viral antigens, tumour-associated antigens, transplantation antigens on allogeneic cells and self-antigens associated with autoimmune disease (Cerottini & Brunner, 1974; Perlmann & Holm, 1969).

The specific adaptive cytotoxic response against transplantation alloantigens was demonstrated to be mediated by cytotoxic T lymphocytes (CTL) via the αß TCR in association with the coreceptors CD4 and CD8 which recognized gene products of the major histocompatibility complex (MHC) class I and II (Allison & Lanier, 1987; Eijsvoogel *et al.*, 1972; Rosenau & Moon, 1964), respectively. It was further demonstrated that CTLs recognized viral antigens (Trinchieri, Aden & Knowles, 1976; Zinkernagel & Doherty, 1974) and tumour-associated antigens (Hellsrom *et al.*, 1968) on target cells only in association with products of self-MHC. These CTL would demonstrate immunological memory on repeat challenge with the antigen.

A non-adaptive, non-MHC restricted cell-mediated "natural" cytotoxic response was also observed. Lymphocytes from healthy normal donors in the absence of known sensitization were found to

spontaneously kill *in vitro* certain cultured tumour cell lines (Ortaldo *et al.*, 1977; Takasugi, Mickey & Terasaki, 1973; West *et al.*, 1977). Initially, it was postulated that the major cell type mediating natural cytotoxicity was the natural killer (NK) cell. NK cells, are functionally defined and unlike CTL have not generally been demonstrated to have clonally distributed specificity, restriction for MHC products at the target cell surface, or immunological memory (Reynolds & Ortaldo, 1987; Ritz *et al.*, 1988).

However, further investigations have demonstrated that different types of lymphocytes including αß TCR+ and γδ TCR+ CTL, and other leucocytes such as macrophages are capable of mediating non-MHC restricted cytotoxicity either spontaneously or upon activation (Hersey & Bolhuis, 1987; Grimm *et al.*, 1983; Porcelli, Brenner & Band, 1991; Reynolds & Ortaldo, 1987). For instance, lymphokine-activated killer cells (LAK) are interleukin-2 (IL-2)-activated lymphocytes that are predominantly NK cells but do also contain some non-MHC restricted T cells (Atzpodien *et al.*, 1987; Grimm *et al.*, 1983; Shau, Gray & Mitchell, 1988). The source of the lymphocytes and the conditions for activation predicate the relative contribution of each cell type (Fitzgerald-Bocarsly *et al.*, 1988).

The accidental discovery in 1984 of the γ chain gene during attempts to isolate and clone the genes for the αß TCR (Lefranc & Rabbitts, 1985; Murre *et al.*, 1985; Saito *et al.*, 1984) resulted in the description of another T lymphocyte class, the γδ T cell, which is capable of cytotoxic responses. Most of the *in vitro* cultured

activated γδ T cells so far described display nonadaptive non-MHC restricted tumour cell cytotoxicity (Fisch *et al.*, 1990a; Porcelli, Brenner & Band, 1991). Although these cells do have the capacity to recognize a heterogeneous array of ligands, including classical and non-classical MHC antigens, heat shock proteins, bacterial products and self-antigens it is not yet possible to establish a paradigm for the development and specificity of the lymphoid γδ cells analogous to the MHC-directed specificity of αß cells (Allison & Havran, 1991; Fisch *et al.*, 1990b; Porcelli, Brenner & band, 1991).

The continued investigation of these different types of cell mediated cytotoxic responses has lead to the development of a generally accepted paradigm for mammalian host response against infection (Janeway, 1989; Welsh & Vargas-Cortes, in press). For instance when a virus parasitizes the host it replicates, produces new virus and spreads infection. The role of the immune response is to block that spread by clearing virus and virus infected cells. Evidence suggests that there are 3 phases in this response. During the immediate phase (<4hrs) there is a non-adaptive nonspecific, innate response which does not involve memory cells or specific T cells but rather is mediated by NK cells. This is followed by the early phase (4-96hrs) which consists of a non-adaptive, nonspecific inducible response involving primarily NK cells which have been activated by lymphokines such as interferon α and ß. The late phase (>96hrs) consists of the adaptive specific memory inducible cell mediated response, involving specific αß TCR+ CTL, and production of specific antibodies by activated B lymphocytes. In addition to

the increased number and activation of NK cells, activated CD3+, TCR+ T cells, displaying a similar antigen-nonspecific cytotoxicity have been documented to be present following certain viral infections in mice. The role of these non-MHC restricted CTL in the immune system has not yet been clarified (Welsh & Vargas-Cortes, 1992). The exact role of $\gamma\delta$ TCR+ cells in this paradigm is still under investigation but preliminary evidence seems to suggest that they may be recruited in the early phase of infection as has been observed in mouse models using intra-peritoneally injected *Listeria monocytogenes* (Carding *et al.*, 1990) as a pathogen. However, with mouse influenza virus infection they did not appear until day 7 of infection (Ohga *et al.*, 1990). Whether this response is nonadaptive or requires some form of memory induction for a small number of common antigens present in pathogens, such as heat shock proteins, is still totally speculative.

The early non-adaptive response has been demonstrated to be important, perhaps essential in many viral infections. It may be pertinent that complete congenital absence of NK cells is a very rare human condition, and in the few cases that have been described the patients often suffer near fatal illnesses from common viral pathogens such as Epstein Barr virus and varicella, requiring intensive medical support to survive (Biron, Byron & Sullivan, 1989; Fleisher *et al.*, 1982). This suggests that NK cells may be essential for survival. In the mouse there are no animal models with congenitally absent NK cells, but mice carrying a homozygous *beige* gene have strongly depressed NK cell function, due to defects

in their lysosomal membrane and granule function (Roder *et al.*, 1979). These mice are more likely to develop fatal infections than normal mice from NK susceptible viruses such as murine cytomegalovirus (MCMV) (Shellam *et al.*, 1981).

The nonadaptive mechanisms of immune defense have also been postulated to be important in the surveillance against tumours. In experimental animals the *in vivo* effect of NK cells against tumours was investigated by evaluating long-term growth of tumours (Kiessling *et al.*, 1976), metastasis formation (Hanna & Burton, 1981), and short-term elimination of radiolabelled tumour cells from the whole animal or from certain organs (Gorelick *et al.*, 1979). These experiments have clearly shown that NK cells are effective *in vivo* and can destroy tumour cells. In humans activated cells of the nonadaptive immune response have been shown to be therapeutically effective when LAK cells, which are comprise activated NK cells as well as αß and γδ T cells, were used to treat renal carcinoma and melanoma (Rosenberg, 1985). Spontaneously occurring tumour infiltrating murine lymphocytes have been reported to be composed of NK cells, and as well as αß and γδ T cells (Karpati *et al.*, 1991; Miescher *et al.*, 1990) In one case selective lysis of an autologous tumour by a δTCS1+ γδ tumour infiltrating lymphocyte from a human lung carcinoma has been reported (Zocchi, Ferrarini & Rugarli, 1990). Freshly isolated TCR γδ T cells do not lyse NK-sensitive tumour targets (Lanier *et al.*, 1986; Faure *et al.*, 1988). However, the use of a mAb-redirected killing assay (or of lectin-dependent cytotoxicity) demonstrated

that these cells constitutively express a partially active lytic machinery (Ferrini *et al.*, 1989b). When bispecific mAb's (produced by the hybrid hybridoma technique) were constructed specific for a tumour-associated antigen, cytolytic activity was triggered by these mAb's against target cells expressing the relevant tumour antigen (Ferrini *et al.*, 1989c). Moreover, TCR $\gamma\delta$+ clones armed by such bispecific mAbs secreted lymphokines (IL-2 and TNF) upon contact with the relevant tumour target. Therefore, it is conceivable that an additional anti-tumour effect *in vivo* may be induced via the release of lymphokines at the tumour site.

There is some evidence to suggest that the cellular mechanisms involved in the nonadaptive immune response may also play a role in the pathogenesis of chronic autoimmune mediated diseases. For instance NK cells have been demonstrated to be activated by rheumatoid factor via the CD16 molecule resulting in lymphokine production (IFNγ and TNFα). It has been postulated that release of these cytokines in a rheumatoid joint perpetuates the chronic inflammation present in rheumatoid arthritis (Hendrich *et al.*, 1990; Werfel, Uciechowsky & Tetteroo, 1898). $\gamma\delta$ T cells have been reported to be elevated in some patients with rheumatoid arthritis and primary Sjogrens syndrome (Brennan *et al.*, 1989; Plater-Zyberg *et al.*, 1989;). Another study reported decreased levels of $\gamma\delta$ cells in the peripheral blood of rheumatoid arthritis patients but with increased levels of δ1 expressing T cell present in the rheumatoid joints (Smith *et al.*, 1990). $\gamma\delta$ cells have also been reported to mediate inhibition of erythropoiesis *in vitro* with

type I autoimmune polyglandular syndrome and pure red blood cell aplasia (Hara *et al.*, 1990). The invasion and destruction of nonnecrotic muscle fibers by γδ cells in a case of polymyositis suggests that γδ T cells may mediate this type of autoimmune disease (Hohlfeld, Engel & Harper, 1991;) as well.

It would appear that the nonadaptive innate immune response characterized by non-MHC restricted cytotoxic effectors plays a significant role in both normal immune responses and if not appropriately regulated may play a role in the pathogenesis of chronic inflammatory diseases. It is therefore important to develop an understanding of the mechanisms involved in the generation and regulation of these non-MHC restricted cytotoxic cells. The present project goes on to examine aspects of non-MHC restricted effector cell activation, specifically NK cells and γδ T cells. To enhance the reader's perspective of the field the following two sections are a brief overview of the present knowledge base concerning these two non-MHC restricted effector cells.

NATURAL KILLER CELLS

INTRODUCTION

A major difficulty in the study of NK cells has stemmed from the fact that they were functionally defined as effector cells mediating natural cytotoxicity (Reynolds & Ortaldo, 1987; Ritz *et al.*, 1988). As already noted many different types of lymphocytes and other leucocytes have been demonstrated to mediate non-MHC

restricted cytotoxicity (Reynolds & Ortaldo, 1987). As well, NK cells generally have not been observed to have clonally distributed specificity, restriction for MHC antigens, or immunological memory (Ritz *et al.*, 1988). NK cells have not been formally assigned to a single lineage based on the definitive identification of a stem cell, a distinct anatomical location of maturation, or unique genotypic rearrangements.

It is possible however, to unequivocally distinguish mature NK cells from T, B, and myeloid cells; distinguish NK progenitors from those of T, B, and myeloid cells; and to demonstrate that NK cells are dependent on intact bone marrow and not on thymus for their differentiation (Lanier *et al.*, 1986a; Trinchieri & Perussia, 1984). NK cells are felt to represent a distinct leucocyte subset, possibly constituting a third lineage of lymphoid cells (Lanier *et al.*, 1986a; Trinchieri & Perussia, 1984). While ongoing investigations continue to characterize the exact nature of this subset an operational definition for NK cells was established at the 1989 International Workshop on Natural Killer Cells in Hilton Head, (North Carolina, USA) that attempted to define and place NK cells among other cell functions and lineages (Fitzgerald-Borcarsly *et al.*, 1989).

NK cells are $CD3^-$ T cell receptor $(\alpha,\beta,\gamma,\delta)^-$ large granular lymphocytes. They commonly express certain cell surface markers such as CD16 and NKH-1 (leu 19) in humans and NK-1.1/NK-2.1 in mice. They mediate cytolytic reactions that do not require expression of class I or class II MHC molecules on the target

cells.

Certain T lymphocytes which are either α/ß+ or γ/δ+ may express, particularly upon activation, a cytolytic activity that resembles that of NK cells. These T lymphocytes should not be termed NK cells. They could be termed either T lymphocytes displaying "NK-like" activity or "non-MHC requiring" cytolysis.

Lymphokine-activated killer (LAK) cells are IL-2-activated lymphocytes in either of the above categories. The relative contribution of the respective cell type depends on the source of lymphocytes and conditions for activation. For instance, lymphocytes from peripheral blood or spleen will produce LAK cells from NK cells in a close to predominant manner.

NK cells besides exhibiting cytotoxic activity have been shown to exert a variety of functions including production of lymphokines, regulatory functions on the adaptive immune system and on haematopoiesis, and natural resistance against microbial infection and tumour growth (Trinchieri, 1989; Trinchieri & Perussia, 1984). Cytotoxicity may or may not represent the most physiologically significant function of NK cells *in vivo*. This discrete subset along with monocytes/macrophages, PMNs and platelets represent an important effector cell type of nonadaptive immunity.

CHARACTERISTICS

The first and most direct study to implicate a defined cell population to NK activity involved the observation that sedimentation of LGLs in discontinuous Percoll gradients coincided with cells having cytolytic activity in this gradient (Timonen, Ortaldo & Herberman, 1981). In general, LGLs are non-adherent cells that possess Fc receptors but lack immunoglobulin- and T-cell receptor-gene rearrangement.Morphologically, these cells are characterized as LGLs with a kidney-shaped nucleus with prominent azurophilic granules. A variety of laboratories have extensively characterized the surface phenotypes of NK cells (Hercend *et al.*, 1983; Lanier*et al.*, 1986; Ortaldo *et al.*, 1981) and demonstrated that human LGLs share both myelomonocytic- (e.g. CD11) and T-cell (e.g. CD2 and CD8) related markers. However, the majority of human NK cell activity is mediated by CD3-, CD16+ CD56+ lymphocytes. However, $CD16^-$ NK cells also exist that have LGL morphology and express markers similar to CD16+ NK cells, including: CD2, CD7, CD11b, CD38, CD45R, CD18, and p75 IL-2R (Fitzgerald-Borcarsly *et al.*, 1989; Nagler *et al.*, 1989). The cells are active cytolytically and respond to IL-2 in terms of proliferation and activation.

The lytic function receiving attention recently is the ability of NK cells to be activated by IL-2 to mediate LAK activity. In contrast to resting NK cells, LAK cells are able to lyse virtually all tumour cells and virus-infected cells with little or no effector activity against non-malignant or uninfected normal counterparts. Analysis of effector cells have been consistent with the view that $CD3^-$ LGLs mediate the majority of LAK activity (Itoh

et al., 1985; Phillips & Lanier, 1986; Ortaldo, Mason & Overton, 1986). The ability of IL-2 to regulate NK cell function has been clarified by the development of mAbs to the ß chain of the IL-2 receptor (Phillips *et al.*, 1989; Ortaldo *et al.*, 1990). In the absence of TAC (IL-2Rα), it is now clear that NK cells spontaneously express high levels of IL-2ßR and thereby exist as a unique population of cells with ability to respond rapidly to IL-2 and IL-2-induced signals.

In addition to the cytolytic functions that have been thoroughly studied, evidence now exists that NK cells exhibit key non-cytolytic functions as well. These functions include a number of important immunoregulatory properties. The best studied non-cytolytic function of NK cells is their ability to control the spread of microbes, especially viral particles. Available data strongly support the conclusion that NK cells play a critical role in eliminating the initial replication of various forms of viruses including human cytomegalovirus, herpes simplex type I, and murine hepatitis virus (Trinchieri, 1989; Welsh, 1985). In the few patients with absolute deficiencies of NK cells viral pathology is very prevalent (Biron, Byron & Sullivan, 1988; Fleisher *et al.*, 1980). These patients have frequent infections with varicella zoster, CMV, EBV and other viruses. As previously noted most likely NK cells, together with IFN and other natural immune mechanisms represent the first line of defense against infection by certain viruses, before adaptive immunity is activated. LCMV infection in mice results in high levels of virus-induced IFN and NK cell

activity (Biron *et al.*, 1984; Stitz *et al.*, 1985; Welsh, 1978). However, NK cells do not appear to play a primary role in protecting the mice against this virus, but their presence in the inflammatory exudate of the cerebrospinal fluid suggests that they may play a role in the pathogenesis of the disease (Allan & Doherty, 1986; Bukowski *et al.*, 1983). For instance, the severity of the encephalopathy induced in mice by intracerebral injection of influenza virus is significantly reduced by elimination of NK cells *in vivo* by using antibodies against NK cells (Wabuke-Bunoti, Bennick & Plotkin, 1986). It would appear that under conditions where NK cells are unable to protect against a virus infection they may contribute to the pathogenesis .

MCMV infection of mice is the most convincing evidence that NK cells can play a role in resistance to virus infection *in vivo*. Injection of antibodies against NK cells increases the virus titre up to 1000-fold (Bukowski, Woda & Welsh, 1984). These results were supported by experiments with adoptive transfer of NK cells (Bukowski *et al.*, 1985).

Also, NK cells have been shown to have direct and indirect effects on some bacteria (Murphy & McDaniel, 1982; Pohajdak *et al.*, 1984) as well as extracellular and intracellular parasites (Fitzgerald & Lopez, 1986). These findings suggest an important role for NK cells in limiting the growth and spread of a variety of microbial infections. Additionally, these observations may indicate that LGLs and their products account for a significant proportion of the host's inflammatory response in their role in the

irradication of microbes.

NK cells have been shown to have a role in the regulation of haematopoiesis (Cudowicz & Hochman, 1979). The observation that immature cells from the bone marrow or thymus were good targets for NK-mediated cytolysis provided support for this hypothesis (Cudowicz & Hochman, 1979; Lotzova, 1986; Trinchieri, 1989; Trinchieri & Perussia, 1984). In recent studies lymphocytes with NK activity have been shown to inhibit the development of bone marrow stem cells *in vitro* and *in vivo*. In many cases the level of NK activity correlated with the ability to reconstitute irradiated bone marrow recipients with donor bone marrow (Scala *et al.*, 1986). Although the basic mechanisms for NK ability to regulate haematopoiesis have not been defined, it possibly relates to the ability of NK cells to produce a variety of cytokines. $CD3^-$ LGLs have been shown to secrete interleukin-1 (IL-1), IL-2, B cell growth factor (interleukin-4; IL-4), interferon α, tumour necrosis factor (TNF) α and ß, various colony stimulating factors, and a macrophage-activating factor and chemotactic factor (Ortaldo & Herberman, 1984; Scala *et al.*, 1986; Trinchieri, 1989). The production of these cytokines suggest that NK cells play a more general regulatory and/or developmental role in the many humoral or cellular responses mediated by T- and B-cells, and other non-lymphocyte populations. In addition to their cytolytic capabilities, the fact that NK cells produce these immunoregulatory molecules suggests that this cell type may play an important role in controlling immune and inflammatory responses.

The role of NK cells in chronic inflammation such as rheumatoid arthritis (RA) is poorly understood. Early studies reported decreased NK activity of synovial fluid lymphocytes (SFL) (Tovar, Pope & Talal, 1986). A recent study demonstrated that CD56+ cells were present in equal proportions in the PBLs and SFLs in RA patients (Hendrich *et al.*, 1990). In contrast, CD16 expression was reduced in the SFL compared to PBL. When fresh PBMC from normal individuals were incubated with rheumatoid factor (RF+) from synovial fluid modulation of CD16 was observed. In fact, purified RF produced the same effect. As previously discussed CD16 is an important activation structure on NK cells (Werfel *et al.*, 1989). These researchers found that activation of CD16 with RF resulted in induction of IFNγ and TNFα specific mRNA. They therefore postulated a new pathophysiological model for RA: 1) CD16+ cells migrate into the synovial fluid where they are activated by RF; 1) this activation results in release of cytokines and modulation of CD16; 3) these cytokines contribute to the perpetuation of chronic inflammation in RA (Hendrich *et al.*, 1990).

As noted previously NK cells are felt to play a significant role in tumour cell defense. Experimental animal studies *in vivo* have clearly shown that NK cells are effective and can destroy tumour cells (Hanna & Burton, 1981; Riccardi *et al.*, 1980). The relationship between NK cell activity and tumour progression has been difficult to establish (Pross & Baine, 1986). In patients with advanced cancer NK cell cytotoxic activity is usually depressed (Kadish *et al.*, 1981) . This depression appears to be secondary to

tumour invasion and due either to interaction of NK cells with tumour cells or to the presence of suppressor cells (Allavena *et al.*, 1981; Herberman *et al.*, 1979; Uchida & Micksche, 1981). There is evidence to suggest that low NK cell activity may have a prognostic value in determining the risk of developing certain types of tumours. In familial melanoma, relatives of the patients, who had an increased risk of developing the tumours, also showed a depressed NK cell cytotoxic activity, suggesting a possible role of NK cells in resistance to tumour growth (Hersey, Honeyman & McMarthy, 1979).

Activation of NK Cells

Activation may involve one or a number of cellular activities, such as proliferation, cytotoxicity and/or cytokine secretion. The process by which NK cells become activated to lyse their targets and secrete cytokines comprise several distinct steps: 1) NK cells must recognized targets presumably by receptor molecules on their surfaces; 2) these molecules, in turn, must transmit a signal across the cell membrane; 3) this signal must activate the cytolytic and secretory machinery and/or result in the alteration of gene expression. In addition, cytokines by virtue of interacting with their respective receptors, can modulate the function of NK cells by transmembrane signalling and the concomitant alteration of gene expression. NK cell activation then is comprised of multiple biochemical steps and may involve many different receptors. Because

of the inherent ambiguity, investigators need to be careful with the term 'NK cell activation". Clearly, the recognition of tumour targets by virgin NK cells results in an activation program which results in the lysis of targets. Treatment of the same cells with IL-2 also activates them. However, surface molecules, as well as the biochemical intermediates responsible for these discrete forms of activation, are not the same. Additionally, the term "activation' does not specify a particular event; it may refer to the generation of second messengers, alteration of gene expression or changes in cytolytic capacity. The precise measure of activation is not inherently apparent. However, despite its potential for ambiguity and confusion, the term activation is convenient, providing one attempts to clarify the receptor that is engaged and the measure of activation utilized. An additional feature to be aware of in the field of NK activation is that the requirements for activating freshly isolated NK cells may differ substantially from those of either NK clones or NK-like lines grown *in vitro*.

The biochemical basis by which engagement of the T-cell receptor (TCR) functions in transmembrane signalling has received considerable attention (Acuto *et al.*, 1985; Goldsmith & Weiss, 1988; Weissman *et al.*, 1989). Although the structure and function of this receptor are likely quite different from those molecules utilized by NK cells to recognize and react to target cells, the two cells do share surprising similarities. In addition, since T-cells, both $\alpha\beta$+ and $\gamma\delta$+, mediate MHC-restricted, as well as non-MHC-restricted cytolysis the majority of data pertaining to

signalling in the CTL has relevance to NK cells. However, NK cells also have a wide range of unique properties which are reflected, in part, by distinct modes of signalling. Such similarities and differences in the surface structures involved in signalling in T- and NK cells are presented in more detail in the following discussion.

A. *Surface structures involved in triggering NK cells*

Resting NK cells recognize and lyse a limited number of tumour cell types. The principle structure(s) involved in this process remain(s) unidentified. However, a number of other cell surface molecules on NK cells have been clearly defined.In contrast to the putative NK receptor, the structure and function of the receptor for the Fc portion of IgG on NK cells has emerged. A second group of accessory and adhesion molecules on NK cells has also been defined. The binding of T-cells to cells expressing antigen is mediated by a number of molecules in addition to the TCR, denoted as accessory molecules. Perturbation of many of these structures activates the T-cell. Analogously, ligation of several molecules on the surface of NK cells, generally with antibodies, also may be an activating stimulus. The predominant function of many of these molecules is to enhance adhesion, though it has become increasingly clear that adhesion molecules are not distinct from signalling molecules(Moretta *et al.*, 1989; Shevach, 1989; Springer, 1990).

1. *FcR (CD16) Antigen*

The ability of NK cells to lyse immunoglobulin-(Ig) coated targets is due to the expression of a receptor for the constant (Fc) portion of Ig on NK cells (FcR). Human haematopoietic cells express various types of FcγR (Fanger *et al.*, 1989; Kinet, 1989; Unkeless, 1989). For instance, monocytes and macrophages express two types of FcγR: a high-affinity receptor (FcRI) able to bind monomeric IgG, and a low-affinity receptor (FcRII, or CDw32), which is also expressed on PMNs and B cells. FcγRIII (CD16) is a low affinity receptor expressed on macrophages, neutrophils, eosinophils, and a subset of T-cells and NK cells.

It would appear that the CD16 molecule is expressed on different cell types with differing levels of glycosylation and with differing lengths of polypeptide backbones (Clarkson & Ory, 1988; Fleitt, Wright & Unkeless, 1982; Ravetch & Perussia, 1989; Simmons & Seed, 1988). The NK cell molecule is 23-28 kDa, while the PMN molecule is 32-36 kDa (Clarkson & Ory, 1988; Ravetch & Perussia, 1989; Simmons & seed, 1988). When the human CD16 molecule was cloned two nearly identical genes were found to encode FcγRIII (Ravetch & Perussia, 1989). By analyzing mRNA encoding for CD16 it has been demonstrated that each gene is exclusively expressed in either NK cell or PMNs.

The PMN CD16 molecule has been shown to be anchored to the cell membrane by a phosphatidylinositol-glycan (PIG) moiety (Simmons & Seed, 1988). Neutrophil CD16 can be released from the membrane surface by treatment with phosphatidylinositol-specific phospholipase (PIPLC) (Huizinga *et al.*, 1988). Experiments with NK

CD16 molecule have indicated that an alternative, PIPLC-resistant form of CD16 is expressed on NK cells (Ravetch & Perussia, 1989; Simmons & Seed, 1988). Therefore, the observed greater size of the NK cell CD16 molecule and the PIPLC resistance indicate that in contrast to PMNs the NK CD16 is a transmembrane protein.

Both PMNs and NK cells spontaneously shed CD16 antigen in the absence of PIPLC treatment. After digestion with N-glycanase, the shed NK CD16 molecule resolves in SDS gels into 23-28 kDa size fragments identical to those precipitated from both PMN supernatant and cells (Ravetch & Perussia, 1989). It would appear that the PMN and NK CD16 antigen might undergo spontaneous proteolytic cleavage at the same position, but unlike in PMNs, the cleaved NK CD16 antigen fails to remain on the membrane as a PIG-associated molecule and is released into the supernatant.

The structural differences between NK and PMN CD16 FcR are reflected in their functional differences. NK cells, but not PMNs, are able to lyse anti-CD16 antibody producing hybrid cells, indicating that CD16 antigen on NK cells functions as a signal-transducing structure in ADCC (Graziano *et al.*, 1989). The PMNs lyse anti-Cdw32 FcRII-producing cells, indicating that in these cell types Cdw32 FcRII functions as the signal-transducing structure in ADCC.

The CD16 antigen has also been demonstrated to be an important signalling molecule on NK cells resulting in stimulation of functional activity. Only in the case of CD16 molecules has activation of NK cell cytotoxic and other functions been shown to

be induced by the natural ligand, immune-complexed IgG (Hendrich *et al.*, 1990). CD16 can trigger activation signals in NK cells resulting in cytotoxicity against NK-resistant targets (Werfel *et al.*, 1989), rise in intracellular Ca^{2+} and induction of cytokine expression including IFNγ, TNFα and GM-CSF (Anegon *et al.*, 1988; Cuturi *et al.*, 1989). The surface expression of CD16 has been shown to be characteristically regulated. For instance, incubation of NK cells with anti-CD16 antibodies or immune complexes modulates the CD16 antigen from the cell surface (Perussia *et al.*, 1983b). As well, studies have demonstrated that phorbol esters induce complete down-modulation by shedding of the antigen as well as drastically decreasing CD16 mRNA in human NK cells (Kolanus *et al.*, 1990; Trinchieri *et al.*, 1984). The mechanisms involved in the transduction and in the regulation of CD16 signal generation are not fully elucidated. Triggering via FcγRIII has generated increases in inositol triphosphate (Casatella *et al.*, 1989). GTP-binding proteins have been shown to regulate signal transduction, second messenger generation and cytolytic functions triggered in NK cells via FcγRIII (Procopio *et al.*, 1990).

Although it was previously believed that the zeta chain was exclusively associated with the TCR essential for optimal transport and expression of the plasma membrane receptor, it has recently been shown that this subunit is expressed in NK cells (Anderson *et al.*, 1989). It was shown that at least one structure that zeta associates with in NK cells is FcγRIII (Lanier, Yu & Phillips, 1989; Anderson *et al.*, 1990). When COS cells were transfected with

the cDNA for both the zeta chain and FcγRIII , this allowed surface expression of the latter. Thus it appears that FcγRIII may have similar rules for receptor assembly as TCR. In addition it appears that zeta chain has critical roles not only in assembly of multimeric receptors but also in signal transduction in the TCR (Ashwell & Klausner, 1990).

2. *Adhesion and accessory molecules on NK cells.*

Approximately 90 % of NK cells react with anti-CD2 antibodies which detect a low affinity receptor for sheep erythrocytes (Perussia *et al.*, 1987) present on all T cells. It is not found on cells of other lineages. This 50-58 kDa glycoprotein functions as an adhesion molecule and is member of the IgG superfamily. The ligand for this molecule is another adhesion molecule LFA3, a 60-70 kDa molecule that is also expressed on NK cells as well as a variety of other cell types. LFA3 exists as a glycan linked and a transmembrane form.

In addition, to its function in adhesion, CD2 appears to have signal transducing functions both in T-cells and in NK cells. The CD2 antigen expresses at least three distinct epitopes: $T11_1$, the erythrocyte binding site; $T11_2$, an epitope with the same cellular distribution as $T11_1$ but with no effect on binding; $T11_3$, an epitope expressed only in activated cells or in cells treated with anti-$T11_2$ antibodies (Muer *et al.*, 1984). In T cells treatment of the cells with anti-$T11_2$ and anti $T11_3$ results in expression of IL-2 receptor, secretion of IL-2 and T cell proliferation (Muer *et*

al., 1984). In T cells CD2 can act as an antigen-dependent pathway of activation although interestingly most data in T-cells point to a requirement for expression of TCR for efficient signalling via CD2 (57). This treatment in NK cells results in expression of IL-2 receptor (Schmidt *et al.*, 1985a), and increased cytotoxic activity of both fresh (Schmidt *et al.*, 1987) and cloned (Schmidt *et al.*, 1988; Siliciano *et al.*, 1985) NK cells. There is no proliferative response observed most likely due to lack of IL-2 production (Ythier *et al.*, 1985). Anti-CD2 treated NK cells demonstrated increased adhesion to target cells and oriented discharge of granules on the area of target cell contact (Schmidt *et al.*, 1988). These studies provide evidence that the CD2 molecule may be an important physiological regulator of functions of NK cells.

CD11/CD18 is a family of three integrin molecules important for their role in cellular adhesion and composed of a common ß subunit (CD18, 95kDa) and different α subunits: CD11a (LFA-1), CD11b (CR3, OKM1) and CD11c (p150) (Springer *et al.*, 1987). All three molecules are expressed on human NK cells (Timonen, Patarroyo & Gahmberg, 1988). CD11a is expressed on all lymphocytes , whereas CD11b and CD11c are expressed on macrophages, PMNs and B cells (Breard *et al.*, 1980; Springer *et al.*, 1987; Timonen, Patarroyo & Gahmberg, 1988). The ligand for LFA1, ICAM-1, is a 90 kDa transmembrane protein and is member of the immunoglobulin gene superfamily. ICAM-1 has homology with the neural cell adhesion molecule (NCAM), an isoform of the NK cell protein, CD56. ICAM-1 is expressed on leucocytes, epithelial cells, endothelial cells, and

fibroblasts. Its expression is regulated by the cytokines, IL-1, IFNγ, and TNFα.

This family of molecules appears to play an important functional role in NK cell killing. Patients with severe deficiencies of either the CD18 or CD11a (LFA-1) chains are deficient in NK cell activity (Kohl *et al.*, 1984; Ross *et al.*, 1985). Anti-CD18 antibodies block binding of NK cells to their targets and prevent cell lysis (Axberg *et al.*, 1987; Mentzer, Krensky & Burakoff, 1986; Timonen, Patarroyo & Gahmberg, 1988). A series of antibodies to various epitopes of the CD11a molecule inhibited both NK cell-mediated and CTL-mediated cytotoxicity by preventing effector-target cell conjugate formation (Mentzer *et al.*, 1986). There is some variability in anti-CD11a inhibition of NK clones depending on the clone and the target cells selected (Hart *et al.*, 1987; Schmidt *et al.*, 1985b). This suggests that CD11a is an important adhesion molecule in the interaction of cytotoxic cells with their targets; the variable requirements for the CD11a molecules in binding of different targets with different clones suggest that either CD11a is one of several receptors that NK cells can use for binding or it has only an accessory function. No evidence has been provided that CD11a is a functional receptor capable of signal transduction and triggering NK cells. The avidity of interaction between ICAM-1 and LFA1 is not simply a passive process however; perturbation of the TCR or CD2 and pharmacological activation of T-cells with phorbol esters results in enhanced LFA1 adhesion (Dustin & Springer, 1989; Springer, 1990; van Kooyk *et*

al., 1989) and this interaction is likely to be analogously regulated in $CD3^-$ LGLs.

A series of antibodies have been produced that bind to most NK cells precipitating a 200-220 kDa molecule and referred to as CD56. The two most commonly used mAbs are NKH-1 and Leu-19. Antibodies to CD56 have been shown to react with immature myeloid cells (Hercend *et al.*, 1985; Lanier *et al.*, 1986b), neurons, neuroblastoma cell lines, and human teratocarcinoma cells (McGarry *et al.*, 1988). More recently, the CD56 antigen has been shown to be an isoform of neural adhesion protein NCAM (Lanier *et al.*, 1989). The latter is a membrane glycoprotein expressed on neural and muscle tissues involved in adhesive interactions in these tissues and is a member of the Ig gene superfamily (Lanier *et al.*, 1989). CD56 is expressed at very low density on peripheral blood NK cells, but its density increases significantly following *in vitro* stimulation of NK cells (Perussia *et al.*, 1987). The subset of PBLs expressing CD56 almost completely overlaps with the expression of CD16 antigen (Lanier *et al.*, 1986b). The CD16-/CD56+ cell represent 2-3% of PBLs and occur in two subsets: CD3- cells which are probably NK cells that do not express the CD16 antigens because of differentiation or activation state; CD3+ cells which are a minor subset of T cells with low but significant non-MHC-restricted cytotoxic ability (Lanier *et al.*, 1986b). CD56 antigen is almost invariably associated with non-MHC-restricted specificity (Ritz *et al.*, 1988), but this is not an exclusive relationship as a few CD56 positive clones have been described without cytotoxic ability (Lanier *et al.*, 1987). The

function of the CD56 molecule is still unknown although anti-CD56 antibodies have synergistic inhibitory effects with anti-LFA1 or anti-LFA3 on NK cell-mediated binding and cytotoxicity.

NK cells also express the well characterized adhesion proteins fibronectin and laminin. Antibodies against these proteins also inhibit NK cell-mediated cytotoxicity (Santoni *et al.*, 1989; Schwarz & Hiserodt, 1988). Thus a variety of adhesion molecules appear to regulate the interaction of NK cells with their targets.

3. *The nature of the "NK receptor": candidate molecules and other molecules with poorly understood function*

Identification and characterization of the NK receptor(s) has been a focal point for a great deal of research. Neither the structure of the molecule(s) mediating recognition of targets susceptible to NK cell killing nor its/their ligand(s) have been convincingly elucidated. NK cells do not rearrange TCR genes (Biron *et al.*, 1987; Lanier *et al.*, 1986; Reynolds *et al.*, 1985; Ritz *et al.*, 1985) nor express CD3 family member proteins on their cell surfaces (Ritz *et al.*, 1985) although, as mentioned previously, the zeta chain is expressed (Anderson *et al.*, 1989). At present, one can only speculate whether the putative "NK receptor" (NKR) should be a polymorphic or monomorphic structure. One would anticipate that a less polymorphic structure would be required for an NKR than the TCR. The issue of the nature of the NKR is further compounded by the observation that upon IL-2 treatment, NK cells and even T-

cells are able to lyse a much broader spectrum of targets. Presumably this phenomena is due either to the expression of a new molecule or the expression of pre-existing molecules at a higher density or possibly an increase in the avidity of binding by the receptor. Since IL-2 activated NK and T cells, both αß+ and γδ+, can mediate virtually identical phenomenum of non-MHC restricted activity, it is not clear if the molecules utilized by these subsets of lymphocytes are similar, or if different cytolytic mechanisms are employed.

Several approaches have been taken to delineate molecules of potential relevance to the recognition and lysis of tumour targets. MAbs capable of influencing NK cell-mediated killing have been generated. Using this method, a molecule involved in transmembrane signalling in NK cells has recently been cloned, NKR-P1 (Giorda *et al.*, 1990). Treatment of NK cells with this antibody results in killing of targets by reverse ADCC. This molecule is a 60kDa disulphide-linked homodimer. It is a lectin-like molecule, which shares homology with asialoglycoprotein receptors, the low affinity Fcε receptor (FcεRII or CD23) and chondroitin sulphate proteoglycan core protein. This molecule also appears to be expressed by neutrophils and to a lesser extent by T-cells.

Another binding structure identified on NK cells includes anti-ID, a surface structure which is recognized by an idiotypic antibody to an anti-adhesion ligand monoclonal antibody (Ortaldo *et al.*, 1989). Ortaldo et al. (1989) have developed an antibody to a K562 tumour cell membrane glycoprotein. This antibody reacts with

surface glycoproteins on K562 and other NK-susceptible target cells, inhibiting binding of NK cells to targets. Hypothesizing that this antibody might structurally resemble an important NK receptor an anti-idiotypic antibody was developed to this first antibody. This anti-idiotypic antibody binds to NK cells, and $\gamma\delta$ T cells as well as $\alpha\beta$ T clones which demonstrate non-MHC restricted killer cell activity. It immunoprecipitates a complex of molecules (80 kDa and 150 kDa) from the surface of NK cells under both reducing and non-reducing conditions. Direct anti-idiotype treatment of NK cells inhibited binding and lysis of both K562 and Molt-4 targets. A novel gene from a NK-specific expressing library, which encodes a 150 kDa cell-surface peptide was cloned using the anti-ID. This structure is a triggering molecule, since antisera blocks NK lysis, mediates reverse ADCC, induces NK cells to release cytokines (IFNγ, TNFα), and reacts specifically with freshly isolated and cloned NK cells. This molecule is, however, lacking on CD3+ TCR $\alpha\beta$+ or $\gamma\delta$+ T-cell clones. These results suggest that the anti-ID molecule is a functional receptor present on cells capable of non-MHC restricted cytotoxicity in NK cells.

As well there is evidence to suggest a surface protein cross-reactive with the Fab part of human IgG may somehow contribute to the orientation of the effector cell Golgi apparatus during the triggering of cytolysis (Timonen, Carpen & Seppälä, 1989). Pretreatment of human LGL with purified rabbit antibodies (F(ab')2) to human IgG/Fab efficiently inhibited NK cytotoxic activity, whereas normal rabbit IgG did not. Antibodies against the other

immunoglobulin classes were also ineffective. The antibodies did not affect the binding of LGL to targets but instead a clear inhibition of postbinding orientation was seen, as evidenced by the diminished polarization of actin-containing microfilaments to the contact area in effector:target conjugates. These antibodies immunoprecipitated in reducing conditions polypeptides of 47 and 195 kDa, and under non-reducing conditions 55- and 92- kDa proteins.

Recently Evans and Harris (Evans *et al.*, 1988; Harris *et al.*, 1991) have described an evolutionarily conserved structure which may function as an NK receptor. They demonstrated that mAb derived against NK-like cells in a lower vertebrate species, the teleost fish reacted with a dimeric complex composed of 43- and 38-kd molecules and was NK-specific in both fish and man. It was found that the mAb inhibited lysis of K562 targets by human NK cells and non-MHC-restricted T cells. It had no effect on cytolysis by antigen specific T cells. There was also some evidence that this antigen was involved in signal transduction functions as crosslinking this receptor with mAbs resulted in increased expression of surface activation antigens such as IL-2 receptor and HLA-DR, secretion of the lymphokine TNF, increased cellular proliferation. This molecule has been identified as vimentin (personal communication).

It has been suggested based on available data that no one NK cell receptor has been found which mediates activation and killing but rather both binding and triggering phases of NK activity are

mediated by heterogeneous surface molecules. The relative importance of these molecules in each case may depend on the type of the target cell (Timonen, 1990).

B. *Cytokines involved in the enhancement of NK cell function*

Cytokines have been demonstrated to immunologically modulate NK cells. IFN has been shown to efficiently enhance NK cell activity (Trinchieri, Santoli & Koprowski, 1978). All three known types of IFN including: fibroblast (ß), species of leucocyte type I (α), and leucocyte type II or immune (γ) are able to enhance NK cell cytotoxicity (Lucero *et al.*, 1981; Perussia, Santoli & Trinchieri, 1980; Weight *et al.*, 1982). Like many other types of lymphocytes NK cells express high affinity receptors for all three types of IFN (Faltynek, Princler & Ortaldo, 1986). Besides enhancing their ability to kill classic NK sensitive targets it enables NK cells to kill efficiently targets that are not very sensitive to kill by resting NK cells (Trinchieri, Santoli & Koprowski, 1980; Perussia, Santoli & Trinchieri, 1980).

IFNs enhance NK cell cytotoxicity by increasing the number of cells able to bind to their target (Timonen, Ortaldo & Herberman, 1982), by accelerating the kinetics of lysis (Targan & Dorey, 1980a; Targan & Dorey, 1980b), and by increasing the recycling ability of the cytotoxic cells (Timonen, Ortaldo & Herberman, 1982). Patients and mice treated *in vivo* with IFN generally have a increase in NK cell number but *in vitro* IFNs do not induce NK cell proliferation (Biron, Sonnenfeld & Welsh, 1984).

Other factors such as TGF-ß (Rook *et al.*, 1986) platelet-derived growth factor (Gersuk *et al.*, 1986) and prostaglandins (Brunda, Herberman & Holden, 1980), have inhibitory effects on NK cell mediated cytotoxicity. Their mechanisms of action have not been determined.

The effect of IL-4 on NK cell cytotoxicity and proliferation is still controversial. IL-4 in humans appears to have no effect on the cytotoxic effect of resting NK cells, but it does inhibit the cytolytic activity of IL-2 activated NK cells while not having any effect on IFN-activated NK cells (Nagler, Lanier & Philips, 1988; Spits *et al.*, 1988).

IL-2 is a potent enhancer of NK cell activity (Henney *et al.*, 1981; Weigent, Stanton & Johnson, 1983). The IL-2 receptor (IL-2R) has recently been shown to consist of two distinct proteins, each with an ability to bind IL-2 (Smith, 1988). In fact the IL-2R can be expressed in three distinct forms: isolated p55 chain (IL-2Rp55) binds IL-2 with low affinity; isolated p75 chain (IL-2Rp75) is of intermediate affinity, and these two individual proteins bound together forms a high affinity receptor. IL-2p55 is not functional in its isolated form, but IL-2Rp75 can function both in isolation and within the high affinity receptor complex (Smith, 1988). Unlike T cells, NK cells have been found to show enhanced proliferation (Le Thi Bich-Thuy, Lane & Fauci, 1984) and cytotoxicity (Kehrl *et al.*, 1988) in response to exogenous IL-2 without prior activation. However, larger doses of IL-2 (100-1000 fold higher) are required to activate NK cells in comparison to T cells. An explanation for

these observations became evident when resting peripheral blood NK cells were shown to constitutively express isolated intermediate affinity IL-2Rp75 without prior activation (Dukovich, *et al.*, 1987). In fact it has recently been shown that resting NK cells when they are divided into subsets based on their level of CD56 expression, CD56-bright cells constitutively express the high affinity receptor as well as IL-2Rp75 (Calgiuri *et al.*, 1990).

The enhancement of cytotoxic activity of NK cells occurs after 3-6 hours of incubation and does not require proliferation (Trinchieri *et al.*, 1984b). After 3-4 days of incubation moderate cellular proliferation is observed. The development of an antibody to IL-2Rp75 which blocks IL-2 binding has been shown to also block cellular activation by IL-2 in a short-term assay (4hr) but it did not inhibit basal levels of cytotoxicity (Ortaldo *et al.*, 1990). IL-2 induces the expression of IL-2Rp55 (CD25) antigen on NK cells after 2-4 days in culture (London, Perussia & Trinchieri, 1985) and anti CD25 antibodies suppress proliferation (London, Perussia & Trinchieri, 1986), suggesting that the expression of the high affinity IL-2 receptor is required for maintenance of proliferation. Other activation antigens such as transferrin receptor, CD38 and MHC class II antigens are induced on NK cells after induction with IL-2 (Perussia *et al.*, 1987).

As this summary indicates considerable progress has been made in recent years moving the investigation of NK cells from the phenomenological study of an activity in peripheral blood cells to delineating the functions of molecules central to the function of

these cells. Advances have been made in understanding the structure and function of two key receptors of NK cells, CD16 and the IL-2R. However, many unanswered questions remain concerning NK cell activation. Whether a unique NK cell receptor or whether a number of cell surface molecules may be involved in the activation of proliferation and/or the cytotoxic event still remain to be determined.

γδ T CELLS

Introduction.

The vast majority of T cells express antigen receptors composed of clonally variable αß heterodimers in association with the invariant components of the CD3 complex (Allison & Lanier, 1987). The αß T cells recognize antigens as peptide fragments in the context of class-I or class-II MHC, express the coreceptors CD8 or CD4, and mediate cytotoxic or helper functions, respectively. The diverse functional αß T-cell receptor (TCR) repertoire is shaped by positive and negative selective events that occur during intrathymic maturation (Fowlkes & Pardoll, 1989).

A minority of T cells in the lymphoid organs express alternative receptors composed of clonally variable γ and δ chains also in association with the CD3 complex. Since the serendipitous

discovery of the γ chain gene during attempts to isolate and clone the genes for the αß TCR (Raulet, 1989) the ontogeny, diversity , and biological function of these cells have been the subject of intense investigation (Bluestone *et al.*, 1991; Porcelli, Brenner & Band, 1991). It is not yet possible to establish a paradigm for the development and specificity of the lymphoid γδ cells analogous to the MHC-directed specificity of αß cells. However, these cells clearly have the capacity to recognize a heterogeneous array of ligands, including classical and nonclassical MHC antigens, heat shock proteins, bacterial products, tumour cell antigens and self-antigens (Bluestone *et al.*, 1991; Born *et al.*, 1990a; Porcelli, Brenner & Band, 1991; Van Kaer *et al.*, 1991). The γδ TCR repertoire is limited by the relatively small number of germline elements available for the construction of functional TCR genes. However, the existence of extensive junctional diversity in the γ and δ chains suggest that the potential repertoire is at least as large as that of αß cells (Allison & Havran, 1991; Lafaille *et al.*, 1990).

γδ TCR gene structure.

As is the case for the immunoglobulin genes, the TCR α, ß, γ and δ loci consist of V, J, or V, D, J, and C gene segments which undergo somatic rearrangement to generate functional genes. The diversity of the rearranged genes results from combinatorial diversity arising from the use of different segments present in the

germline, junctional diversity arising from differential trimming of the termini of the recombining gene segments by an exonuclease, and additional junctional diversification by insertion of templated independent nucleotides by terminal transferases (Tonegawa, 1983). Additional, diversity may be generated by the use of tandem D segments in the δ chain (Lafaille *et al.*, 1990).

The genomic organization of the $\gamma\delta$ TCR genes in both mouse and human are shown schematically in Figure 1. (Raulet, 1983). The murine γ genes are located on chromosome 13 (Raulet, 1983). The γ locus consists of four Jγ genes, each associated with a Cγ gene, and seven Vγ genes (Garman, Doherty & Raulet, 1986; Iwamoto *et al.*, 1986). The interspersal of V and J-C gene segments is reminiscent of the λ light chain locus and differs from most other receptor gene loci , including the human γ gene. Cγ3 is a pseudogene and is deleted in some strains (Iwamoto *et al.*, 1986). The Vγ genes seem to rearrange to the Jγ segments in three functional sets: Vγ5, Vγ2, Vγ4, and Vγ3 with Jγ1-Cγ1; Vγ1.2 with Jγ2-Cγ2; and Vγ1.1 with Jγ4-Cγ4 (nomenclature is that of Garman, Doherty & Raulet, 1986. See Raulet, 1989 for concordance with other systems).

The human γ genes, located on chromosome 7 (Rabbitts *et al.*, 1985) are organized in a fashion similar to that of the ß chain genes with two neighbouring JγCγ gene clusters flanked on their 5' sides by an array of Vγ genes (Lefranc & Rabbitts, 1985). Currently 14 human Vγ genes have been identified, of which only 7 or 8 are potentially functional (Dialynas *et al.*, 1986; Forster *et al.*, 1987; Huck, Dariavach & Lefranc, 1988; Lefranc *et al.*, 1986;

Quertermous *et al.*, 1986). Each human Cγ gene is preceded by two (Cγ2) or three (Cγ1) J segments (Lefranc, Forster & Rabbitts, 1986a). All five Jγ gene segments have been reported to be functional (Huck, Dariavach & Lefranc, 1988). The sequences of the two human Cγ regions are very similar overall consisting of three exons, although heterogeneity in the second exon, the membrane proximal connector region of the Cγ2 gene results in significant differences in the structure of the two Cγ regions. The Cγ2 gene, but not the Cγ1 gene, includes two or three homologous tandem copies of the second exon which makes the Cγ2 connector region larger than that of Cγ1 (Lefranc, Forster & Rabbitts, 1986b). Due to genetic polymorphisms, different individuals have two (b and c) or three (a,b and c) copies of the second exon. The second exon of Cγ1 encodes a cysteine residue required for disulfide linkage to the δ chain, while the none of the three Cγ2 exons encodes a cysteine. Therefore, this chain cannot form a covalent linkage with the δ chain (Krangel *et al.*, 1987). These differences in the two Cγ chains results in the production of three structurally different forms of γδ TCRs (Fig. 2) (Brenner, Strominger & Krangel, 1988). The heterogeneity in the size and sequence of the connector region of the human γ chain may have functional consequences as is discussed later.

The murine Cδ, Jδ, and Dδ gene segments are located on chromosome 14 between the Vα and Jα gene segments (Fig.1) (Raulet, 1989). There are about 10 Vδ genes (Bluestone *et al.*, 1988; Chien *et al.*, 1987a; 1987b; Elliott *et al.*, 1988; Korman *et al.*, 1988;

Takagaki *et al.*, 1989b). Some Vα genes, or genes very similar to Vα , are used in functional δ genes. The δ locus has two D and two J elements upstream of a single C gene. The human δ locus, located on chromosome 14, is also located between Jα and Vα gene segments (Greisser *et al.*, 1988; Satyanarayana *et al.*, 1988). Similar to the murine locus, three Jδ and two Dδ gene segments are located 5' to Cδ. Three human Vδ gene segments account for the majority of the γδ T cells and only 6 differnet V genes have been reported to date (Porcelli, Brenner & Band, 1991).

While the TCR γ and δ loci contain many fewer V and J segments than the α and ß locus, extensive junctional diversity has been observed in rearranged γ and δ genes, especially in the adult murine thymus (Elliott *et al.*, 1988). If, as proposed the junctional regions are located in the putative third complementarity determining region and are involved in recognition of antigenic peptides, the capacity of γδ cells to recognize diverse antigen might be remarkably large. There is evidence for the role of junctional diversity influencing antigen specificity. Two alloreactive clones, LKD1, specific for the I-Ad class II MHC molecule, and LBK5, specific for I-EK,b,s Ia molecules expressed receptors encoded by rearranged Vγ1.2,Jγ1, and Vδ5, Dδ2, Jδ1 gene elements differing in sequence only in the V(D)J junctional regions (Rellahan *et al.*, 1991). However, as will become evident on further discussion of the predominant forms of γδ T cells present in epithelial tissues there is preferential pairing of specific Vγ and Vδ chains and extremely limited junctional diversity.

γδ T-cell development.

Murine.

In the murine system γδ cells are the first T cells to appear in ontogeny (Havran & Allison, 1988; Pardoll *et al.*, 1987) detectable by day 14 of gestation. They comprise the major population of TCR+ cells in the thymus until day 18, after which they represent about 1% of the total thymocyte population as αß T cells emerge (Havran & Allison, 1988; Itohara *et al.*, 1989). γδ cells first appear in the spleen between birth and three weeks of age (Pardoll *et al.*, 1988).

It became apparent that there is a programming of γδ TCR appearance during ontogeny. Cells bearing different γδ TCR appear during ontogeny as a series of overlapping waves (Fig. 3) (Allison & Havran, 1991). The first TCR+ cells to appear express Vγ3 followed sequentially by Vγ4, Vγ2 and finally cells expressing Vγ5 and other γδ TCR. In the adult thymus Vγ2-Jγ1/Cγ1 and Vδ5 predominate (Takagaki *et al.*, 1989b; Itohara *et al.*, 1989), with some Vγ1.1-Jγ4/Cγ4 present. The composition of the adult spleen is very similar but it also expresses Vδ2, Vδ4 and Vδ6 (Ezquerra *et al.*, 1990; Pardoll *et al.*, 1988). Another striking difference in fetal and adult TCRs is the extensive junctional diversity which occurs in the adult thymus and spleen giving these cells the potential to interact with a wide array of antigens. In contrast, essentially no junctional diversity appears in the Vδ1, Vγ3, or Vγ4 genes in the fetal thymus (Elliott *et al.*, 1988; Lafaille *et al.*, 1990).These non diverse junctional sequences are referred to as

canonical fetal sequences.

A single Vγ gene segment is used in each epithelial tissue except the lung (Table 1) (Allison & Havran, 1991). Analyzing the relevant cells in a number of mice bearing TCR transgenes indicated that tissue tropism via the TCR was not involved. For example , the intestinal and the epidermal cells of mice transgenic for Vγ2/Vδ5 TCR of the type expressed by most adult γδ thymocytes were found to bear TCR encoded by the transgenes , rather than Vγ5 or Vγ3 (Bonneville *et al.*, 1990). Instead there is evidence to suggest that the skin, vaginal epithelium and tongue are colonized by early thymic emigrants. The analysis of Vγ3, Vγ4 and Vδ1-Dδ2-Jδ2 rearrangements in the fetal thymus revealed canonical sequences for each which were the same as those found in the γδ cells in the above mentioned epithelia. The intestinal γδ cells use V segments not present early in development, raising the possibility that the intestine is seeded in another manner (Takagaki *et al.*, 1989a).

Thy-1+ cells can be detected in the skin of athymic nu/nu mice, an observation consistent with an extrathymic origin of these cells (Stingl *et al.*, 1987; Tschachler *et al.*, 1983). However, these Thy-1+ epidermal cells in nude mice do not express detectable cell -surface CD3 or associated γδ TCR, and the TCR genes in these cells were found to be unrearranged (Stingl *et al.*, 1987). These results suggested that the thymus was required for generation of the skin Thy-1+ γδ cells. Engraftment of adult nude or newborn euthymic mice with fetal thymic lobes or isolated Vγ3+ fetal thymocytes results in the appearance of donor type Vγ3+ cells in

the skin (Havran & Allison, 1990). These results support the idea that the absence of mature skin γδ cells in nude mice is due to the lack of a functional thymus. Furthermore, the skin of 4-month-old mice which had been exposed to anti-Vγ3 antibodies in utero contained no Vγ3+ cells (Havran & Allison, 1990). These data support the hypothesis that at least skin γδ cells arise from fetal thymic precursors and cannot be generated in the adult mouse. Most likely the intestinal γδ cells arise in a different manner. The V gene segments used in intestinal γδ cells are not abundant in fetal thymus and the junctions exhibit extensive diversity characteristic of rearrangements occurring in the adult. In addition, they are found in athymic nude mice (Bonneville *et al.*, 1990; De Geus *et al.*, 1990; Klein, 1986) and intestinal epithelial γδ cells of donor type arise following bone marrow reconstitution of irradiated thymectomized mice (LeFrancois, 1990). These results support the hypothesis that unlike the skin γδ cells the intestinal γδ cells can arise in the adult in the absence of thymic influence. It would therefore appear that in the mouse development of some γδ cells are thymus dependent while others are not.

Human.

Analyses of T cell development are limited by the difficulty in obtaining tissue samples from fetal sources. By 7 to 8 weeks gestation the human thymus is colonized by T-cell precursors. Triple negative thymocytes (CD2-CD3-CD4-/CD8-) can differentiate in culture into mature γδ T cells, expressing a TCR repertoire that resembles thymic γδ cells. This process was found to be dependent on IL-4 but not on thymic epithelial cells (Haas, Kaufmann & Martinez-A, 1990).

In humans, two major γδ T cell subsets have been identified. The Vδ 1 subset, which expresses Vδ1 chains (recognized by the δTCS1 mAb), are noncovalently associated with various VγCγ2/1 chains (Bottino *et al.*, 1988; Casorati *et al.*, 1989). They predominate in the thymus throughout life. The Vδ2 subset, which express Vδ 2 chains (recognized by the BB3 mAb), are linked by disulphide bridges to Vγ9Cγ1 chains (Borst *et al.*, 1989; Bottino *et al.*, 1988; Casorati *et al.*, 1989; Triebel *et al.*, 1988). They become predominant in the periphery within the first month of life in most individuals. The predominance of the Vδ2 subset in the peripheral blood is thought to result from the selective expansion of these cells in response to peripheral stimuli such as bacterial antigens. They appear to be thymus independent as they accumulate in the periphery of a patient with complete thymic aplasia (Haas, Kaufmann & Martinez-A, 1990). The Vδ1 cells predominate in the blood of some individuals with no obvious genetic contributing

factor (Parker *et al.*, 1990) as well as in the tonsils and the intestine of normal donors (Haas, Kaufmann & Martinez-A, 1990). Their numbers are increased in the intestine of patients with coeliac disease (Halstensen, Scott & Brandtzaeg, 1989; Savilahti, Arato & Verkasalo, 1990). There is no information on whether their development is thymus dependent.

Other.

In other species, counterparts of the murine fetal γδ T cell subsets have not been found. In chickens and in humans there is no predilection of γδ cells for the skin. In the ruminants epidermal γδ cells are abundant, but it is not known yet whether these express the homogeneous TCR like the mouse. Intestinal γδ cells have been found in all species but the preferential localization to the epithelial layer observed in mice and chickens is less obvious in cattle, sheep and humans (Haas, Kaufmann & Martinez-A, 1990).

Lineage relationship of γδ cells and αß cells.

The present available evidence suggests that γδ TCR cells do not differentiate to αß TCR cells. First, surveys of cloned αß T cell lines have revealed that many lack functional rearrangements of all known γ genes (Heilig & Tonegawa, 1987; Reilly *et al.*, 1986). A comparable examination of δ rearrangements in most mature αß T cells is impossible since δ genes are deleted from chromosomal DNA by Vα-Jα rearrangements (Chien *et al.*, 1987a). This deleted DNA segment is present in thymocytes as extrachromosmal circular DNA.

When over 400 such DNA clones were examined there was no evidence of δ gene rearrangement. It appears that neither δ rearrangements nor productive γ rearrangements have occurred in the precursors of most αß cells. Most likely αß and γδ T cells are distinct T cell lineages, likely arising from common precursor thymocytes. The TCR γ and δ genes undergo rearrangement and expression first, under developmental controls that are not known. Some insight into the lineage relationship has come from the analysis of various TCR transgenic mice. αß T cells develop normally in γδ TCR transgenic mice if the γ transgene includes a silencer (Ishida *et al.*, 1990). Thus commitment to the γδ cell lineage seems to determined not by productive γ and δ gene rearrangements, but by nuclear proteins which control γ and perhaps δ, chain expression.

Human γδ T cell distribution and phenotype.

One of the phenotypic hallmarks of TCR γδ cells is the lack of reactivity with mAb directed against TCR αß (Brenner *et al.*, 1986; Weiss, Newton & Crommie, 1986). These monoclonal antibodies include BMA031 (Borst *et al.*, 1990) which detects framework determinant of the CD3-αß complex which is not present on the CD3-γδ complex. The mAb WT31 displays a less clearly defined reactivity as it is felt to bind some epitope present on CD3 complex which apparently is more accessible on the αß cells (Tax *et al.*, 1983). It binds strongly to the surface of αß cells but under certain conditions WT31 can bind at low levels to TCR γδ cells (Van de Griend *et al.*,

1988).

A number of mAb's which react specifically with γδ cells have been developed. These include TCRδ1 (Band *et al.*, 1987) which recognizes a framework determinant on the δ chain; and the more specific mAb Ti-γA (Jitsukawa *et al.*, 1987) which reacts with the Vγ9 variable domain which usually occurs only on the δ2 subset of γδ cells (Borstet *al.*, 1989; Bottino *et al.*, 1988; Casorati *et al.*, 1989; Parker *et al.*, 1990; Triebel *et al.*, 1988) the mAb BB3 which recognizes a Vδ2 epitope (Ciccone *et al.*, 1989); and the mAb δTCS1 which recognizes a Vδ1 epitope (Wu *et al.*, 1988). Using these reagents it has been determined that TCR-γδ lymphocytes ranging between >0.5 and 16% of CD3+ cells were found in fetal and postnatal thymus, fetal and adult peripheral lymphoid organs, and adult peripheral blood. Generally they comprise a small subset (4%) of the CD3+ cells but occasionally they are greater than 10 to 16% of the CD3+ population (Groh *et al.*, 1989). The more specific mAb's have been useful to define the Vδ1 subset as previously mentioned commonly occurring in the thymus throughout life while the Vδ2 subset is predominant in the periphery.

A striking feature of these γδ cells is that many of the cells display a double negative phenotype (CD4-/CD8-) (Brenner *et al.*, 1986; Groh *et al.*, 1989). CD8 has been found on greater than 25% of peripheral blood TCR-γδ+ cells and on about 50% of the γδ T cells found in splenic sinusoids, but at levels approximately one-tenth that found on CD8+ TCR-αß cells (Groh *et al.*, 1989). CD4 has been detected on very few γδ cells (Groh *et al.*, 1989).

γδ cells in the peripheral blood are reported to be either negative or express low levels of CD5 (Groh *et al.*, 1989; Jitsukawa *et al.*, 1987; Lanier & Weiss, 1986). CD2 is generally expressed at levels comparable to αß cells (Groh *et al.*, 1989; Jitsukawa *et al.*, 1987; Lanier & Weiss, 1986). CD16, the IgG Fc receptor has been found on cultured γδ cells using mAb VD2 (Borst *et al.*, 1987), although the majority of γδ cells do not react with other CD16 mAb such as B73.1 (anti-Leu-11c) (Van de Griend *et al.*, 1987). Resting γδ cells lack the B cell antigen CD19, but express CD7 (Lanier & Weiss, 1986). CD56 (NKH1), usually present on natural killer cells has been found on several clones (Jitsukawa *et al.*, 1987) and greater than 70% of human peripheral γδ T cells (Inghirami *et al.*, 1990).

Human γδ cells populate both organized lymphoid tissues (thymus, tonsils, lymph nodes and spleen) as well as the gut and skin associated lymphoid systems at similar frequencies with out obvious tropism for epithelial microenvironments. γδ cells tend to be located within a given organ wherever αß cells are found (Groh *et al.*, 1989).

γδ T lymphocyte specificity.

Despite the similarity between the γδ heterodimer and the αß TCR, antigen-specific reactivity of γδ cells has been difficult to demonstrate. At the present time putative ligands for the γδ TCR include MHC class-1-like proteins, mycobacteria and heat shock proteins (HSP). αß T cells recognize antigens in association with

self MHC class I or class II encoded proteins. They also readily develop clones reactive to specific foreign MHC class I (Ciccone *et al.*, 1989; Rivas *et al.*, 1989) and class II proteins (Bosnes *et al.*, 1990). Also, there is a description of human γδ T cells clones to tetanus toxoid which appeared to be HLA-DR4 restricted (Kozbor *et al.*, 1989). However not all HLA-DR4+ individuals could present this antigen and it was suggested that a gene closely associated and in linkage disequilibrium with it. A more common finding with γδ clones was observed by Born et al. (1990) when a large panel of T cell hybridoma were tested against a panel of allogeneic cells, alloreactive αß hybridomas were easily detected but not a single alloreactive γδ T-cell hybridoma among 2000 combinations tested was found.

There is some evidence to suggest that γδ cells recognize antigens that are presented by the nonclassical MHC proteins, such as the TL-region-encoded antigens (Van Kaer *et al.*, 1991) or members of the CD1 family of proteins (Porcelli, Brenner & Band, 1991; Porcelli *et al.*, 1989). Fairly conclusive evidence for the recognition of a TL gene product by a γδ TCR was demonstrated when a γδ T-cell hybridoma KN6 was found to recognize fibroblasts that were transfected with one of the cDNA clones that was isolated with a pan-class-I probe from the embryonal carcinoma cell line PCC3 (Van Kaer *et al.*, 1991). The cDNA originated from a new TL gene that belongs to a previously undescribed TL region cluster in B6 mice. This new TL gene was found to be expressed in many tissues and by many cells, while other TL genes have a more restricted

tissue distribution. It is possible that different TL-region-encoded proteins might serve as antigen presenting molecules for different $\gamma\delta$ cell subsets in different tissues.

Heat shock proteins are a highly conserved group of proteins found in all organisms. They are constitutively expressed in cells and play a vital role in maintaining normal cell functions (Lindquist & Craig, 1988). Induction of HSP's occurs following many environmental stresses such as temperature changes, inflammation and fever, irradiation, viral infection, malignant transformation, exposure to oxidizing agents, ethanol and anoxia. They are divided into families of related proteins based on size, antigenic crossreactivities and sequence homologies. They are divided into the "small" HSPs, the GroEL-related HSPs (HSP-60 group), the HSP-70 group, the HSP-90 group and the HSP-110 group (Lindquist & Craig, 1988). Members of the GroEL family share homology with the GroEL protein of *Escherichia coli* and are found in both prokaryotes and eukaryotes. In eukaryotes they are normal components of the mitochondrial matrix, although they are encoded in the nucleus and synthesized in the cytoplasm. These HSP's appear to function as accessory factors in facilitating the folding of proteins and association of subunits with protein complexes (Cheng *et al.*, 1989; Hemmingsen *et al.*, 1988). The subcellular location of mycobacterial 65 kDa HSP has not been clearly defined. This protein has been found in insoluble cell fractions, in the cytoplasm and as a major culture filtrate protein during zinc-deprived growth. GroEL-related proteins have been identified as major antigens in various

bacterial infections, including Q fever, syphilis, tuberculosis and leprosy (Abo *et al.*, 1990; Mink, Gatrill & Kaufmann, 1990; Porcelli, Brenner & Band, 1991; Watson, 1989). Mycobacterial HSP-65 is a frequent target of antibodies and T cells suggesting it has an immunodominant role. Multiple HSP-65 epitopes recognized by mouse and human T and B cells have been identified (Anderson, Barry & Buchanan, 1988; Born *et al.*, 1990b; Kabelitz *et al.*, 1990; Mehra, Sweetser & Young, 1986; Oftung *et al.*, 1988; Ottenhoff *et al.*, 1988; Shinnick *et al.*, 1987).

In humans, $\gamma\delta$ cell lines reactive with mycobacterial purified protein derivative (PPD) have been generated from synovial fluid of a rheumatoid arthritis patient (Holoshitz *et al.*, 1989), leprosy skin lesions (Modlin *et al.*, 1989) and a healthy individual immune to PPD (Haregewoin *et al.*, 1989). These human $\gamma\delta$ cells which proliferate after stimulation predominantly express Vγ9/Vδ2 gene products (Porcelli, Brenner & Band, 1991). In limiting dilution cultures roughly half of all $\gamma\delta$ cells in peripheral blood proliferate after mycobacterial stimulation. 60 % of Vγ9/Vδ2 clones generated by polyclonal activation recognize *Mycobacterium tuberculosis*-pulsed antigen presenting cells (Kabelitz *et al.*, 1990; Porcelli, Brenner & Band, 1991). Both the mycobacterial responsive and non-responsive clones exhibit marked junctional diversity (Porcelli, Brenner & Band, 1991). A minority of the mycobacterial reactive human $\gamma\delta$ T cells appear to recognize mycobacterial HSP-65 (Kabelitz *et al.*, 1990; Porcelli, Brenner & Band, 1991). Many recognize an antigen that is induced by a

protease-resistant low molecular weight (<3kDa) fraction of mycobacterial lysates (Pfeffer *et al.*, 1990). This could of course represent a heat shock protein fragment.

A tumour cell line, Daudi, has also been described as capable of γδ T cell induction (Fisch *et al.*, 1990b) in humans and has been used to produce Daudi responsive clones. It preferentially stimulates Vγ9 expressing clones and evidence is presented which suggests that a member of the GroEL-HSP family is possibly involved in this response. As well Vγ9 expressing γδ cells have been shown to recognize staphylococcal enterotoxin A coated cells in cytotoxic assays but not in proliferation assays (Rust *et al.*, 1990).

The human observations presumably depend on antigen-driven expansion of peripheral γδ cells. However, Born et al. found γδ cells reactive with mycobacterial antigen in the neonatal thymus, that is, at a time in ontogeny and at a site where antigen-driven expansion has probably not yet occurred (Born *et al.*, 1990a; 1990b; O'Brien *et al.*, 1989). 10% of these TCR γδ-bearing hybridomas generated from fusions with neonatal thymocytes were reactive with PPD. Among αß hybridoma produced in the same fusions, no PPD reactive cells were found. Unlike the humans more than half the PPD reactive hybridomas were also stimulated by purified recombinant *M. bovis* HSP-65. In fact, to clarify the nature of the antigen, these γδ hybridomas were stimulated with small synthetic peptides corresponding to different portions of the *M. leprae HSP-65* and a specific linear epitope that was recognized by these γδ cells (Born *et al.*, 1990b). It would appear that γδ cells may see at least

GroEL HSP as presented peptides.

Another unusual feature of these mouse γδ T cell hybridomas was that over a third of them produced IL-2 "spontaneously" in the absence of added antigens, and IL-2 production required the participation of the γδ receptors (Born *et al.*, 1990a; O' Brien *et al.*, 1989). These data suggested that some γδ TCRs recognize an antigen expressed by the T-cell hybrids themselves. The hybrids that "spontaneously" produced IL-2 also responded to PPD and HSP-65. One interpretation of this is that the self-antigens is a murine stress protein, and that the T-cell hybrids can present self-stress protein determinants.

Generally it has been observed that there was no restriction to polymorphic MHC molecules for human or murine mycobacteria-reactive γδ cells (Porcelli, Brenner & Band, 1991), although there is one report where a human HSP-65 reactive γδ cell line apparently requires antigen presentation in the context of self MHC (Haregewoin *et al.*, 1989). Where antigen presentation is required by γδ cells, a number of studies as mentioned above have demonstrated that γδ cells with specificities to the non-polymorphic class Ib molecules (CD1, TL and Qa) are more likely to be involved than the polymorphic MHC class Ia or class II molecules (Porcelli, Brenner & Band, 1991; Van Kaer *et al.*, 1991). An alternative explanation for γδ stimulation by PPD or HSP-65 is that these proteins could behave as superantigens (Fisch *et al.*, 1990b; Porcelli, Brenner & Band, 1991; Rust *et al.*, 1990). The molecular requirements for superantigens are not fully defined for cells but

they appear to act as polyclonal activators for cells expressing particular Vß genes (Herman *et al.*, 1991). Since in humans all the γδ cells reactive to HSP-65 express the same Vγ region, Vγ9, and in the mouse they express Vγ1, it is possible this protein is a superantigen for these cells.

The murine dendritic epidermal cells (DEC), Thy-1+ γδ cells, which have been previously discussed are a unique quite sessile cell with little lateral mobility and a very restricted γδ TCR repertoire. It is likely that each individual DEC encounters only those few keratinocytes that surround it. Thus, the function of DEC may be to recognize damage-induced self-antigens induced in keratinocytes, rather than the agent that induces the cellular damage (Allison & Havran, 1991). Although there is no evidence that DEC respond to HSP-65 (they also do not express Vγ1) there is evidence that DEC recognize self-antigens. Freshly isolated DEC, as well as DEC clones, respond to cultured keratinocytes by IL-2 secretion and proliferation (Allison & Havran, 1991). The stimulatory capacity of the keratinocytes is increased by heat shock or by exposure to sodium arsenite, suggesting that stress-related antigens might be involved in the response. The stimulation is specific, in that cells of non-epidermal origin are not recognized by the DEC, and γδ cells that express TCR other than the DEC Vγ3/Vδ1 combination do not respond to keratinocytes. The response is blocked by Fab fragments of anti-γδ TCR antibodies, and transfection of the DEC TCR genes into a nonresponsive human lymphoma confers reactivity. The stimulatory antigen appears to be

a peptide, since live or fixed fibroblasts incubated with tryptic digests of cultured keratinocytes, but not other cells, can stimulate IL-2 release by DEC. These observations suggest that DEC have the capacity to recognize stress-induced keratinocyte specific self-peptides. There was no obvious role for products of the MHC observed in the DEC keratinocyte interactions.

γδ T lymphocyte function.

γδ T cells exhibit various functional capabilities similar to those of αβ T cells. TCR γδ cells can be activated by perturbations of the TCR-CD3 complex using calcium and inositol phosphate-mediated pathways (Krangel *et al.*, 1987; Pantaleo *et al.*, 1987). Production of T cell -derived lymphokines has also been documented. IL-2 production in response to activation with mitogens or anti-CD3 antibodies has been reported for human and murine γδ cell lines (Bluestone *et al.*, 1988; Ferrini *et al.*, 1987; Patel *et al.*, 1989), murine Thy-1+ DEC lines (Allison & Havran, 1991; Nixon-Fulton *et al.*, 1987), and T-hybridomas derived from adult murine thymic γδ cells (Marusic-Galesic *et al.*, 1988). Early fetal thymic γδ cells may produce both IL-2 and IL-4 (Tentori *et al.*, 1988). An alloreactive murine γδ cell line has been documented to produce both GM-CSF and γ-interferon (Bluestone *et al.*, 1988). Measurements of IL-2, -4, -5, TNFα, GM-CSF, γ-interferon production after anti-CD3 antibody or lectin stimulation demonstrate that all of these cytokines can be synthesized and secreted by γδ T cell clones (Porcelli, Brenner & Band, 1991). However, most of these cell lines

exhibit both lymphokine production and cytolytic activity suggesting that for the present unlike αß cells γδ cells cannot be categorized into cytotoxic and helper subsets.

One of the most notable features of γδ cells is their ability to carry out cell-mediated lysis of target cells. They are able to carry out redirected lysis (Spits *et al.*, 1985), tumour lysis (Borst *et al.*, 1987) and MHC-linked lysis (Matis *et al.*, 1989a). In redirected lysis, anti-CD3 mAb bind and presumably signal effector cells, which then conjugate in part through the anti-CD3 mAb binding IgG Fc receptors on target cells (Leeuwenberg *et al.*, 1985; Spits *et al.*, 1985). These experiments did not define the nature of the antigens which may be recognized by γδ cells but they did demonstrate the potent cytotoxic capability of these cells.

In vitro cultured although not fresh γδ cells (Porcelli, Brenner & Band, 1991) from various sources, peripheral blood (Borst *et al.*, 1987; Ferrini *et al.*, 1987), pleural effusion (Borst *et al.*, 1987), fetal blood (Moingeon *et al.*, 1986), and thymus (Lanier *et al.*, 1987) have been found to exhibit spontaneous non-MHC restricted cytolytic activity against tumour targets. In some cases, lysis of tumour targets showed broad reactivity while others were more selective. They were able to lyse targets which did not express MHC I or MHC II molecules such as K562, and antibodies to the MHC molecules did inhibit kill in systems where the targets did express MHC molecules. It is not clear if this tumour lysis is a TCR-receptor mediated, antigen specific interaction, as mAb to CD3 blocked killing in some cases (Borst *et al.*, 1987; Moingeon *et al.*,

1986) and in others it did not (Lanier *et al.*, 1987).

However, there is a report of γδ clones recognizing allogeneic targets (Matis *et al.*, 1989a,b; Rellahan *et al.*, 1991). Balb/c nu/nu mice (H-2^d) were immunized *in vivo* with irradiated B10.BR spleen cells (H-2^k). A γδ cell line derived from these mice proliferated to and lysed concanavalin A T cell blasts from several H-2^k strains of mice. However, the pattern of reactivity was broad (including H-2^b and H-2^q targets) and the specificity was felt to correlate with the recognition of nonclassical MHC-linked antigens, possibly TLa (Matis *et al.*, 1989a). This demonstrates allogeneic reactivity that appeared to be MHC-linked, further supporting the idea that in some circumstances as previously noted γδ cells may be capable of self-non-self discrimination.

Although these functional studies demonstrate that γδ cells are activated by mechanisms and display effector capabilities that are similar to those of αß cells the physiological role of γδ cells is still not clearly established. There is data accumulating which suggest that γδ cells may have a protective role in infectious diseases. Increased numbers of γδ cells have been reported in peripheral blood of patients with measles infection (Haas, Kaufmann & Martinez-A, 1990), HIV-1 infection (DePaoli *et al.*, 1991; Margolick *et al.*, 1991), malaria (Ho *et al.*, 1990) and mononucleosis (DePaoli *et al.*, 1990), in granulomatous lesions of leprosy patients (Modlin *et al.*, 1989), in the peritoneal cavity of mice infected with *Listeria monocytogenes* (Ohga *et al.*, 1990) and in the *in vitro* proliferation of PBMCs to various bacteria (Mink,

Gatrill & Kaufmann, 1990). An accelerated appearance of γδ T cells in the intestine of young parasitized chickens (Haas, Kaufmann & Martinez-A, 1990) and an accumulation of mucosal γδ cells five days after intranasal infection of mice with influenza virus (Carding *et al.*, 1990) have been reported. γδ cells may also have a role to play in the pathogenesis of various autoimmmune diseases and diseases of unknown origin. Elevated levels of γδ cells have also been reported in disease such as rheumatoid arthritis (Brennan *et al.*, 1989; Plater-Zyberk *et al.*, 1989), multiple sclerosis (Freedman, Ruijs, Selin & Antel, 1991; Mix *et al.*, 1990), pulmonary sarcoidosis (Balbi *et al.*, 1990; Tamura *et al.*, 1990), coeliac disease (Halstensen, Scott & Brandtzaeg, 1989; Savilahti, Arato & Verkasalo, 1990), thyroiditis (Teng *et al.*, 1990), polymyositis (Hohlfeld *et al.*, 1990) and various hematological disorders (Carbonari *et al.*, 1990; Hara *et al.*, 1990; Lehmann, Sawyer & Donabedian, 1989; Morio *et al.*, 1990).

One plausible theory concerning the physiological role of γδ cells which encompasses much of the information already presented has been proposed by Born et al. (Born *et al.*, 1990). They suggest that: 1) a large portion of γδ cells (as opposed to a minor fraction of αß cells) may recognize autologous HSPs, and individual cell populations (characterized by different receptors and different anatomical locations) may be specific for different stress proteins; 2) antigenic epitopes for γδ cells may coincide with the evolutionarily more conserved portions of HSPs; 3) HSP reactive γδ cells may require different and less polymorphic

antigen-presenting molecules than αß cells with similar specificities.

These postulates imply that γδ cell recognition would normally be confined to a limited number of autologous indicator peptides. Certain autologous peptides might appear on potential γδ targets, depending on the kind of stress-inducing stimuli and on the genetic makeup of these cells. Different γδ subsets might respond to certain stress signals in particular ways, for instance by direct activation, inactivation or destruction of their targets, or alternatively by relay of the primary signals to other components of the immune system. Therefore, reactivity with autologous HSPs could be a basis for several previously postulated γδ functions, including immune surveillance of epithelia (Allison & Havran, 1991; Janeway, Jones & Hayday, 1988), regulation of lymphocyte growth and development (Owen *et al.*, 1988; Ferrick *et al.*, 1989), and perhaps macrophage activation (Born *et al.*, 1990).

The present data suggest that γδ antigens are limited in diversity and that they can also recognize heterologous HSP peptides which are highly evolutionarily conserved. The recognition of conserved antigens should not require junctional variability of the γδ receptor (Janeway, Jones & Hayday, 1988)). This variability may be of no functional significance or it could increase the chances of generating cells with higher affinities for heterologous antigens and may represent a partial adaptation of some γδ subsets to conventional immune functions (Asarnow *et al.*, 1988). The large numbers of cells with the same or similar specificities may provide

a pool for rapid immune responses to certain critical antigens without the need for prior clonal expansion. γδ cells could act as a first line of defense, that could complement the αß T cell and B cell responses which may take more time to develop (Janeway, Jones & Hayday, 1988; O'Brien & Born, 1989).

To postulate that γδ cells are reactive to autologous HSPs would assume that normal unstressed cells have special properties or there are other regulatory immune elements which prevent uncontrolled autoaggressive responses. However, crossreactivity with heterologous HSPs might break this tolerant state, and lead to autoimmune states. This could explain why HSP-65 reactive γδ and αß clones are present in rheumatoid arthritis patients (Holoshitz *et al.*, 1989).

As this brief review indicates there are still a great many unanswered questions concerning γδ activation by specific ligands or lymphokines, the nature of their interaction with MHC molecules, and their physiological role in the normal immune response.

THE PRESENT INVESTIGATION

Aim and Strategy of the present investigation.

The nonadaptive innate immune response characterized by non-

MHC restricted cytotoxic effectors appears to play a significant role in host cellular immunity against both infectious diseases and tumours. As well, evidence suggests that these normal immune responses perhaps when inadequately controlled may be involved in the pathogenesis of chronic autoimmune diseases such as rheumatoid arthritis. It is therefore important to understand the mechanisms involved in the generation and regulation of these effector cells. This information could lead not only to a better understanding of host response but to methods of enhancing or inducing responses which might cure tumours or to methods of intervention which could break the cycle of inflammation in chronic autoimmune diseases.

The most significant feature of this early innate immune response is the activation of non-MHC restricted cytotoxicity. Generally NK cells have been felt to be synonymous with non-MHC restricted killer activity (Ortaldo *et al.*, 1977; Takasugi, Mickey & Terasaki, 1973; West *et al.*, 1977). However, it has become evident that T cells, both $\gamma\delta$ and $\alpha\beta$ T cells can also demonstrate this function (Hersy & Bolhuis, 1987; Grimm *et al.*, 1983; Porcelli, Brenner & Band, 1991; Reynolds & Ortaldo, 1987). As previously noted, whether and how these nonspecific T cells participate in the innate response is currently still under investigation.

The mechanisms involved in the activation and expansion of these non-MHC restricted effector cells have not been fully defined. Activation may involve one or a number of cellular activities, such as proliferation, cytotoxicity and/or cytokine secretion. Because of the inherent ambiguity, investigators need to

be careful with the term "activation". Clearly, the recognition of tumour targets by non-MHC restricted killer cells results in an activation program which results in the lysis of targets. Treatment of the same cells with IL-2 also activates them. However, surface molecules as well as the biochemical intermediates responsible for these discrete forms of activation are not the same. Additionally the term "activation " does not specify a particular event; it may refer to the generation of second messengers, alteration of gene expression or changes in cytolytic capacity. The precise measure of activation is not inherently apparent. However, despite its potential for ambiguity the term activation is convenient, providing one attempt to clarify the receptor that is engaged and the measure of activation utilized.

The objective of the present project was to examine potential events leading to the activation of the early non-adaptive immune response specifically assessing the induction of non-MHC restricted killing. The term "activation" in the present context of the present studies refers to the induction of proliferation and cytolytic potential in cells of the non-MHC restricted population. However, as stated earlier there are recognition events which occur during tumour target cell interaction with non-MHC restricted killer cells which result in an activation program resulting in the lysis of targets. This activation of the lytic machinery in non-MHC restricted killer cells, especially concerning the NK phenotype has been extensively studied. However, to date the "NK receptor" has not been identified. For $\alpha\beta$ T cells the TCR has been identified as

an important molecule involved in MHC-restricted antigen specific cytotoxicity but whether or not this receptor is the only structure on T cells involved in non-MHC restricted cytotoxicity is yet to be determined. The exact nature of receptor-ligand interaction with γδ T cells is also not yet clearly defined. For both T cell types certainly the TCR and the CD3 complex are important in the activation of not only lysis but also cellular proliferation and cytotoxic potential. Once again for NK cells these molecules have not been identified.

It is possible that the recognition event in tumour target cell interaction with non-MHC restricted killer cells results not only in an activation program resulting in the lysis of targets but also of a program resulting in effector cell proliferation and enhanced cytotoxic potential. It was therefore decided to take a critical look at whether there is a relationship between the role tumour cells may play not only as targets but as potential inducers of activation. Studies of this kind could potentially give more insight into possible factors needed for activation of non-MHC restricted killer cells.

As outlined in the first paper a systematic examination of different tumour cell lines revealed a differential ability of the lines to induce effectors, both NK cells, and T cells (αß+ and γδ+). Since all these tumour lines were targets of the effectors it suggested that the inducing structures and target structures were unlikely to be the same. The study then went on to examine if there were any common elements or properties shared between different

stimuli. It became apparent that lymphoblastoid B cell lines were the most effective inducers of both proliferation and cytotoxic activity. Different LBL alone or in conjunction with IL-2 were able to stimulate non-MHC restricted cytotoxic activity in NK cells, $\gamma\delta$ and $\alpha\beta$ T cells. All three phenotypes generally coexisted with all stimuli, they were not mutually exclusive. However, the predominant phenotype would vary depending on the specific LBL used in the induction system as well as the presence or absence of IL-2. This suggested that these three effector phenotypes displayed differing induction requirements.

The second paper addresses further the issue of what common elements in LBL resulted in non-MHC restricted activity. Specifically, the role of EBV infection of the LBL, and the role of cellular contact was examined. The presence of EBV infection was found to significantly enhance LBL cytotoxic and proliferation inductive capacity as well as the proportion of CD16+ cells. However, these results suggested that the role of EBV was to enhance IL-2 secretion spontaneously by the PBMCs. Specific EBV uninfected lines could induce NK cells and $\gamma\delta$ T cells with potent cytotoxic activity in the presence of exogenous IL-2. These results suggested there were specific factors other than EBV present on LBL which led to the activation of either NK, or $\gamma\delta$ T cell effectors. As almost all LBL activated NK cells and only two activated $\gamma\delta$ T cells the activating factor is most likely different for these two effector types.

Consistent with the hypothesis that a LBL cell surface

molecule was involved in the induction cellular contact was found to be essential. As well antibodies to 3 classes of adhesion molecules (CD2, CD18 & CD29) were found to inhibit LBL induction.

In the third paper the induction systems of γδ T cells by two LBL, RPMI 8226 and Daudi were explored. They both induced predominantly the Vγ9 subset of T cells. There was no clear common characteristic between RPMI 8226 or Daudi which might assist in further identifying the factor which results in γδ T cell activation. There also were some differences in the nature of the effectors induced by these tumours. When the effectors induced by RPMI 8226 were examined at the clonal level they were found to be heterogeneous in terms of cell surface markers, patterns of proliferation and cytotoxic responses. The role of HSP58 which has been suggested as an inducing molecule in this system was examined. However, based on these results until the stimulating structure(s) are identified it is unclear whether the same or a similar element present on these two tumour cell lines is involved in the γδ T cell activation process.

Collectively the results suggest that some LBL cell surface stimulus can induce an activation and expansion of non-MHC restricted killer cells, both NK and T cells (αß and γδ). This inductive capacity appears to relate in part to viral infection and in part to the phenotypic properties of the inducer. The nature of the stimulus is still unclear at this time but these results do suggest that there is a clear distinction between target susceptibility and inductive capacity.

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PAPER I.

Induction of Non-MHC Restricted Killer Cells: Differential Induction of Effector Populations

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SUMMARY

Non-MHC restricted killer cell activity is an essential part of the normal host defense against viral infection and tumour growth. These same cell population(s) may be involved in the pathogenesis of autoimmune diseases. An understanding of the mechanisms involved in their activation and growth is pertinent. In order to study the nature of the potential cellular interactions during activation of these nonspecific effectors, a panel of human tumour cell lines were screened for their ability to induce non-MHC restricted killer cell activity in peripheral blood mononuclear cells (PBMC). Lymphoblastoid B cell lines (LBL) were the most effective stimuli of non-MHC restricted killer cell activity as they induced both proliferation and effector activity in the responder populations. Different LBL alone or in conjunction with IL-2 were able to selectively stimulate non-MHC restricted cytotoxic activity in NK cells, $\gamma\delta$ or $\alpha\beta$ T cells. The phenotype(s) which was induced was dependent on the specific LBL used in the induction system as well as the presence or absence of exogenous IL-2.

Purified populations of the activated CD16+, $\alpha\beta$ and $\gamma\delta$ T cells all displayed non-MHC restricted killer cell activity. On a per cell basis the CD16+ cells were more potent effectors than the $\alpha\beta$ T cells. Both these cell populations were able to lyse all targets tested. Although, the $\gamma\delta$ T cells were able to lyse targets in a non-MHC restricted manner they were more restricted in their range

of targets.

These results indicate that B cells, either virally infected or transformed B cells express properties which induce non-MHC restricted killer cell activity. These properties appear to be unique to B cells as other tumour cell types were not effective inducers.

INTRODUCTION

The nonadaptive immune response plays a significant role in host cellular immunity to infectious diseases and tumours (Trinchieri, 1989; Welsh, 1986; Pross & Baines, 1986). During a viral infection the role of the immune response is to block viral spread. The response occurs in three phases which include: an immediate response (<4 hrs) consisting of a nonadaptive, nonspecific cytotoxic response predominantly mediated by NK cells; followed by an early phase (4-96 hrs) which consists of a non-adaptive, nonspecific but inducible response involving primarily NK cells activated by lymphokines such as IFN-α and -β; the late phase (>96 hrs) consists of an adaptive, specific, memory inducible cell mediated response, involving specific $\alpha\beta$ TCR+ cytotoxic T cells and the production of specific antibodies by activated B cells (Janeway, 1989; Welsh & Vargas-Cortes, 1992). The earlier phases are important and perhaps essential in controlling the infection by lysis of infected cells and by production of lymphokines which

recruit other inflammatory cells to the site of infection. The late phase may be more effective in the clearance of virus.

An important feature of the early innate immune response is the activation of non-MHC restricted cytotoxicity. Although NK cells are felt to be the predominant non-MHC restricted killer effectors, there is evidence that T cells, both γδ and αß T cells can mediate this function (Hersey & Bolhuis, 1987; Grimm *et al.*, 1983; Porcelli, Brenner & Band, 1991; Reynolds & Ortaldo, 1987; Welsh & Vargas-Cortes, in press). However, the role of these non-MHC restricted cytotoxic T cells in the early response is unknown.

The nonadaptive mechanisms of immune defense have also been postulated to be important in the surveillance of tumours. Spontaneously occurring tumour infiltrating murine lymphocytes have been reported to be composed of NK cells, as well as αß and γδ T cells (Karpati *et al.*, 1991; Miescher *et al.*, 1990). Experimental animal models have clearly shown that NK cells are effective *in vivo* and can destroy tumour cells (Gorelick *et al.*, 1979; Hanna & Burton, 1981; Kiessling *et al.*, 1976). In humans activated cells of the nonadaptive immune response have been shown to be therapeutically effective when lymphokine activated killer cells (LAK), which are comprised of activated NK cells as well as αß and γδ T cells, were used to treat renal cell carcinoma and melanoma (Aparicio *et al.*, 1990; Grimm *et al.*, 1983; Hersey & Bolhuis, 1987; Rosenberg, 1985). One case of selective lysis of an autologous tumour by a δTCS1+ γδ tumour infiltrating lymphocyte from a human lung carcinoma has been reported (Zocchi, Ferrarini & Rugarli,

1990).

Conversely, the non-MHC restricted cytotoxic response may also contribute to the pathogenesis of chronic autoimmune diseases such as rheumatoid arthritis. Freshly isolated cells from rheumatoid patients display enhanced non-MHC restricted killer cell activity (Goto & Zvaifler, 1985). NK cells have been demonstrated to be activated by rheumatoid factor via the CD16 molecule resulting in lymphokine production (IFNγ and TNFα). It has been postulated that release of these cytokines in a rheumatoid joint perpetuates the chronic inflammation present in rheumatoid arthritis (Hendrich *et al.*, 1990; Werfel, Uciechowsky & Tetteroo, 1989). γδ T cells have been reported to be elevated in some patients with rheumatoid arthritis and primary Sjogrens syndrome (Brennan *et al.*, 1989; Plater-Zyberg *et al.*, 1989). Another study reported decreased levels of γδ cells in the peripheral blood of rheumatoid arthritis patients but with increased levels of δ1 expressing T cells present in the rheumatoid joints (Smith *et al.*, 1990).

The mechanisms involved in the activation and expansion of these nonspecific effector cells have not been fully defined. Lymphokines such as IL-2 are reported to trigger NK cells and a small percentage of T cells into cell cycle and to maintain their expansion (Brooks, Holschen & Urdal, 1985; London, Perussia & Trinchieri, 1986; Trinchieri *et al.*, 1984). NK cells and γδ T cells both express the intermediate IL-2 receptor (75Kd) which can directly bind IL-2 (Porcelli, Brenner & Band, 1991; Aparicio *et al.*, 1989; Ortaldo *et al.*, 1990). The majority of αß T cells do not

spontaneously express this surface molecule but require activation before they become IL-2 responsive (Wang & Smith, 1987). IFN-α and -ß have also been demonstrated to induce potent non-MHC restricted killing by both NK cells and T cells but not to enhance their proliferation *in vitro* (Brooks, Holschen & Urdal, 1985; Perussia, Santoli & Trinchieri, 1980; Trinchieri, 1989). These results suggest that lymphokines are sufficient to activate NK cells and $\gamma\delta$ T cells and some lymphokines such as IL-2 can expand these populations.

Triggering of non-MHC restricted activity via direct cellular contact, activation via specific cell surface receptors has not been addressed. For $\gamma\delta$ T cells the TCR has been demonstrated to have a role in cell activation (Porcelli, Brenner & Band, 1991) but the identity of the target structure(s) is still unknown. For NK cells the search for a unique NK receptor continues. Although there is some evidence that NK cells can be activated via the cell surface molecules CD16 and CD2 (Trinchieri, 1989).

In order to further examine events leading to the activation of the early non-adaptive immune response the role(s) which tumour cells may play not only as targets but as potential inducers was studied. It is possible that cytotoxic responsiveness to their corresponding targets also implies a capacity of the targets to induce the effector population. In the present study an examination of different tumour cell lines did demonstrate a differential ability of tumour cell lines to induce effectors. The properties and characteristics which made tumour cell lines into effective

inducers were examined as well as the nature of the effector populations.

MATERIALS AND METHODS

Induction of non-MHC restricted killer cells.

Ficoll hypaque purified normal PBMC's (10^6/ml) were cultured in the presence of the indicated irradiated continuous cell lines (50Gy; $3x10^4$/ml) with or without highly purified recombinant human IL-2 (rhIL-2) (50U/ml; Cetus, Emeryville, Calif.) (Rosenberg *et al.*, 1984) in AIM V serum free media (Gibco, Grand Island, NY). The cells were subcultured on day 6 in AIM V media containing rhIL-2 (50 U/ml). At day 10 the cells were harvested, counted and used for immunofluorescence studies, cytotoxicity or proliferation assays.

Cell lines.

Continuous human cell lines K562 (erythroleukemia), Colo 205 (adenocarcinoma), Daudi (Burkitt's lymphoma), Raji (Burkitt's lymphoma), JY (B cell leukemia), Jurkat (T cell leukemia), Molt-3 (T cell leukemia), Molt-4 (T cell leukemia), A549 (lung carcinoma), U937 (histiocytic lymphoma), SKW-13 (B cell leukemia), Ramos (Burkitt's-like lymphoma), U266 (myeloma), IM-9 (myeloma), Hela (cervical carcinoma) and RPMI 8226 (myeloma) were obtained from the American Type Culture Collection (Rockville, MD). RPMI 8866 (B cell leukemia) was purchased from ABS (Buffalo, NY). BJAB and BJAB-P3

were kindly provided by Dr. Louis Qualtiere (University of Saskatchewan, Saskatoon, Saskatchewan). The JR-2 cell line was an EBV transformed B lymphoblastoid generated in our laboratory (Wilkins *et al.*, 1983). These cells were maintained on 10% fetal calf serum (FCS) with RPMI 1640. All continuous cell lines were negative for mycoplasma contamination as defined by culture and/or Hoescht staining.

mAbs and Immunofluorescence.

The cells were labelled with mAbs reactive against CD3 (OKT3), CD2 (OKT11) and CD18 (ß2) produced by hybrid cell lines obtained from the American Tissue Culture Collection (ATCC, Rockville, Md); CD16 (Leu 11b), CD4 (Leu3a PE), CD8 (Leu2a PE), HLA-DR obtained from Becton Dickinson Co. (Mountainview, Calif.); CD56 (NKH-1 PE) obtained from Coulter Immunology (Hialeah, Florida); CD29 (ß1,JB1) and VLAα5 (JBS5) were produced and purified from hybridomas developed in this laboratory (Shen *et al.*, 1991); Diversi-T γV2(a) (Vγ9) and Identi-T pan-TCRαß obtained from T Cell Sciences, Inc (Cambridge, MA). The VLAα4 antibody from Telios Pharmaceuticals Inc. (San Diego, California). The mAb TCRδ1 which recognizes a framework determinant on all δ chain subsets, was kindly provided by Dr. M. Brenner, Dana-Farber Cancer Institute, Boston, MA) (Band *et al.*, 1987). The anti-MHC class II antibody was obtained from Cedarlane Laboratories (Hornby, Ont., Can.). For direct fluorescence, cells were labelled with mAbs conjugated with phycoerythrin (PE) in PBS (30 min at 4^0C)and washed. For indirect

fluorescence, cells were exposed to mAbs, subsequently incubated with FITC-F(ab')$_2$-conjugated goat anti-mouse antibody (Cedarlane Laboratories, Hornby, Ont., Can).

Purification of CD16+ and γ/δ T cells.

To obtain purified CD3+ and CD16+ populations from day 10 RPMI 8866 stimulated PBMCs the cells were positively sorted using anti-CD16 (Leu 11b) or CD3 (OKT3) by flow cytometry (Coulter Epics C). Purified γδ T cells (95%) were positively sorted from RPMI 8226 stimulated cultures using anti TCRδ1 mAb and a fluorescent-activated cell sorter (Coulter Epics C). Cells were rested for at least 4 days in 10% AB+ Rh- human serum plus RPMI 1640 and penicillin/streptomycin before being assayed for proliferative and cytotoxic responses.

Proliferation assay

Purified γδ T cells. 2X10^4 responder cells were cultured with various stimuli: irradiated cell lines (2X10^4/well) RPMI 8226 (human myeloma), RPMI 8866 (human B cell leukaemia), Daudi (human lymphoma), and Colo 205 (human colonic adenocarcinoma); or PHA (0.5 μg/ml). Stimuli were added either alone, or in combination with rhIL-2 (50U/ml) or with rhIL-2 (50U/ml) plus autologous APC's (2X10^4/well). The cultures were maintained for 4 days in 10% AB+Rh- human serum plus RPMI 1640 (penicillin/streptomycin) and pulse labelled with ^{3}H-Tdr (0.2 μCi/well; Amersham, Oakville, Ont.,Can.) for the final 18 hrs of culture.

Purified CD16+ Cells. $1X10^5$ PBMC's were cultured in round bottomed 96 well plates with various irradiated (50Gy) continuous cell lines ($4x10^3$) alone or in the presence of rhIL-2 (50U/ml). The cultures were maintained for 5 days and pulse labelled with ^{3}H-Tdr for the final 18 hrs of culture. Purified CD16+ cells were cultured in the presence of various stimuli including RPMI 8226, RPMI 8866 and PHA (1/1000 dilution) alone or with the presence of irradiated autologous antigen presenting cells (APC)($5x10^4$). These stimuli were added either alone, or in combination with rhIL-2 (50U/ml). The cultures were maintained for 4 days and pulse labelled with ^{3}H-Tdr for the final 18 hrs of culture.

Cytotoxicity Assay.

The cytotoxic activity of the effector cells was determined in a chromium-release assay as previously described (Rosenberg *et al.*, 1974)). The target cells were labelled by incubating $2x10^6$ cells in 0.5 ml RPMI-1640, 10% FCS, containing 300 μCi $Na_2{}^{51}CrO_2$ (Amersham, Oakville, Ontario, Canada) for 90 min at 37^0C. The cells were washed twice in RPMI-1640 and resuspended to a final concentration of $2.5x10^4$ cells/ml. Per cent specific lysis was calculated as 100x[(CPM released with effectors - CPM spontaneously released) / (total CPM - CPM spontaneously released). In all assays spontaneous release was less than 15%. These results were further quantified by calculating the number of lytic units (LU) per 10^7 cells with the use of a linear regression equation (exponential fit) (Pross, Callewaert & Rubin, 1986). One lytic unit was defined as the number

of effector cells necessary to induce 20% release from the target cells during the assay period.

RESULTS

Induction of non-MHC restricted killer cells by continuous cell lines. Of the cell lines tested (9) only the 3 lymphoblastoid B cell lines were capable of inducing non-MHC restricted killer cells. Daudi, Raji or RPMI 8866 consistently induced effector cells capable of lysing NK sensitive target, K562 and NK resistant targets, Daudi and Colo 205 (Fig.1, A-C). Cell lines of other origins, T cell leukemias (Jurkat, Molt 3), histiocytic lymphoma (U937), chronic myelogenous leukemia (K562), colonic adenocarcinoma (Colo 205) or lung carcinoma (A549) did not induce non-MHC restricted killer cells. RhIL-2 also induced non-MHC restricted killing although, the level of cytotoxicity was lower than that observed with LBL induction.

There was an analogous observation in the effects of these cell lines on PBMC proliferation (Fig. 1D). Only the LBL were effective inducers of proliferation. In comparison, IL-2 was a poor inducer of PBMC proliferation.

LBL induction of non-MHC restricted killer cells. In order to further examine the role of LBL in the induction of non-MHC restricted killer activity a larger panel of LBL were screened for this ability. Four LBL, RPMI 8866, IM-9, Daudi and Raji induced

high levels of non-MHC restricted killer activity while 3 other lines Ramos, RPMI 8226 and BJAB were less effective inducers of cytotoxic activity (Table 1) . When exogenous IL-2 was added to RPMI 8866 and IM-9, there was no apparent further enhancement in induction of non-MHC restricted killer activity. However, IL-2 synergized with the three cell lines which were weak inducers resulting in 30-190 fold increases in cytotoxic activity (Table 1).

Similar results were also obtained in PBMC proliferation assays (Table 1). IL-2 was able to enhance proliferation induced by the potent inducer IM-9 by 4 fold but not RPMI 8866. IL-2 did synergize with the LBL to enhance proliferation by the poor inducer lines.

Effector phenotypes induced by LBL.

Stimulation of PBMCs with inducers resulted in the generation of mixed populations. However, depending on the stimulus used different effector populations were generated. Stimulation with Daudi, RPMI 8866, and IM-9 resulted in a marked increase in the proportion of CD16+ cells in the final culture (Table 2). In some cases, such as Experiment 1 it was found that IL-2 could also act as a weak inducer resulting in a significant increase in the percentage of CD16+ cells. However, unlike the LBL induced cultures there was much less proliferation in those cultures receiving only IL-2. Thus LBL caused the growth of CD16+ cells whereas there was little evidence of proliferation in the IL-2 cultures. Exogenous IL-2 did not further enhance the proportion of CD16+ cells induced

by the potent inducer LBL.

The cell lines RPMI 8226 and Ramos, which induced low levels of cytotoxic activity generated predominantly CD3+ αß TCR+ cells. However, addition of exogenous IL-2 resulted in an enhancement of the CD16+ population by Ramos and the γδ TCR+ T cells by RPMI 8226 (Table 2).

These results suggested that a major portion of the non-MHC restricted killer activity was mediated by either CD16+ NK cells or γδ T cells. In order to test this correlation PBMCs were stimulated with cell lines and examined for CD16+ NK cell and γδ T cell generation. These experiments were done in the presence of exogenous IL-2 as some LBL only induced potent cytotoxic activity in its presence. Nine of 10 LBL tested in this manner were efficient inducers of CD16+ cells (Fig. 2A). While only two LBL, RPMI 8226 and Daudi induced γδ T cells (Fig. 2B). Daudi was the only LBL which was able to induce both types of lymphocytes. The non B cell lines were ineffective inducers of CD16+ cells except for the cell line K562 and Jurkat which were able to increase the proportion of CD16+ cells in the presence of IL-2. However, they did not enhance cellular proliferation. The response to Jurkat appeared to have some individual variability.

Characteristics of CD16+ and CD3+ cells induced by RPMI 8866.

The above experiments demonstrated that various LBL could induce non-MHC restricted killer activity resulting in cultures in which there were distinct population phenotypes. The induced

populations were predominately either CD16+ NK cells, αß or γδ T cells. Purified populations of CD16+, CD3+ or γδ+ cells were isolated to determine the cytotoxic characteristics of these generated populations. Purified CD16+ and CD3+ cells were assessed for their cell surface markers after purification with positive cell sorting (Table 3a). The CD16+ purified population was 96% CD16+, 99% CD56+, and 3% CD3+. These cells were 51% CD8+ and were CD4-. The CD3+ purified population was 95% CD3+, 4% CD16+ cells, 10% TCRδ1+ and 90% αß TCR+.

In the IL-2 stimulated cultures the CD16+ sorted cells were 75% CD16+, 90% CD56+ and 2% CD3+ while the CD3+ sorted cells were 98% CD3+, 43% TCRδ1+ and 50% αß TCR+.

The purified CD16+ cells derived from an LBL stimulated population were extremely potent effectors against all three targets (Table 3B). Since the LBL-stimulated CD3+ cells were predominantly αß TCR+ these results were interpreted to reflect their cytotoxic ability. They were effective against all three targets, but not at levels comparable to the CD16+ population. In the case of CD16+ and CD3+ purified cells derived from IL-2 stimulated populations, CD16+ cells were more potent than the CD3+ cells, although both populations were capable of killing all three targets. The IL-2 stimulated CD16+ cells were not as potent in their killing activity as the LBL induced cells. The IL-2-stimulated populations contained equal amounts of αß and γδ TCR+ cells. These data therefore reflect the cytotoxic activity of both these populations.

The purified CD16+ cells were also assessed for their proliferative response to various stimuli including the original LBL stimulus, RPMI 8866. They had a low proliferative response to IL-2 alone (Fig. 3). They did not respond to the original stimulus RPMI 8866 either alone or in the presence of IL-2. The only stimulus that the cells proliferated in response to was autologous antigen presenting cells when combined with IL-2 or with PHA.

Characteristics of γδ T cells induced by RPMI 8226.

Purified populations of γδ cells were obtained by sorting the RPMI 8226 stimulated cells. The predominant γ receptor expressed by these cells is Vγ9 (82%) (Fig. 4). These cells express the common T cell antigens CD3 and CD2 and CD8 (14%). They also expressed HLA-DR (97%) a molecule normally present on many activated cells. They coexpressed integrin molecules ß1 and ß2, but not ß3 (ß2 and ß3 data not shown). Some γδ cells also coexpressed the CD56 (42%) antigen which is most commonly associated with NK cells.

Positively selected γδ T cells proliferated in response to the original stimulus RPMI 8226 with a moderate response to Daudi (Fig 5a). This response was dependent upon the presence of exogenous rhIL-2. In contrast there was no effect of other tumour cell lines tested.

The enriched γδ T cells lysed both the B cell lines, Daudi and RPMI 8226, with moderate to poor lysis of NK targets K562 and RPMI 8866. They failed to display significant lysis of two other activated NK cell targets, Raji and Colo 205 (Fig 5b).

DISCUSSION

The present studies demonstrate that different potential target cell lines have distinctive capacities to induce non-MHC restricted effector cell activity. Consistent with previous reports lymphoblastoid B cell lines were effective inducers of effectors (Perussia *et al.*, 1987). Their growth promoting activity has been recognized for some time and been highly utilized in propagation of clonal lymphocyte populations (Hercend *et al.*, 1982; London, Perussia & Trinchieri, 1986; Van de Griend *et al.*, 1984). However, a number of nonlymphoid and T cell lines failed to stimulate their production. Although the underlying mechanism(s) of non-MHC effector generation is unknown, a contributory role for IL-2 in their production has been indicated (Brooks, Holschen & Urdal, 1985; London, Perussia & Trinchieri, 1986; Perussia *et al.*, 1987; Porcelli, Brenner & Band, 1991). The present study supported these findings as IL-2 was able to induce effectors but it alone was not sufficient to obtain maximal responses. As well the addition of exogeneous IL-2 did enhance inductive capacity of some but not all of the less potent inducers. These results would suggest that stimuli in addition to IL-2 are required for non-MHC restricted effector induction.

Analysis of the effectors led to the observation that there appeared to be heterogeneity in tumour cell line inductive potential. The effector populations induced by different stimuli

were not pure containing CD16+, αß and γδ TCR+ cells, although depending on the stimulator cell line there clearly was a strong bias towards one population. Purified populations of these effectors indicated that they all had some cytotoxic ability.

Some LBL were able to induce potent cytotoxic activity which was found to correlate with an ability to increase the proliferation of CD16+ NK cells as well as to increase cytotoxic specific activity of each CD16+ cell, that is CD16+ cells stimulated with LBL were much more efficient killers on a per cell basis than those stimulated by IL-2 alone. Other LBL, such as Ramos and RPMI 8226, which were weak inducers of cytotoxic activity were found to induce predominantly CD3+ αß TCR+ cells which can display cytotoxic activity but this was generally of a lower level than that of CD16+ cells. The addition of IL-2 to cultures stimulated with the Ramos and RPMI 8226 resulted in enhanced cytotoxicity mediated by an increase in CD16+ cells and γδ cells respectively. Two other cell lines K562 (a myelogenous leukemia) and Jurkat (T cell leukemia) were able to induce an increase in percentage of CD16+ cells when cocultured in the presence of IL-2 but not alone. K562, however did not enhance proliferation nor cytotoxic activity either alone or in the presence of IL-2 (Data not shown). Jurkat did increase PBMC proliferation, increasing CD16+ cells, as well as enhancing cytotoxic activity in the presence of IL-2 but there appeared to be variability depending on the individual. K562 alone (Hercend *et al.*, 1982; Phillips & Lanier, 1985; Ythier *et al.*, 1985) or in the presence of IL-2 (Perussia *et al.*, 1987) has been

previously reported to have some ability to induce CD16+ cells. The response to these two tumour cell lines which are not B cell in origin suggests that under other circumstances perhaps in the presence of other lymphokines tumour cells of differing origin may have the capacity to induce cytotoxic effectors.

These results clearly indicate that the requirements for induction differ from those necessary for effector activation, that is a good target does not necessarily equal a good inducer. The results observed with Il-2 addition would imply that effective induction is not necessarily related to IL-2 production alone, although IL-2 clearly plays a role. It would appear from these results that B cells lines are the most efficient in this process inducing all three types of effectors NK cells, $\gamma\delta$ and $\alpha\beta$ T cells depending on the circumstances. It is difficult to believe this is circumstantial. It is possible this could be part of a surveillance mechanism for clearance of abnormal B cells either ones infected with viruses such as EBV or B cell tumours from the immune system. It also suggests the possibility that there maybe an important communication between activated B cells and the non-adaptive immune response. It is possible that these nonadaptive immune mediators are maintained in an activated state by activated or aberrant B cells in autoimmune processes without the presence of the instigating antigen.

Exactly how the LBL are able to activate non-MHC restricted killer cells has not been addressed in the present investigation. The two most obvious mechanisms for induction are either their

presence leads to the production by PBMC's of a cytokine(s) which results in the induction of NK, γδ and αß T cells, or that actual cellular contact between cell surface molecules is required for this activation to occur. Kobayashi *et al.* (1989) were able to isolate a factor produced by stimulated RPMI 8866 cells which has been identified as IL-12 (D'Andrea *et al.*, 1992). This factor was able to enhance IL-2 induced non-MHC restricted killer activity, PBMC proliferation and IFN-γ production but did not increase CD16+ cell numbers. We have evidence to suggest that cellular contact is required although as can be seen by the present evidence IL-2 is required to be present with the LBL which are weak inducers. These issues are addressed further in other manuscripts (Selin *et al.*, submitted; Wilkins *et al.*, 1992). These systems provide a means to examine the growth and activation requirements including evaluation of the role of specific cytokines or cellular ligands for different non-MHC restricted effector populations.

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Figure 1. Induction of non-MHC killer cells by continuous cell lines. A-C demonstrate the lysis of three different targets. D demonstrates the proliferative response to various cell lines. The bracketed number above each bar in panel A represents the number of times each stimulator was tested.

Figure 2. Induction of A) CD16+ cells and B) $\gamma\delta$+ cells by continuous cell lines in the presence of IL-2 (50U/ml). [a] LBL ; [b] cell lines other than B cell origin.

Figure 3. Proliferative responsiveness of purified CD16+ cells induced by RPMI 8866. These data are representative of two similar experiments.

Figure 4. Surface phenotype of purified RPMI 8226 stimulated $\gamma\delta$ T lymphocytes. The first panel shows both CD3 (shaded) and the FITC control (unshaded). The per cent positive cells and the mean fluorescence for each antibody is expressed in its respective panel.

Figure 5. (a) The proliferation of purified γδ T cells in response to various tumour cell lines (RPMI 8226 (8226), RPMI 8866 (8866), Daudi and Colo 205 (COLO), and PHA. The γδ T cells were cultured with these stimuli alone (■), or in combination with rhIL-2 (□), or rhIL-2 plus autologous APC's (□). These data are representative of results obtained with 3 positive sorts for γδ T cells. CONT is the control group. (b) The cytotoxic ability of the purified γδ T cells against 6 targets (E:T, 20:1). This is a representative experiment of 3 individuals tested from the γδ positive sorts.

Figure 1.

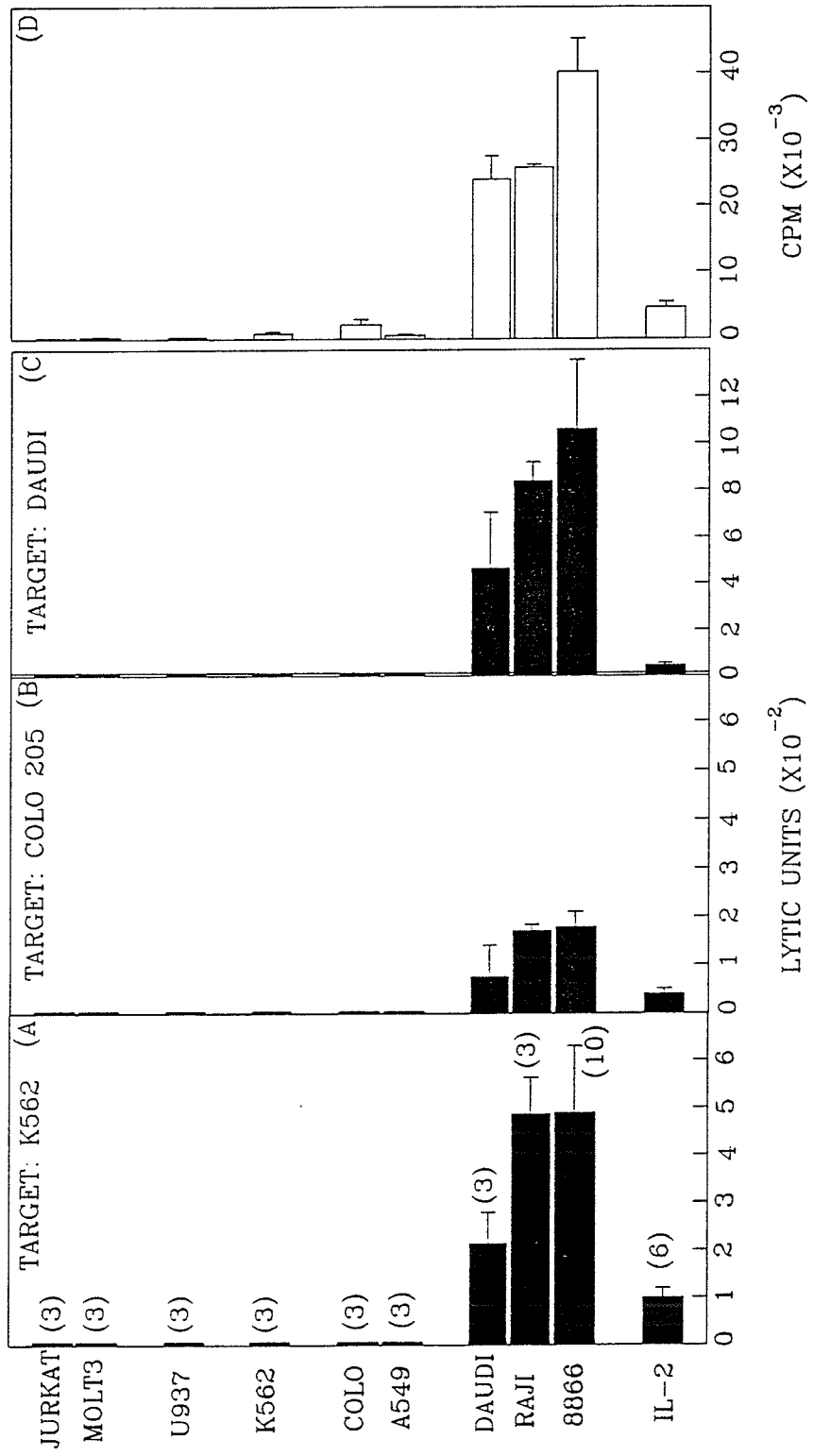
(A
TARGET: K562
(B
TARGET: COLO 205
(C
TARGET: DAUDI
(D
JURKAT
MOLT3
U937
K562
COLO
A549
DAUDI
RAJI
8866
IL-2
(3)
(3)
(3)
(3)
(3)
(3)
(3)
(3)
(10)
(6)
0 1 2 3 4 5 6
0 1 2 3 4 5 6
0 2 4 6 8 10 12
0 10 20 30 40
LYTIC UNITS (X10^{-2})
CPM (X10^{-3})

Figure 2.

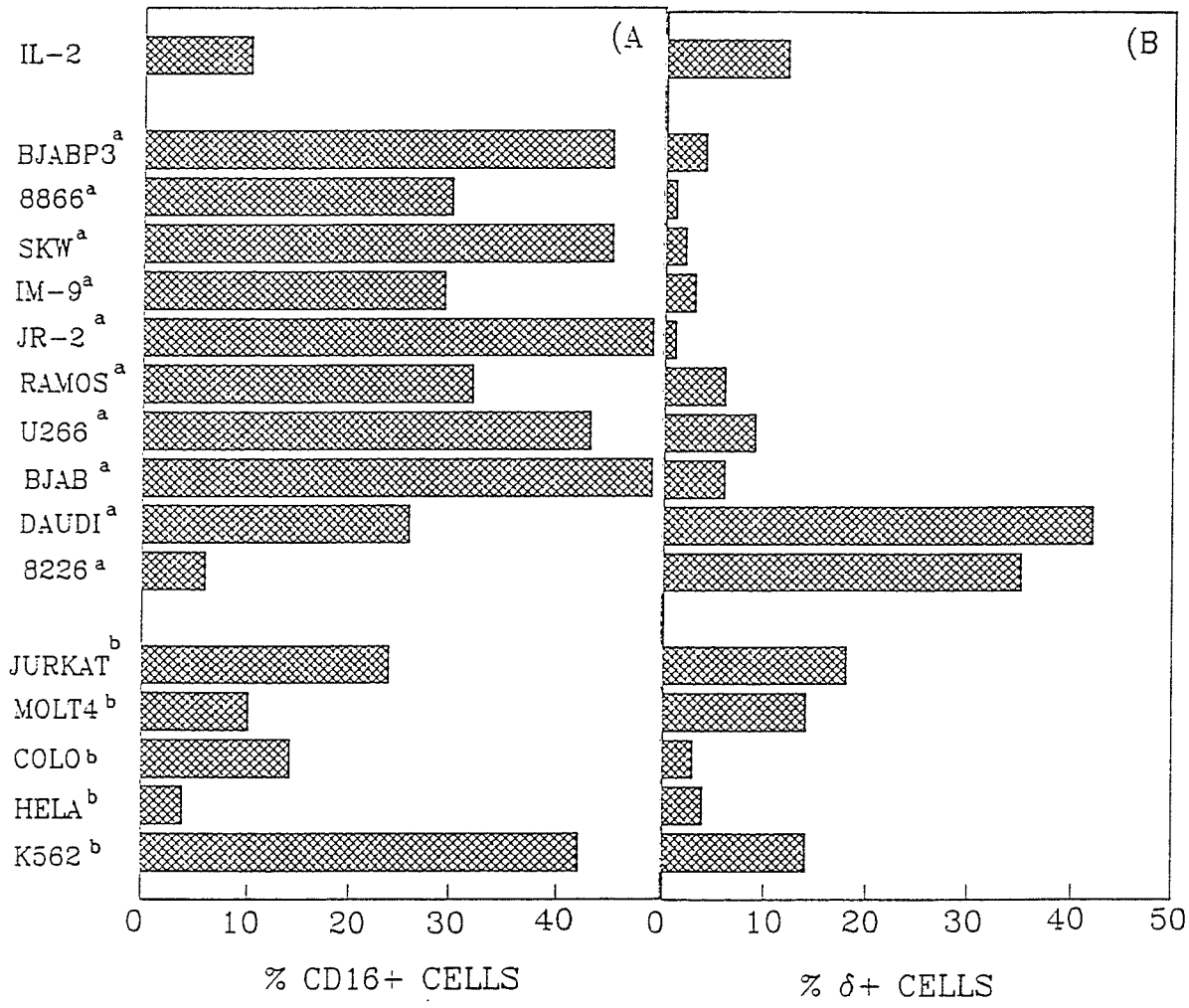
(A
(B
IL-2
BJABP3[a]
8866[a]
SKW[a]
IM-9[a]
JR-2[a]
RAMOS[a]
U266[a]
BJAB[a]
DAUDI[a]
8226[a]
JURKAT[b]
MOLT4[b]
COLO[b]
HELA[b]
K562[b]
0 10 20 30 40
0 10 20 30 40 50
% CD16+ CELLS
% δ+ CELLS

Figure 3.

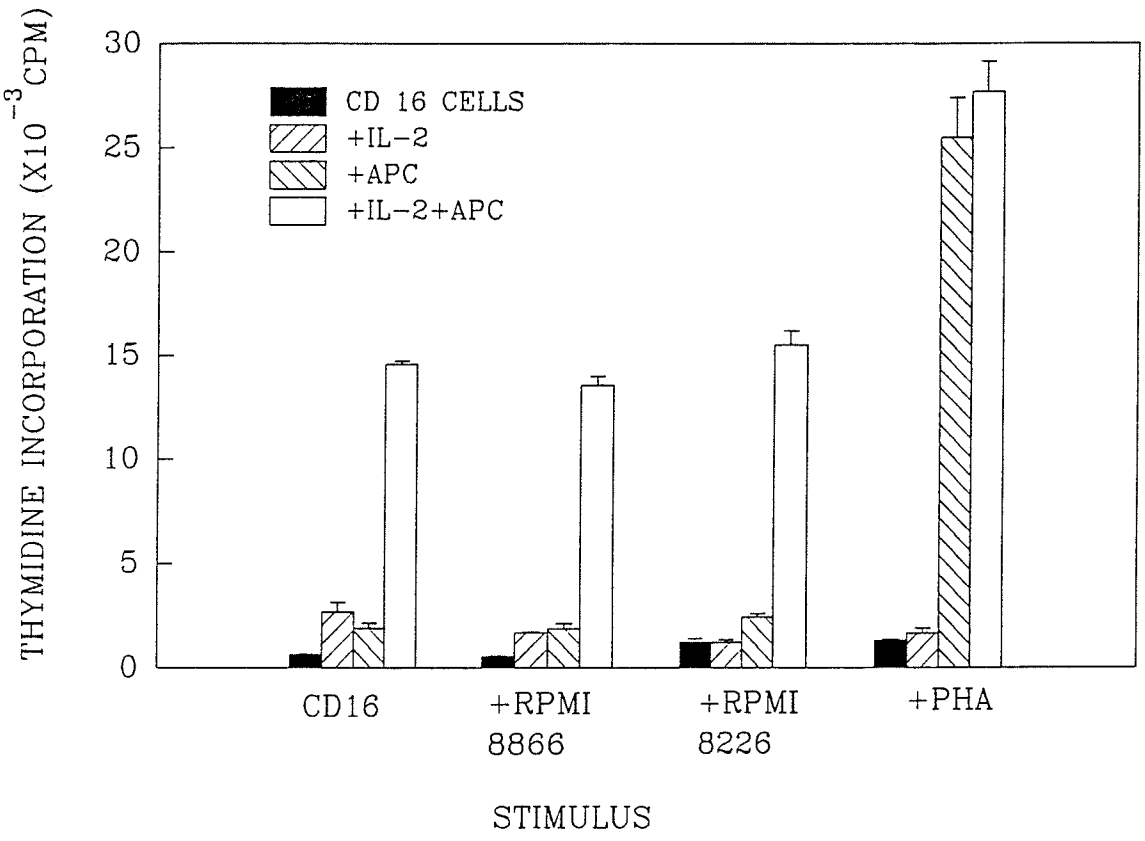
THYMIDINE INCORPORATION (X10^{-3}CPM)
30
25
20
15
10
5
0
CD 16 CELLS
+IL-2
+APC
+IL-2+APC
CD16
+RPMI 8866
+RPMI 8226
+PHA
STIMULUS

Figure 4.

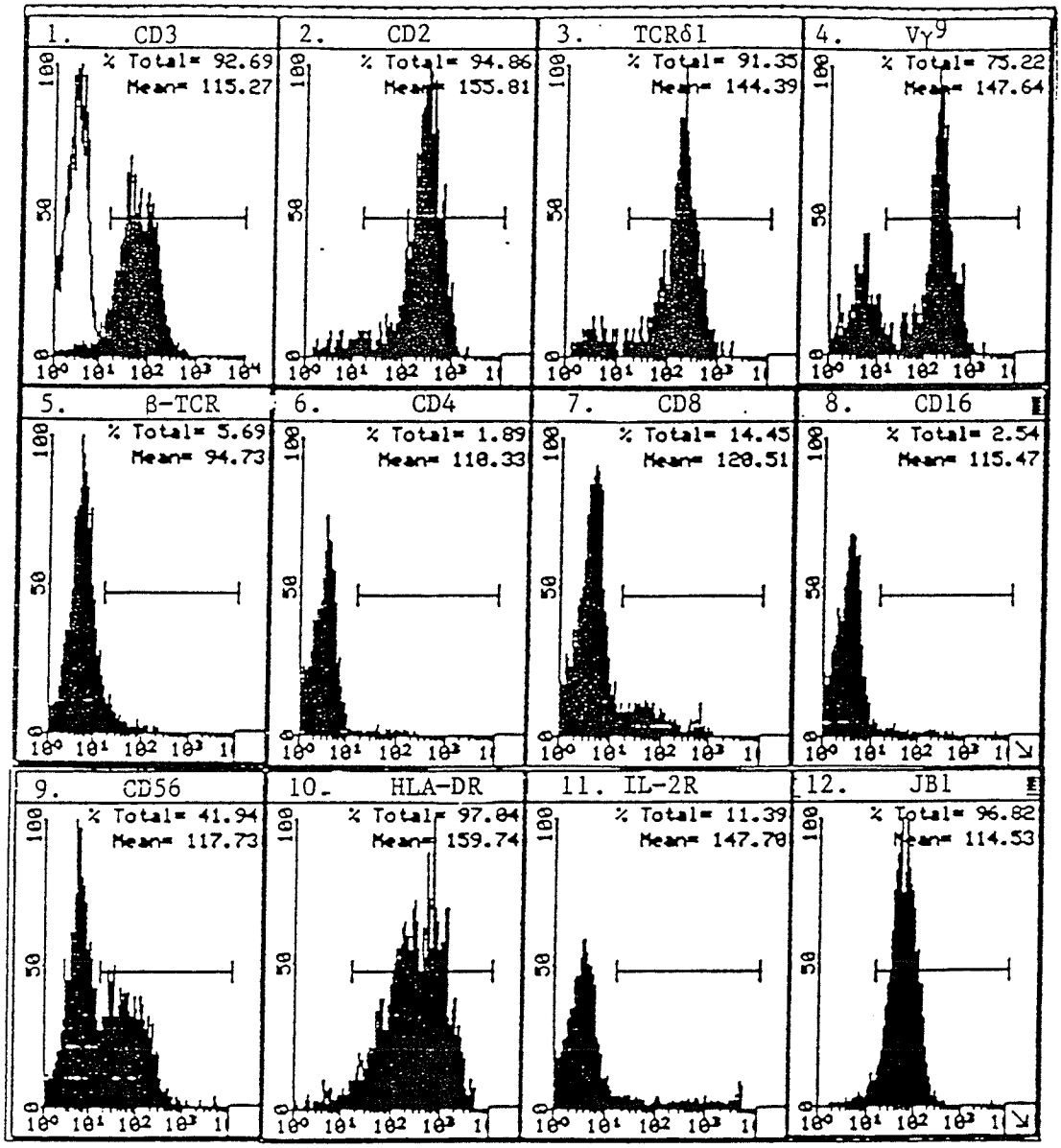
1. CD3
% Total= 92.69
Mean= 115.27
2. CD2
% Total= 94.86
Mean= 155.81
3. TCRδ1
% Total= 91.35
Mean= 144.39
4. Vγ9
% Total= 75.22
Mean= 147.64
5. β-TCR
% Total= 5.69
Mean= 94.73
6. CD4
% Total= 1.89
Mean= 110.33
7. CD8
% Total= 14.45
Mean= 120.51
8. CD16
% Total= 2.54
Mean= 115.47
9. CD56
% Total= 41.94
Mean= 117.73
10. HLA-DR
% Total= 97.84
Mean= 159.74
11. IL-2R
% Total= 11.39
Mean= 147.70
12. JB1
% Total= 96.82
Mean= 114.53

Figure 5.

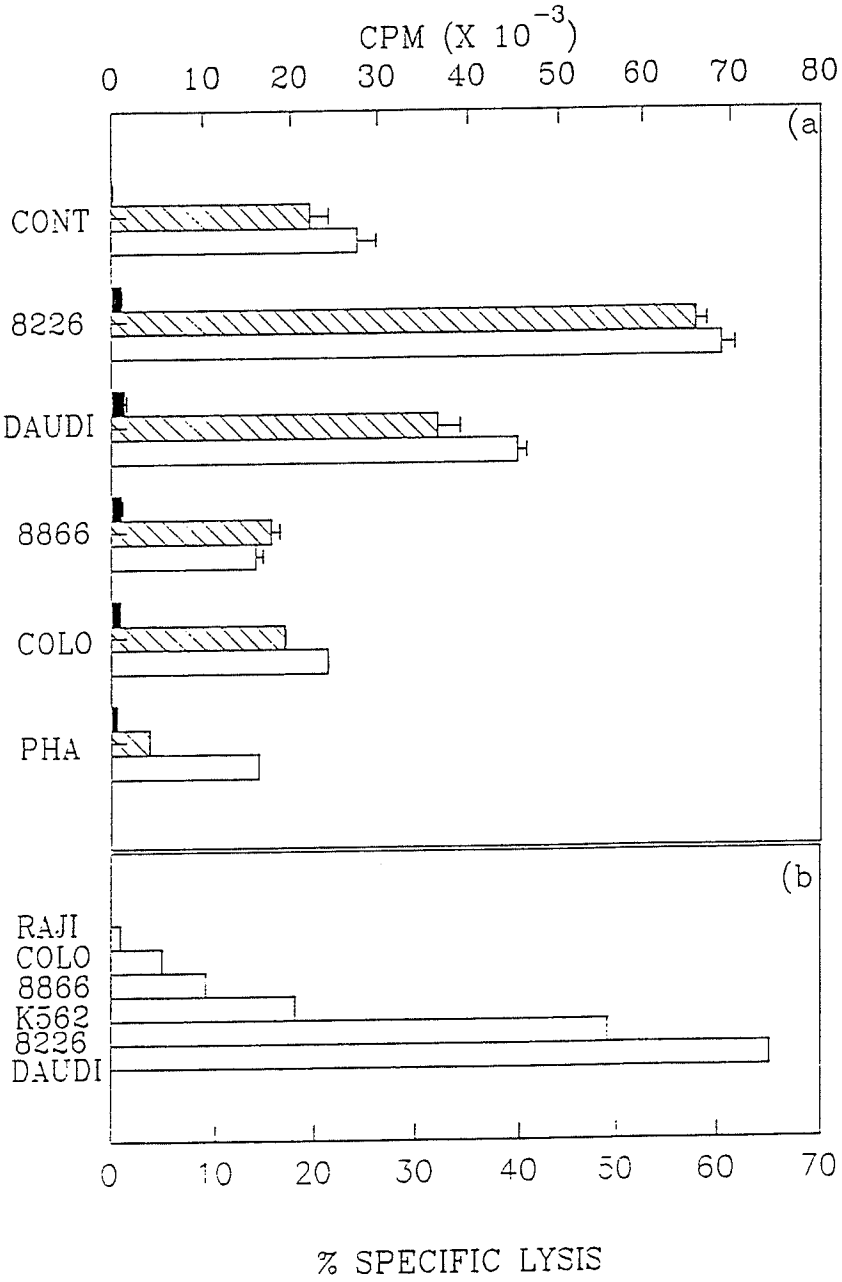
CPM (X 10^{-3})
0 10 20 30 40 50 60 70 80
(a
CONT
8226
DAUDI
8866
COLO
PHA
(b
RAJI
COLO
8866
K562
8226
DAUDI
0 10 20 30 40 50 60 70
% SPECIFIC LYSIS

Table 1. Stimulation of non-MHC restricted killer cells by different LBL.

A. STIMULATOR CELL LINE	IL-2*	NON-MHC RESTRICTED KILLER ACTIVITY: TARGETS: (LU)			PROLIFERATION (CPM)
		K562	COLO	DAUDI	
IL-2 (6)	+	99±20	41±11	44±12	4,582±974
RPMI 8866 (8)	-	286±49	140±25	490±140	40,223±5700
(3)	+	314±122	177±75	534±126	45,054±3970
IM-9 (3)	-	2587±217	1254±119	2400±108	10,423±555
(3)	+	2540±61	1525±117	3125±518	44,212±5848
DAUDI (4)	-	222±49	95±50	486±174	24,056±3438
RAJI (3)	-	485±77	169±14	837±82	25,738±519
RAMOS (8)	-	34±15	39±27	42±28	1,823±501
(3)	+	1342±457	877±416	1371±558	6,835±376
RPMI 8226 (11)	-	20±6	3±2	8±4	2,889±494
(5)	+	1395±316	311±136	2932±666	22,569±4172
BJAB (3)	-	35±6	10±6	41±8	1,179±405
(3)	+	721±341	394±175	493±702	19,422±10058

() The number in brackets represents the number of times each stimulator was tested.
* +/- indicates whether coculture was done in the presence of IL-2 (50 U/ml).

Table 2. Phenotype of populations induced by different LBL.

	EXP. NO.	CD3	TCRδ1	CD16
DAY 0				
PBMC	1	61	3	6
	2	79	4	9
	3	69	6	13
	4	76	3	10
DAY 10				
IL-2	1	64	13	30
	2	73	11	6
	3	74	13	13
	4	72	12	6
RPMI 8866	1	56	6	45
	4	64	6	37
RPMI 8866 +	1	59	7	41
IL-2	4	71	7	30
IM-9	3	30	3	49
	4	42	3	40
IM-9 +	3	38	4	36
IL-2	4	46	4	35
DAUDI	2	66	37	35
	4	59	35	39
DAUDI+IL-2	2	60	35	30
	4	64	41	26
RAMOS	1	80	3	17
	3	93	9	13
RAMOS+IL-2	1	42	10	61
	3	64	15	35
RPMI 8226	2	92	6	4
	3	88	16	12
RPMI 8226 +	2	92	40	16
IL-2	3	92	66	8

The numbers represent the % of the total populations generated.

Table 3. A) Phenotype of the purified CD16+ and CD3+ cells induced by RPMI 8866.

A.)	PERCENTAGE OF POSITIVE CELLS	
	RPMI 8866 STIMULATED:	
MARKER	CD16+ SORT	CD3+ SORT
CD16	96	4
CD56	99	-
CD3	3	95
TCRδ1	1	10
αß TCR	2	90
CD4	1	15
CD8	51	71
CD29 (ß1)	99	97
CD18	98	98
VLA α4	99	89
VLA α5	98	78

B) Cytotoxicity of purified CD16+ and CD3+ cells induced by RPMI 8866 or IL-2.

	CYTOTOXICITY (LU)		
	TARGETS:		
	K562	COLO	DAUDI
RPMI 8866 STIMULATED:			
CD16+	7,316±900	4,617±437	11,735±1200
CD3+	914±129	433±41	987±211
IL-2 STIMULATED:			
CD16+	613±33	490±55	773±44
CD3+	52±11	98±17	56±11

These data are representative of three experiments.

PAPER II.

Preferential Induction of CD16+ Natural Killer Cells by Lymphoblastoid Cell Lines: Role of Epstein Barr Virus (EBV) and Cellular Contact

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Footnotes

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SUMMARY

Lymphoblastoid cell lines (LBL) have been shown to display features which rendered them effective inducers of CD16+ NK cells. The present investigation undertook to identify some of the inducer cell properties. The ability of LBL to induce CD16+ non-MHC restricted effector cells related partially to the presence of Epstein Barr virus (EBV). Coculture of PBMCs with EBV infected LBL (EBV^+ LBL) resulted in the induction of IL-2 production by PBMCs. IL-2 alone however was not sufficient to induce analogous levels of CD16+ effectors. IL-2 was found to synergize with some EBV uninfected LBL (EBV^- LBL) to induce CD16+ NK cells. These results suggested that two parameters were involved in the EBV^+ LBL induction process, the presence of a stimulating antigen on the B cell lines which specifically stimulates CD16+ cells a second element which results in the induction of IL-2. Neither parameter was sufficient alone.

Cellular contact was found to be essential in LBL induction of CD16+ NK cells as this event 1) required coculture in the same vessel (not effective in transwell); 2) involved trypsin sensitive structures; 3) was not transferable by LBL supernatants. These data were consistent with the hypothesis that a LBL cell surface molecule was involved in the induction process. Antibodies to three classes of adhesion molecules (CD2, CD18, & CD29) were found to inhibit LBL induction of non-MHC restricted killer cell activity. These results could suggest that all these molecules are important

in maintaining cell contact while the stimulatory ligand triggers the NK cell. It is however, possible that ligands to CD2 or CD18 may be able to trigger activation of NK cells.

INTRODUCTION

One of the features of the early host response to infections is the appearance of lymphocytes capable of non-MHC restricted killing (Biron, Byron & Sullivan, 1989; Janeway, 1989; Herberman *et al.*, 1975; Ortaldo *et al.*, 1977; West *et al.*, 1977). It is postulated that these cells act as cytotoxic effectors against cells modified as a consequence of viral infection or malignant transformation (Trinchieri, 1989). Additionally these cells may produce cytokines involved in the recruitment and activation of other lymphoid cells at the inflammatory site (Cuturi *et al.*, 1989; Hendrich *et al.*, 1990). Although natural killer (NK) cells have been described as the primary effectors of non-MHC restricted activities, it is clear that T cells, both $\gamma\delta$ T cell receptor positive (TCR+) (Fisch *et al.*, 1990; Porcelli, Brenner & Band, 1991) and αß TCR+ cells can also express this type of activity (Hersey & Bolhuis, 1987; Thiele & Lipsky, 1989).

Non-specific, non-MHC restricted effector cells may play a significant role in both normal immune responses and if not appropriately regulated in the pathogenesis of chronic inflammatory diseases (Trinchieri, 1989). An understanding of the mechanisms involved in their activation and expansion may provide insight into

the disease process. In the case of NK cells these processes are only partially defined (Trinchieri, 1989). Cytokines such as IL-2 have been shown to be sufficient to both activate and expand NK cell populations (Dukovich *et al.*, 1987; Le Thi Bich-Thuy, Lane & Fauci, 1984). Other cytokines such as IFN-α and -ß are apparently able to activate NK cells but not induce proliferation in vitro (Lucero *et al.*, 1981; Perussia, Santoli & Trinchieri, 1980; Weight *et al.*, 1982; Trinchieri & Santoli, 1987). This pattern of activation is unlike that which occurs with αß T cells where two separate signals are required for activation and proliferation (Aparicio *et al.*, 1989; Allison & Lanier, 1987; Wang & Smith, 1987). Activation occurs via the CD3-TCR complex resulting in expression of high affinity IL-2 receptor making the cells responsive to IL-2 and only then capable of proliferation.

The nature of non cytokine mediated NK cell triggering events , such as contact with certain cellular ligands present on activated or altered cells are poorly defined. So far the search for a specific NK cell receptor analogous to the TCR has been unsuccessful. There are molecules reported to be present on the surface of NK cells such as CD16 (Anegon *et al.*, 1988; Werfel *et al.*, 1989), CD2 (Schmidt *et al.*, 1985a; 1987; 1988), p80 molecule (Ortaldo *et al.*, 1989), and a family of lectin-like receptors (Giorda & Trucco, 1991) which can be triggered to activate the lytic machinery and induce signal transduction in NK cells. Only in the case of CD16 molecules has activation of NK cell cytotoxic and other functions been shown to be induced by their natural ligand,

immune-complexed IgG (Cassatella *et al.*, 1989; Hendrich *et al.*, 1990). Other molecules like CD18 (Axberg *et al.*, 1987; Mentzer, Krensky & Burakoff, 1986), CD29 (Santoni *et al.*, 1989) and laminin (Schwarz & Hiserodt, 1988) have been demonstrated to have some role in binding of the effector and target. The majority of this research looking for a unique NK cell receptor has focused on a receptor involved in the recognition of targets and activation of the lytic event. Most recently it has been suggested based on available data that since no one NK cell receptor has been found which mediates activation and killing both binding and triggering phases of NK activity may be mediated by a heterogenous group of surface molecules, the relative importance of these molecules in each case being dependent on the type of target cell (Timonen, 1990; Trinchieri, 1989).

It is possible that such a receptor-ligand interaction between NK cells and tumour cell targets could result in activation of their cytotoxic potential as well as triggering resting NK cells into cell cycle. Such an observation would be significant as it would provide a basis for activation and expansion of NK cells in addition to cytokines. A previous examination of tumour cell capacity to induce activation and expansion of non-MHC restricted killer cells indicated that lymphoblastoid cell lines were selective inducers (Selin, Stewart & Wilkins, manuscript submitted). The effectors were heterogeneous in terms of phenotype including CD16+ NK cells, $\alpha\beta$+ and $\gamma\delta$+ T cells. These results were consistent with the previous reports that some LBL could induce

either CD3+ cells or NK cells (Matera *et al.*, 1988; Perussia *et al.*, 1987). As well their growth promoting activity has been recognized for some time and been highly utilized in propagation of clonal lymphocyte populations (Hercend *et al.*, 1982). However, the recognition events involved and the mechanisms underlying these abilities of LBL including the contribution of cytokines are still essentially undefined. The present investigation under takes to further characterize the responses to the different LBL in an attempt to address some of the parameters involved in this induction process including the role of EBV, soluble mediators and the need for cellular contact.

MATERIALS AND METHODS

Induction of non-MHC restricted killer cells.

Ficoll hypaque purified normal PBMC's (10^6/ml) were cultured in the presence of various irradiated continuous cell lines (50Gy; $3x10^4$/ml) with or without highly purified recombinant human IL-2 (rhIL-2) (50U/ml; Cetus, Emeryville, Calif.) (Rosenberg *et al.*, 1984) in AIM V serum free media (Gibco, Grand Island, NY). The cells were subcultured on day 6 in AIM V media containing rhIL-2 (50 U/ml). At day 10 the cells were harvested, counted and used for immunofluorescence studies, cytotoxicity or proliferation assays.

Cell lines.

Continuous human cell lines K562 (erythroleukemia), Colo 205 (adenocarcinoma), Daudi (Burkitt's lymphoma), Raji (Burkitt's lymphoma), JY (B cell leukemia), Jurkat (T cell leukemia), Molt-3 (T cell leukemia), Molt-4 (T cell leukemia), A549 (lung carcinoma), U937 (histiocytic lymphoma), SKW-13 (B cell leukemia), Ramos (Burkitt's-like lymphoma), U266 (myeloma), IM-9 (myeloma), Hela (cervical carcinoma) and RPMI 8226 (myeloma) were obtained from the American Type Culture Collection (Rockville, MD). RPMI 8866 (B cell leukemia) was purchased from ABS (Buffalo, NY). BJAB and BJAB-P3 were kindly provided by Dr. Louis Qualtiere (University of Saskatchewan, Saskatoon, Saskatchewan). The JR-2 cell line was an EBV transformed B lymphoblastoid generated in our laboratory (Wilkins *et al.*, 1983). These cells were maintained on 10% FCS with RPMI 1640. Mycoplasma contamination was excluded in all continuous cell lines used by culture and/or Hoescht staining.

mAbs and Immunofluorescence.

The cells were labelled with mAbs reactive against CD3 (OKT3), CD2 (OKT11) and CD18 (ß2) produced and purified from hybrid cell lines obtained from the American Tissue Culture Collection (ATCC, Rockville, Md); CD16 (Leu 11b), CD4 (Leu3a PE), CD8 (Leu2a PE), HLA-DR and WT31 (αß TCR) obtained from Becton Dickinson Co. (Mountainview, Calif.); CD56 (NKH-1 PE) obtained from Coulter Immunology (Hialeah, Florida); mAb to CD29 (ß1,JB1), and VLAα5 (JBS5) were produced and purified from a hybridoma developed in our

laboratory (Shen *et al.*, 1991). The polyclonal CD29 antibody was obtained from (Telios Pharmaceuticals Inc., San diego, CA). The mAb TCRδ1 which recognizes a framework determinant on all δ chain subsets, was kindly provided by Dr. M. Brenner, Dana-Farber Cancer Institute, Boston, MA (Band *et al.*, 1987). The anti-MHC class II antibody was obtained from Cedarlane Laboratories (Hornby, Ont., Can.). The mouse anti-chlamydia antibody was kindly provided by Dr. R. Brunham, University of Manitoba, Winnipeg, Manitoba. for direct fluorescence, cells were labelled with mAbs conjugated with phycoerythrin (PE) in PBS (30 min at 4^0C)and washed. For indirect fluorescence, cells were exposed to mAbs, subsequently incubated with FITC-F(ab')$_2$-conjugated goat anti-mouse antibody obtained from Cedarlane Laboratories (Hornby, Ont., Can).

Proliferation Assay.

1X10^5 PBMC's were cultured in round bottomed 96 well plates with various irradiated (50Gy) continuous cell lines (4x10^3) alone or in the presence of rhIL-2. The cultures were maintained for 5 days and pulse labelled with ^{3}H-Tdr for the final 18 hrs of culture.

Cytotoxicity Assay.

The cytotoxic activity of the effector cells was determined in a chromium-release assay as previously described (Rosenberg *et al.*, 1974). The target cells were labelled by incubating 2x10^6 cells in 0.5 ml RPMI-1640, 10% FCS, containing 300 μCi $Na_2{}^{51}CrO_2$ (Amersham,

Oakville, Ontario, Canada) for 90 min at 37^0C. The cells were washed twice in RPMI-1640 and resuspended to a final concentration of $2.5x10^4$ cells/ml. Per cent specific lysis was calculated as 100x[(CPM released with effectors - CPM spontaneously released) / (total CPM - CPM spontaneously released). In all assays spontaneous release was less than 15%. These results were further quantified by calculating the number of lytic units (LU) per 10^7 cells at 20% specific lysis with the use of a linear regression equation (exponential fit) (Pross, Callewaert & Rubin, 1986). One lytic unit is the number of effector cells necessary to lyse 20% of the target cells during the assay period.

Pretreatment of LBL.

Cycloheximide and Trypsin treatment of RPMI 8866: Cells were incubated for 30 min. at 37^0C in RPMI 1640 plus 2% FCS +/- trypsin (1mg/ml) or cycloheximide (10μg/ml) (Gromkowski *et al.*, 1985). Cells were then rinsed three times in RPMI 1640 plus 10% FCS; the first rinse contained 1 mg/ml soybean trypsin inhibitor (Sigma) when trypsin was pretreatment was done. Viability by trypan blue exclusion exceeded 95%.

Paraformaldehyde pretreatment of RPMI 8866: Cells were treated as described above except they were fixed for 20 min. with 1% paraformaldehyde at room temperature.

Antibody blocking studies. Cell cultures were set up as described above using either RPMI 8866, or BJAB as a stimulus. At day 0

various antibodies (CD2, CD18, CD29, JBS5, anti-MHC class II, and as control anti-chlamydia mAb) were added to each culture at 1/20 and cultured as previously described.

The effect of these antibodies on cytotoxic activity was assessed by pretreating the effector cells for 30 min. prior to addition of the target cells.

Cell contact experiments. RPMI 8866 induction cultures were initiated in 12 well transwell filter chambers (Costar) with .22 μm filters. PBMCs ($0.5X10^6$) were placed in the upper chamber and $1.5X10^6$ cells in the lower. The RPMI 8866 were added in the appropriate wells at a 1:30 ratio with the PBMC. The cells were harvested at day 6 counted, characterized. A portion of these cells were cultured for an additional 4 days in 12 well plates at $2.5X10^5$/ml in AIM V media with 50U rhIL-2/ml and examined by flow cytometry.

RESULTS

Induction of CD16+ cells by continuous cell lines. As previously reported, some LBL when cocultured with PBMCs were more efficient inducers of CD16+ cells (Fig. 1A). The generation of CD16+ cells correlated with the level of cytotoxic activity of the cultures produced with these various stimuli (Fig. 1B). Furthermore proliferative responses correlated with inductive capacities. In

the case of BJAB where increased CD16+ cells were generated there was little proliferation (Fig 1C) or cytotoxic activity (Fig. 1B) induced by these cells. The cytotoxic and proliferative responses were assessed at day 6 of culture. At day 6 the significant increase in proportion of CD16+ cells which occurs with the potent stimulator LBL was not usually clearly evident. This observation was only made at day 10 of culture, the cells having been harvested at day 6 and fed with fresh media and rhIL-2 (10 U/ml). The cultures induced by the potent LBL were able to continue growing until day 10 without the exogenous IL-2, however the cultures induced by the poor inducer cell lines were not able to do so. Therefore a low level of IL-2 (10U/ml) was added to the all the cultures to maintain them and determine the percentage of CD16+ cell growth. It would appear that BJAB although alone was not able to induce significant effectors nor PBMC proliferation when low doses of IL-2 were added 6 days into culture these cell were able to generate some level of CD16+ proliferative response with an enhancement in effector and proliferative responses. The other two poor inducers RPMI 8226 and Ramos did not demonstrate this ability to induce CD16+ cells when IL-2 was added late in culture.

A comparison of the properties of the LBL indicated that the most notable differences between CD16 inducers or non-inducers was the presence of EBV in the inducer LBL. It was therefore questioned if EBV infection was the stimulus for the CD16+ cell generation.

The role of Epstein Barr Virus (EBV). To test this hypothesis a

larger panel of LBL (Fig. 2A) was examined for CD16+ cell induction. A direct correlation was observed between the presence of EBV and induction of CD16+ cells.

The EBV^- line BJAB is a poor inducer of CD16 activity. However, a derivative of this line BJAB-P3 has been infected with EBV (Patel & Menezes, 1982). This allowed for a direct examination of effect of EBV infection on CD16+ cell induction. As previously observed BJAB was a poor inducer of non-MHC restricted killer cell activity. In contrast BJAB-P3 was a strong inducer of CD16+ non-MHC restricted killer activity (Fig. 2B) and proliferation (Fig. 2C). Thus results indicate that EBV infection can confer a capacity to generate CD16+ effectors.

Role of IL-2. Preliminary results indicated that EBV+ lines were effective inducers of IL-2 production (data not shown). This raised the possibility that the differences in capacity to generate CD16+ cells totally related to the ability to stimulate PBMC IL-2 synthesis in the primary cultures.

The culture of PBMC with IL-2 alone resulted in induction of effector populations but this was substantially less than that observed with a strong inducer line (Fig. 3). The addition of IL-2 (50 U/ml) to poor inducer lines led to marked synergy in the generation of effectors and a modest increase in proliferation. These results suggest that IL-2 alone is not sufficient and requires additional stimuli for effective proliferation.

Requirements for induction of CD16+ cells. The cell line RPMI 8866 was selected as the stimulus to further characterize the requirements for induction.

1) Dose response. RPMI 8866 was optimally effective at inducing killer cells in a wide range from 5:1 (PBMC:RPMI 8866) to 125:1 (Fig. 4). Even at 625:1 there was some evidence of killer cell induction. Proliferation of PBMC's was only optimal between 5:1 to 25:1 (Fig. 4). Although proliferation was still stimulated at 125:1. Higher concentrations of stimulators such as 1:1 which have been used in mixed lymphocyte reactions was not stimulatory to PBMC proliferation or to killer cell induction. These results suggest that the molecules involved in induction of CD16+ cells are very potent inducers. The potency of the response suggested that cell contact may not be essential and lymphokines may be involved. The fact that effectors were induced under conditions of low proliferation suggest that effector induction and proliferation may be dissociable events with different requirements.

2) Soluble mediators. Two approaches were utilized to address the issue of the role soluble mediators might play. It was found that addition of culture supernatants from LBL as well as coculture supernatants of LBL plus PMBCs at various time periods in culture were not successful at generating CD16+ effectors (data not shown). It was still possible that a labile mediator was involved or the appropriate kinetics had not been assessed. Therefore, coculture experiments using separated populations in transwells were performed (Fig. 5). No response was observed if the inducer and

effector populations were separated. If PBMCs were incubated together with RPMI 8866 in the upper chamber and PBMCs were alone in the lower chamber this resulted in the production of an enhanced number of CD16+ cells which demonstrated potent non-MHC restricted killer activity in the PBMCs in the upper chamber. Although the PBMCs alone in the lower chamber demonstrated some moderate killer activity they were predominantly CD3+ cells. These results suggest that the induction of CD16+ effectors is not mediated by soluble factors alone. There is an apparent need for cell contact to induce the CD16+ population. The induction of CD3+ effectors is most likely due to the induction of IL-2 by EBV^+ LBL.

Factors responsible for activation. Although there was a suggestion that surface structures directly activate CD16+ effectors it is possible that cellular contact mediated local production of factors could result in activation.

Pretreatment of RPMI 8866 cells with cycloheximide, an inhibitor of *de novo* protein synthesis did not affect the capacity of these cells to induce non-MHC restricted killer cell activity (Fig. 6A) or PBMC proliferation (Fig. 6B). In contrast, exposure of cells to trypsin markedly reduced their induction capacity for cytotoxicity (approximately 75% inhibition) (Fig. 6A). However, the effect on proliferation was very slight, again suggesting that the two processes have distinct requirements (Fig. 6B).

RPMI 8866 fixation with 1% paraformaldehyde inhibits the induction response implying that viable cells are required or that

fixation in some way modified the structures involved in the induction responses as it impacted on both proliferative (Fig. 6B) and cytotoxic activity (Fig. 6A).

Cell surface structures involved in induction. MHC class II molecules are reported to be an important ligand in the mixed lymphocyte response which can generate non-MHC restricted killer cells (predominantly CD3+ cells) (Perussia *et al.*, 1987). MHC class II antigen expression has also been reported to be enhanced by EBV infection of B cell lines (Robinson & Miller, 1982). Since cellular contact appears to be important in the induction of CD16+ non-MHC restricted killer cell activity it was possible that MHC class II antigens were involved in this process. Antibody blocking studies using a polyclonal antibody to framework determinants on the MHC class II molecule demonstrated that an EBV^+ LBL, such as RPMI 8866 minimally inhibited the induction of non-MHC restricted killer cells and PBMC proliferation (Fig.7A). This same antibody totally inhibited induction of the much lower levels of cytotoxic activity and PBMC proliferation induced by EBV^- LBL, BJAB (Fig. 7B). These results support the concept that the predominantly CD3+ effectors induced by EBV^- LBL without exogenous IL-2 are analogous to the effectors induced during an MLR which is known to involve the MHC class II molecules. The MHC class II molecules appear to a play a minimal role in effector induction by EBV^+ LBL.

Since cellular contact appears to be required for CD16+ non-MHC restricted killer cell induction antibodies to other adhesion

molecules were assessed. Antibodies to CD2 and CD18 markedly reduced induction of effectors (Fig. 8A) and PBMC proliferation (Fig. 8B). A polyclonal antibody to CD29 may have a minor effect on induction especially of cells capable of killing the NK resistant cell lines Colo 205 and Daudi. It however, had no effect on PBMC proliferation. These observations were further supported by evidence that a monoclonal anti-CD29 antibody (JB1) had the ability to inhibit the induction of killer cells by RPMI 8866 (data not shown).

The effects of these antibodies on effector function were also examined (Fig. 8C). The results indicated that these are not the predominant molecules necessary for cell adhesion in the killing process by these effectors. Only anti-CD18 partially inhibited the effectors.

DISCUSSION

These results indicate a heterogeneity of non-MHC restricted killer cell inductive potential by continuous cell lines. Previous studies have indicated that susceptible targets are not necessarily good inducers (Selin, Stewart & Wilkins, submitted for publication). The LBL were examined in order to attempt to define the basis for their inductive capacity.

It was demonstrated that not all LBL are equally effective inducers of CD16+ non-MHC restricted killer cell activity. The presence of EBV infection was found to significantly enhance LBL

cytotoxic and proliferation inductive capacity as well as the proportion of CD16+ cells in the cultures. This is consistent with previous observations that EBV^+ LBL have a unique growth promoting activity which has been highly utilized in propagation of clonal lymphocyte populations (Hercend *et al.*, 1982). It would appear from the present studies and the work of others that EBV^+ LBL are strong inducers of IL-2 production. IL-2 is known as an effective inducer of non-MHC restricted killer cell activity and some CD16+ cell growth.

However, IL-2 was not sufficient in the present system to induce analogous levels to EBV^+ LBL of cytotoxic function or PBMC proliferation nor CD16+ cell growth. Instead IL-2 was found to synergize with some EBV^- LBL to generate potent effectors of the CD16+ phenotype. These results would suggest that the basis for this potent stimulation of CD16+ effector cells by EBV^+ LBL involved at least two parameters. It required the presence of a stimulating antigen or cell surface structure present on B cell lines which specifically stimulates CD16+ cells and a second element which results in the induction of IL-2. Neither parameter alone was sufficient. It would appear that many LBL whether infected or not have the cell surface structure(s) which result in CD16+ cell induction. However, only the EBV^+ LBL have the ability to induce significant IL-2 secretion. When exogenous IL-2 is added to the EBV^- LBL they also become potent inducers of CD16+ cells.

Infection of human PBMCs *in vitro* with viruses such as measles (Casali *et al.*, 1981), EBV and rubella (unpublished data),

as well as murine *in vivo* infection with cytomegalovirus and lymphocyte choriomeningitis virus (Welsh & Vargas-Cortes, 1992) have been reported to induce the innate non-MHC restricted NK cell response. It is possible that the induction of non-MHC restricted killer cells may not only be a property of EBV infected cells but other virus infected cells may directly stimulate CD16+ effectors .

The requirement for cell contact for this activation process to occur appears to be absolute. The implication of the present studies is that cytokines are not sufficient. In an attempt to identify the targets structures used during this interaction antibodies to both MHC class II molecules and adhesion structures were used.

The expression of MHC class II molecules have been reported to be enhanced by EBV infection of B cells (Robinson & Miller, 1982). These molecules are considered an important stimulatory ligand in MLRs which are reported to generate non-MHC restricted killer cells predominantly of CD3 origin (Perussia *et al.*, 1987). As well, in some circumstances LBL expression of MHC class II antigens has been found to be important in non-MHC restricted killer cell induction as has the ability of these cells to induce IL-2 production (Santoli *et al.*, 1986). These types of findings would suggest that the LBL interaction with PBMC's is analogous to a MLR . However, the present results demonstrated that anti-MHC class II framework antibodies were unable to inhibit the EBV^+ LBL, RPMI 8866 induction of non-MHC restricted killer activity or PBMC proliferation.

However, they were effective in inhibiting the EBV^- LBL, BJAB the low level but consistent induction of non-MHC restricted killer activity and PBMC proliferation. This type of response is more analogous to the MLR also resulting in predominantly CD3+ effectors. MHC class II does not appear to be a major factor in LBL-EBV^+ induction of non-MHC restricted killer cells.

The adhesion molecules CD2, CD18 and CD29 have all been reported to have some role as potential recognition structures in the cytolytic process by NK cells (Axberg *et al.*, 1987; Mentzer, Krensky & Burakoff, 1986; Santoni *et al.*, 1989; Schmidt *et al.*, 1985; 1987; 1988; Trinchieri *et al.*, 1989;). As well, NK cells have been reported to be activated via the CD2 surface molecule (Schmidt *et al.*, 1985; 1987; 1988). Lines of B cell origin often express high levels of both $ß_1$ and $ß_2$ integrins (Stupack *et al.*, 1991). The present evidence demonstrates that CD2 and CD18 are involved in the inductive process. This may reflect the need for PBMC contact and presumably activation for the amplification of this response. It does not necessarily indicate that these molecules play a direct role in activation of the CD16+ effectors. It has been suggested for the lytic event that since no one NK cell receptor has been found that a heterogeneous group of surface molecules maybe involved, the relative importance of these molecules based on the particular target cell. Perhaps the same is true for LBL induction of CD16+ NK cells, that multiple antigens maybe activators depending on the circumstances, and therefore no one antibody significantly influences the response.

It would appear that target viability or at least antigen accessability was required as trypsin and paraformaldehyde fixing prevented the induction process. This does not however, directly indicate if one or more stimuli are capable activating the cells.

Collectively the results suggest that some LBL cell surface stimulus can induce an activation and expansion of non-MHC restricted killer cells. In the present studies the expansion of CD16+ effectors was examined while in other studies $\gamma\delta$ T cell effectors have been examined (Selin *et al.*, 1992). This inductive ability of LBL appears to relate in part to viral infection and in part to the phenotypic properties of the inducer. The nature of the stimulus is still unclear at this time but these results do suggest that there is a clear distinction between target susceptibility and inductive capacity.

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Figure 1. Induction potential of EBV infected and uninfected LBL. A) The percentage of CD16+ cells present in culture at day 10. B) The lysis of 3 different target cells at day 6 in culture. B. PBMC proliferative response at day 6 in culture. (The bracketed number represents the number of times this tumour cell was tested). The non-stimulated control cultures did not induce any cytotoxicity or proliferation.

Figure 2. A) Induction of CD16+ cells by continuous cell lines. This is representative of data obtained with 3 different individuals. Cell lines 8226, U266 and IM-9 originate from myelomas; Daudi, Ramos, BJAB, and BJABP3 from Burkitt's lymphomas; 8866 and SKW from B cell leukemias; JR-2 is an EBV transformed B cell line. B,C) Enhancement of LBL inducing potential by EBV infection. B) Lysis of 3 different targets. C) PBMC proliferative response. These data are representative of 3 similar experiments.

Figure 3. Enhancement of EBV uninfected LBL inducing potential by exogenous IL-2. A) Lysis of 3 different target cells. B) PBMC proliferative response. This data is a mean value for 3 similar experiments.

Figure 4. Effect of stimulator cell concentration on non-MHC restricted killer cell activity PBMC proliferation.

Figure 5. Effect of cell contact on induction of non-MHC restricted killer cells. The results demonstrated depict the findings for the lower chamber cells. The numbers in brackets represent the percentage of each cell phenotype present under those culture conditions at Day 10 in the lower chamber.

Figure 6. Effect of various pretreatments of RPMI 8866 on its ability A) to induce non-MHC restricted killer cell activity and B) PBMC proliferation.

Figure 7. Effect of anti-MHC class II on non-MHC restricted killer cell induction and PBMC proliferation by A) RPMI 8866, an EBV^+ LBL and B) BJAB, an EBV^- LBL.

Figure 8. Effect of antibodies to adhesion molecules on: A. induction of non-MHC restricted killer cells; B) PBMC proliferation upon stimulation with RPMI 8866; C) effector cell cytotoxic function.

Figure 1.

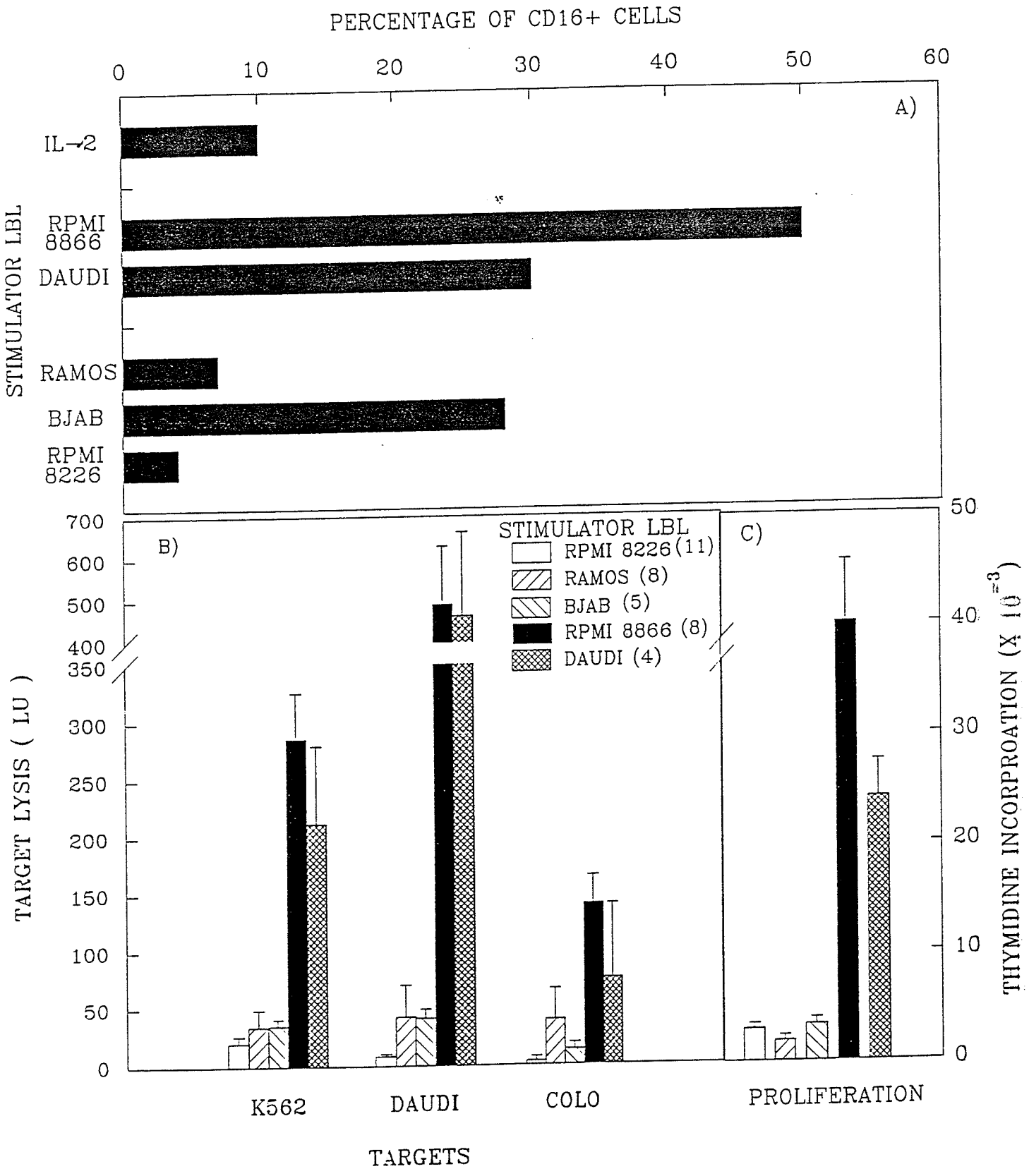

PERCENTAGE OF CD16+ CELLS
0
10
20
30
40
50
60
A)
IL-2
RPMI 8866
DAUDI
RAMOS
BJAB
RPMI 8226
STIMULATOR LBL
B)
C)
STIMULATOR LBL
RPMI 8226 (11)
RAMOS (8)
BJAB (5)
RPMI 8866 (8)
DAUDI (4)
TARGET LYSIS (LU)
700
600
500
400
350
300
250
200
150
100
50
0
50
40
30
20
10
0
THYMIDINE INCORPROATION (X 10^-3)
K562
DAUDI
COLO
PROLIFERATION
TARGETS

Figure 2.

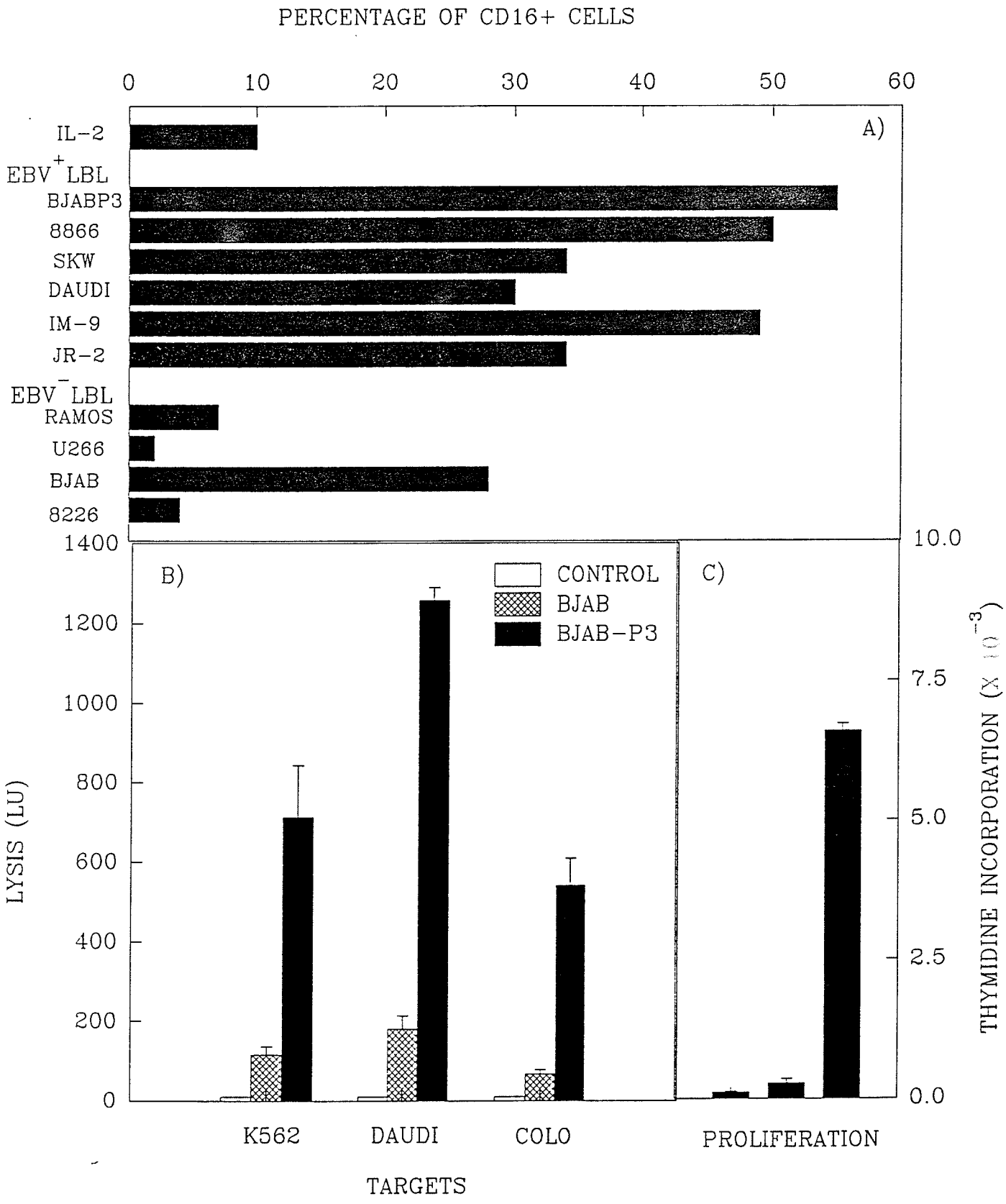

PERCENTAGE OF CD16+ CELLS
0
10
20
30
40
50
60
A)
IL-2
EBV+ LBL
BJABP3
8866
SKW
DAUDI
IM-9
JR-2
EBV- LBL
RAMOS
U266
BJAB
8226
B)
CONTROL
BJAB
BJAB-P3
LYSIS (LU)
1400
1200
1000
800
600
400
200
0
K562
DAUDI
COLO
TARGETS
C)
PROLIFERATION
10.0
7.5
5.0
2.5
0.0
THYMIDINE INCORPORATION (X 10-3)

Figure 3.

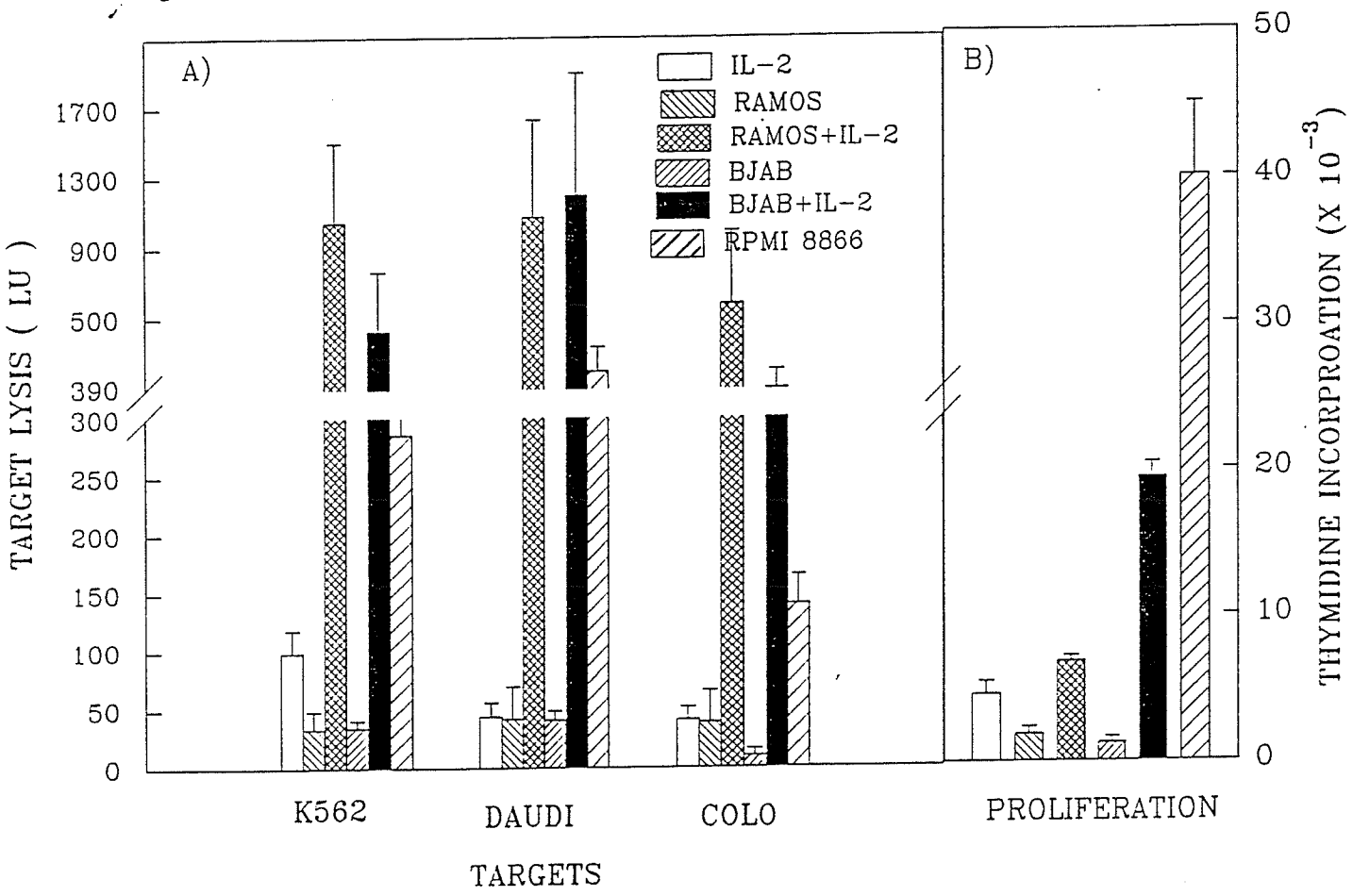

A)
B)
IL-2
RAMOS
RAMOS+IL-2
BJAB
BJAB+IL-2
RPMI 8866
TARGET LYSIS (LU)
THYMIDINE INCORPROATION (X 10^{-3})
1700
1300
900
500
390
300
250
200
150
100
50
0
50
40
30
20
10
0
K562
DAUDI
COLO
PROLIFERATION
TARGETS

Figure 4.

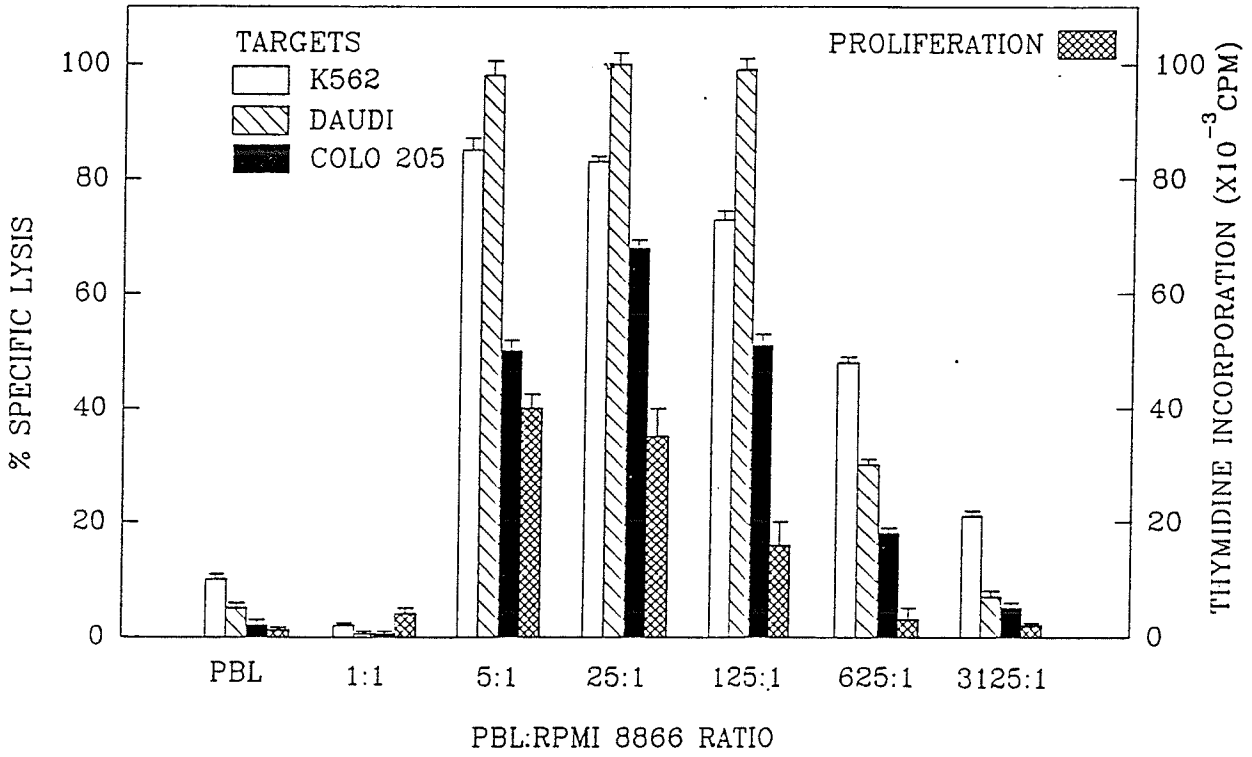

TARGETS
K562
DAUDI
COLO 205
PROLIFERATION
% SPECIFIC LYSIS
THYMIDINE INCORPORATION (X10^{-3}CPM)
0
20
40
60
80
100
PBL
1:1
5:1
25:1
125:1
625:1
3125:1
PBL:RPMI 8866 RATIO

Figure 5.

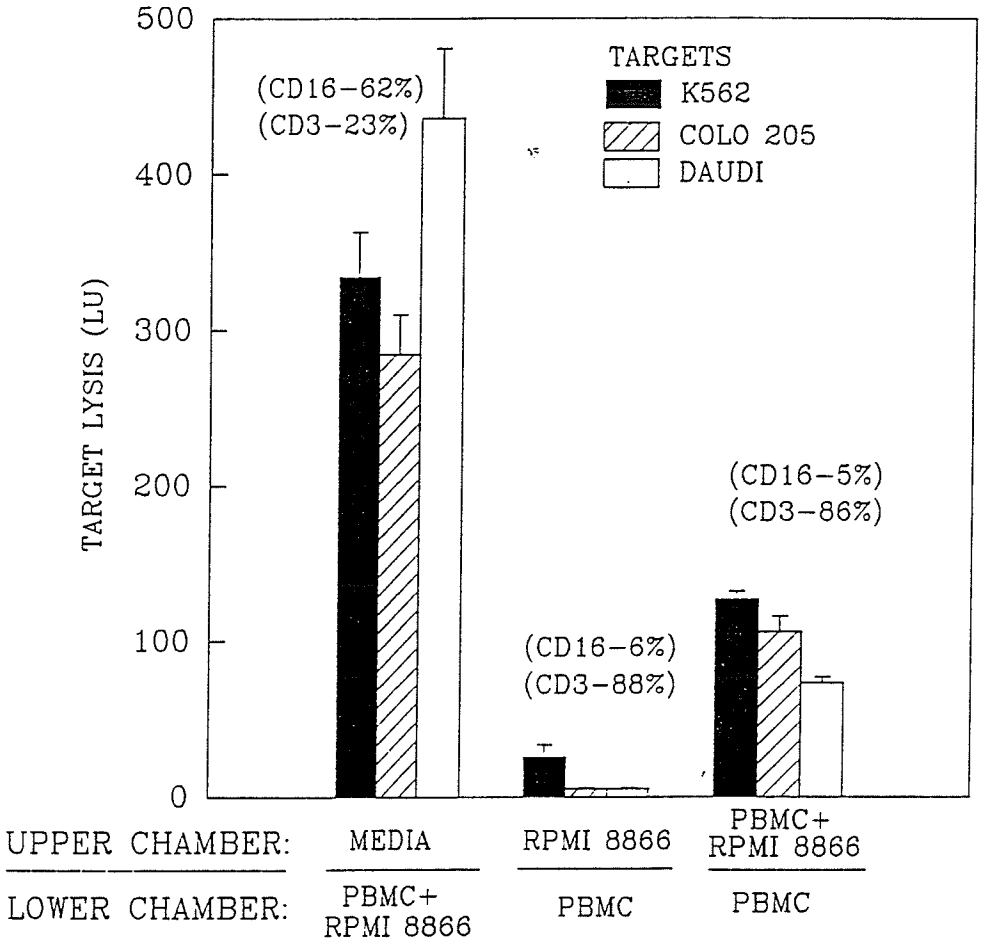

500
400
300
200
100
0
TARGET LYSIS (LU)
TARGETS
K562
COLO 205
DAUDI
(CD16-62%)
(CD3-23%)
(CD16-6%)
(CD3-88%)
(CD16-5%)
(CD3-86%)
UPPER CHAMBER:
MEDIA
RPMI 8866
PBMC+ RPMI 8866
LOWER CHAMBER:
PBMC+ RPMI 8866
PBMC
PBMC

Figure 6.

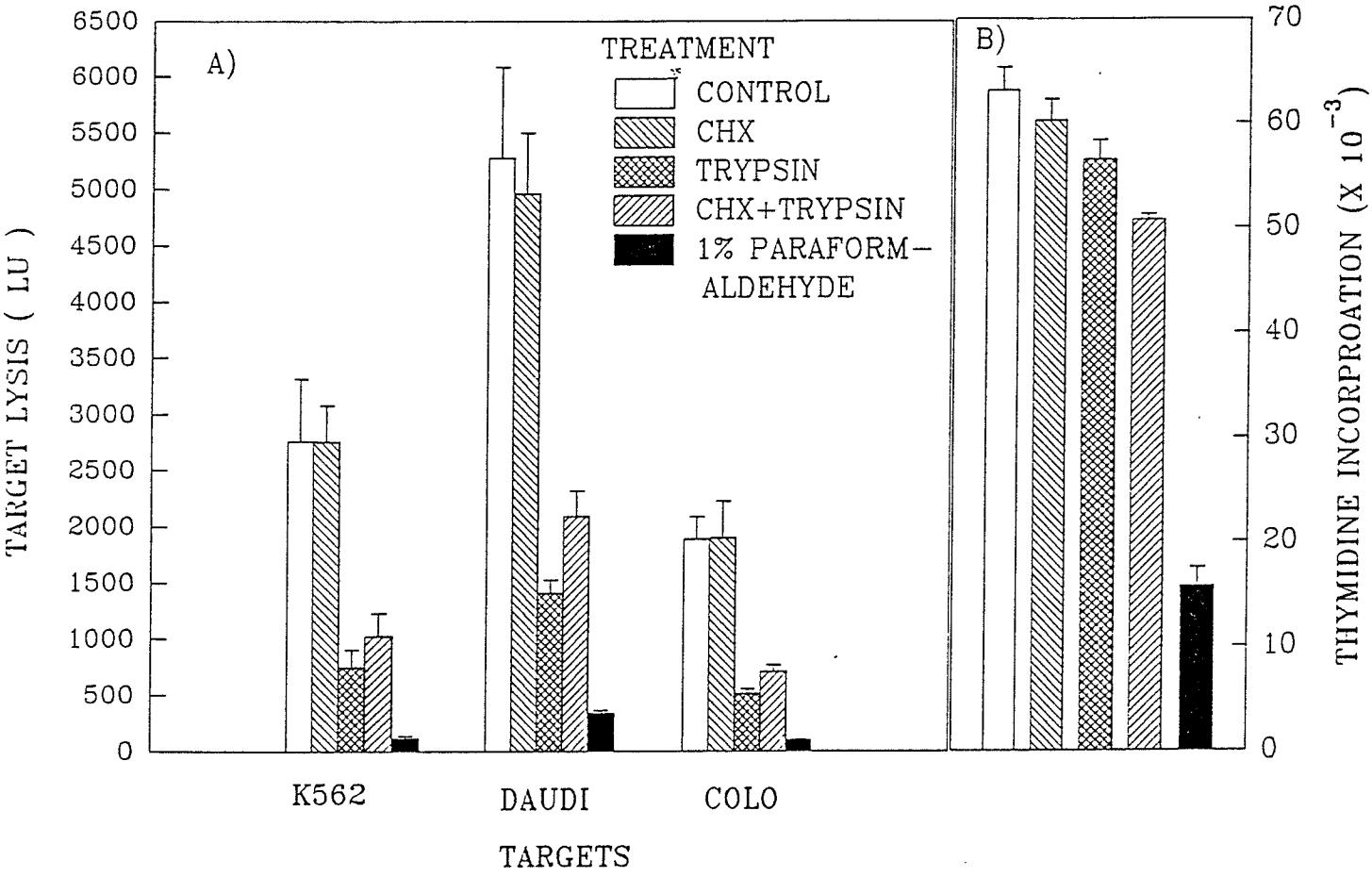
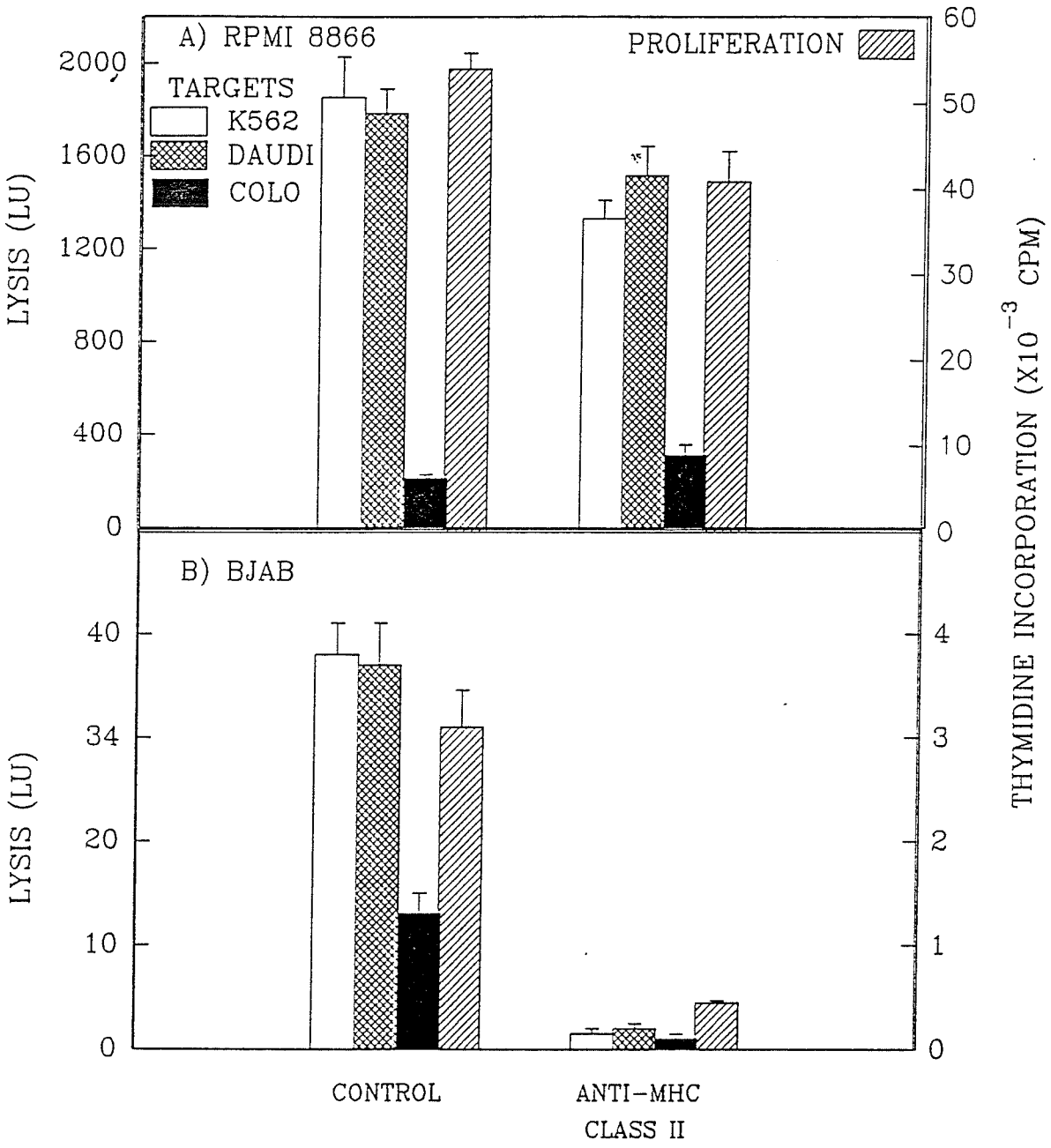
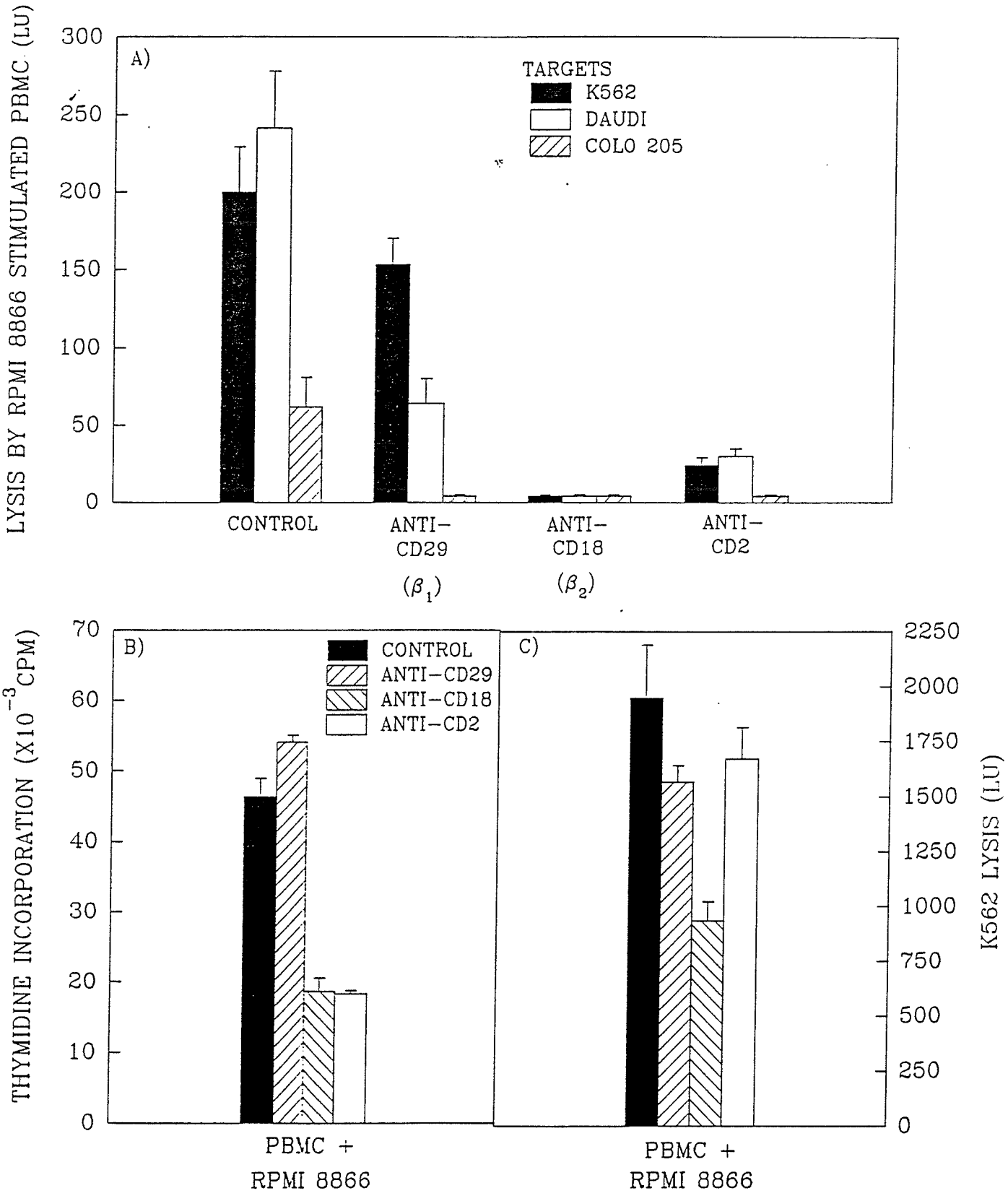

A)
B)
TREATMENT
CONTROL
CHX
TRYPSIN
CHX+TRYPSIN
1% PARAFORM-
ALDEHYDE
TARGET LYSIS (LU)
THYMIDINE INCORPROATION (X 10^{-3})
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
70
60
50
40
30
20
10
0
K562
DAUDI
COLO
TARGETS

Figure 7.


A) RPMI 8866
PROLIFERATION
TARGETS
K562
DAUDI
COLO
LYSIS (LU)
2000
1600
1200
800
400
0
60
50
40
30
20
10
0
B) BJAB
LYSIS (LU)
40
34
20
10
0
4
3
2
1
0
THYMIDINE INCORPORATION (X10^{-3} CPM)
CONTROL
ANTI-MHC CLASS II


A)
LYSIS BY RPMI 8866 STIMULATED PBMC (LU)
300
250
200
150
100
50
0
TARGETS
K562
DAUDI
COLO 205
CONTROL
ANTI-CD29 (β_1)
ANTI-CD18 (β_2)
ANTI-CD2
B)
THYMIDINE INCORPORATION (X10^{-3}CPM)
70
60
50
40
30
20
10
0
CONTROL
ANTI-CD29
ANTI-CD18
ANTI-CD2
PBMC + RPMI 8866
C)
2250
2000
1750
1500
1250
1000
750
500
250
0
K562 LYSIS (LU)
PBMC + RPMI 8866

PAPER III.

Reactivity of γδ T Cells Induced by the Tumour Cell Line RPMI 8226: Functional Heterogeneity of Clonal Populations and Role of GroEL Heat Shock Proteins.

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From The Rheumatic Disease Unit Research Laboratory[*], Department of Internal Medicine[‡] , Department of Medical Microbiology[†], University of Manitoba, Canadian Red Cross, Blood Transfusion Service[#], Winnipeg, Manitoba, Canada and Shanghai Medical University[¶], Shanghai, China.

ABSTRACT

The human tumour cell lines RPMI 8226 and Daudi are potent inducers of Vγ9 expressing T cells. The inducing element of RPMI 8226 has not been defined but evidence suggests that a member of the groEL HSP family (HSP 58) may have a role in the induction by Daudi cells. The present study examined the reactivity patterns of γδ T cell clones generated in response to RPMI 8226 and addressed the possible role of HSP 58 in this process.

RPMI 8226 induced a population of Vγ9 TCR+ cells which were heterogeneous in terms of their cell surface markers, patterns of proliferation and cytotoxic responses. All clones expressed CD3, CD2, CD18 and CD29. They demonstrated variability in expression of CD56, CD8 and HLA-DR. RPMI 8226 stimulated proliferation in purified bulk γδ cultures and clones. Daudi was also capable of inducing these cells to proliferate while mycobacterial products were not effective. The clones demonstrated a limited non-MHC restricted cytotoxicity pattern with some evidence of clonal heterogeneity. Although both Daudi and RPMI 8226 were sensitive to lysis by the clones cold inhibition experiments indicated differential activity towards these targets.

Anti-HSP 58 was inhibitory to γδ T cell induction by RPMI 8226, Daudi and mycobacterial products. However, the anti-HSP 58 antibody appears to bind to the surface of at least 6 different tumour cell lines with no correlation to their ability to induce γδ

T cells and the anti-HSP 58 inhibited non γδ responses.

Footnotes

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Human peripheral blood γδ T cells show a remarkably high frequency of reactivity to antigens of mycobacteria (1). Although γδ T cell clones specific for mycobacterial HSP 65, a member of the GroEL HSP family (2,3) have been identified most γδ T cells responding to mycobacterial antigens appear not to react to the intact HSP 65. The frequency of peripheral blood γδ cells responding to a killed *Mycobacterium tuberculosis* preparation ranged from 5% to 50%, whereas the frequency of γδ cells responding to purified mycobacterial HSP 65 was 10 to 20 fold lower (4). The majority of the cells stimulated by mycobacterium bear the Vγ9/Vδ2-encoded receptor and display extensive junctional diversity (1). The apparent innate mycobacterial reactivity of Vγ9 TCR+ cells and the high precursor frequency for mycobacteria among γδ T cells suggests a mode of recognition analogous to that of superantigens by αß T cells (1).

Heat shock proteins (HSP), a group of highly conserved proteins expressed in all organisms, have been suggested as possible ligands not only in γδ T cell responses to mycobacteria but also in their response to "stressed" cells (5-9). Induction of

HSPs can occur following many environmental stresses. They are divided into families based on size, antigenic crossreactivity and sequence homologies. Members of the GroEL HSP (HSP 60) family share homology with the GroEL protein of *Escherichia coli* and are found in both prokaryotes and eukaryotes. In eukaryotes HSP 60 is a normal component of the mitochondrial matrix, and functions as an accessory factor in facilitating the folding of proteins (10,11). GroEL proteins have been identified as major immunogenic antigens in various bacterial infections, including Q fever, syphilis, tuberculosis and leprosy (1,12-14).

Molecules related to HSPs have been reported to be present on the surface of human cell lines (15-17) and there is evidence that they may function as target molecules for γδ T cells. γδ T cell lines derived from patients with systemic lupus erythematosus proliferated in response to autologous B-lymphoblastoid cell lines. These responses could be significantly inhibited by monoclonal antibodies specific for mycobacterial HSP 65, and human HSP 60 (18). The Burkitt's lymphoma cell line Daudi have been reported to induce Vγ9/Vδ2 T cells in a manner suggestive of a superantigen type response (15). Antibodies reactive with the human HSP 58, which is the human homologue of the mycobacterial HSP 65, inhibited the Daudi induction of γδ T cells. These studies suggest that autologous HSP recognition could be a prominent function of γδ T cells, perhaps forming part of a mechanism for immune surveillance that would eliminate stressed cells from the body.

The human myeloma RPMI 8226 is also a potent inducer of Vγ9

expressing T cells (19). While the inducing element of RPMI 8226 has not been defined evidence suggests that the inducing element present on Daudi cells maybe a member of the GroEL HSP family (15). As RPMI 8226 and Daudi induce similar populations of $\gamma\delta$ cells the possibility exists that they may express similar inducing antigen(s). The present study examines the reactivity patterns of $\gamma\delta$ clones generated in response to RPMI 8226. The possible role of HSP 58 in this process is specifically addressed.

MATERIALS AND METHODS

T Cell Lines and Clones.

Ficoll hypaque purified normal PBMC's (10^6/ml) were cultured in the presence of irradiated RPMI 8226 cell line (50Gy; $3x10^4$/ml) and highly purified recombinant human IL-2 (rhIL-2) (50U/ml; Cetus, Emeryville, Calif.) (20) in AIM V serum free media (Gibco, Grand Island, NY). The cells were subcultured on day 6 in AIM V media containing rhIL-2 (50 U/ml). Day 10 the cells positively sorted using the $\gamma\delta$ TCR framework mAb anti-TCRδ1 Mab (21) (kindly provided by Dr. M. Brenner, Dana-Farber Cancer Institute, Boston, Mass.) by flow cytometry (Coulter Epics C). The cells were then cloned at limiting dilution in 10% pooled AB, Rh negative human serum with RPMI 1640, containing penicillin/streptomycin and rhIL-2 (50 U/ml) in the presence of irradiated feeder cells (PBMC's at $1x10^5$/well and RPMI 8226 at $3x10^3$/well). The clones were maintained by feeding

twice weekly with fresh media and rhIL-2. Every two weeks they were given irradiated feeders (PBMC's, RPMI 8226 and RPMI 8866) and PHA (0.5 μg/ml). Bulk sorted uncloned γ/δ T cells were also maintained in 10% pooled human serum with RPMI 1640 and rhIL-2 (50U/ml).

Cell lines.

Continuous cell lines K562 (erythroleukemia), Colo 205 (adenocarcinoma), Daudi (lymphoma), Raji (lymphoma), JY (B cell leukemia), Jurkat (T cell leukemia), Molt 4 (T cell leukemia) and RPMI 8226 (myeloma) were obtained from the American Type Culture Collection (Rockville, MD). RPMI 8866 (B cell leukemia) was purchased from ABS (Buffalo, NY). BJAB and BJAB-B95 were kindly provided by Dr. Louis Qualtiere (University of Saskatchewan, Saskatoon, Saskatchewan). These cells were maintained on 10% FCS with RPMI 1640. Mycoplasma contamination was excluded in all continuous cell lines used by culture and/or Hoescht staining.

mAbs and Immunofluorescence.

For immunofluorescence studies the cells were labelled with mAbs reactive against CD3 (OKT3), CD2 (OKT11) and CD18 (ß2) produced by hybrid cell lines obtained from the American Tissue Culture Collection (ATCC, Rockville, Md); CD16 (Leu 11b), CD4 (Leu3a PE), CD8 (Leu2a PE), HLA-DR obtained from Becton Dickinson Co. (Mountainview, Calif.); CD56 (NKH-1 PE) obtained from Coulter Immunology (Hialeah, Florida); CD29 (ß1,JB1) was produced and purified from a hybridoma developed in our laboratory (22);

Diversi-T γV2(a) (Vγ9) and Identi-T pan-TCRαß obtained from T Cell Sciences, Inc (Cambridge, MA); rabbit polyclonal anti-HSP 58 was kindly provided by Dr. W. Welch, Lung Biology Centre, UCSF, San Francisco, Calif) (23); and the γδ TCR framework mAb anti-TCRδ1 was kindly provided by Dr. M. Brenner, Dana-Farber Cancer Institute, Boston, Mass. (21). For direct fluorescence, cells were labelled with mAbs conjugated with phycoerythrin (PE) in PBS (30 min at 4^0C) and washed. For indirect fluorescence, cells were exposed to mAbs, subsequently incubated with FITC-$F(ab')_2$-conjugated goat anti-mouse antibody obtained from Cedarlane Laboratories (Hornby, Ont., Can).

Labelling, extraction and immunoprecipitation of cell surface proteins.

Immunoprecipitates of ^{125}I-labelled stimulated PBMC cell lysates were resolved by SDS-PAGE as previously described (21,24). Briefly, cells ($1X10^7$) were surface labelled with 0.5 mCi (=18.5 MBq) ^{125}I using lactoperoxidase. The cells were extracted in 1% Triton X-100, 1.0 mM PMSF in PBS for 20 min and the supernatants collected by centrifugation. Antibodies were added to the supernatants and incubated overnight. The immune complexes were precipitated with *S. aureus* Cowan I which had been preloaded with rabbit anti-mouse Ig. The pellets were washed with the extraction buffer and the precipitated membrane proteins recovered by boiling for 3 min in 0.1 M Tris-HCl, 2% SDS, 10% glycerol, 5% 2-ME. The supernatants were separated on 10% polyacrylamide gels under reducing and non-reducing conditions.

Proliferation Assay.

$2x10^4$ responder cells were cultured in flat bottomed 96 well plates with various stimuli including the irradiated (50Gy) cell lines ($2x10^4$) RPMI 8226, RPMI 8866, Colo 205, Daudi, sonicated extract from killed *M.tb.* H37Rv (25) (100μg/ml) (Difco Laboratories, Inc., Detroit, MI) in the presence of irradiated autologous antigen presenting cells (APC)($2x10^4$), and APC's alone. These stimuli were added either alone, or in combination with rhIL-2 (50U/ml). The cultures were maintained for 4 days and pulse labelled with ^{3}H-Tdr for the final 18 hrs of culture.

Cytotoxicity Assay.

The cytotoxic activity of the effector cells was determined in a 4 hr. chromium-release assay as previously described (26). The target cells were labelled by incubating $2x10^6$ cells in 0.5 ml RPMI-1640, 10% FCS, containing 300 μCi $Na_2{}^{51}CrO_2$ (Amersham, Oakville, Ontario, Canada) for 90 min at 37^0C. The cells were washed twice in RPMI-1640 and resuspended to a final concentration of $2.5x10^4$ cells/ml. Per cent specific lysis was calculated as 100x[(counts/min released with effectors - counts/min spontaneously released) / (total counts/min - counts/min spontaneously released). In all assays spontaneous release was less than 15%. In the cold competition assays unlabelled targets were added to the wells at the indicated ratios of cold targets to a constant number of labelled targets.

Anti-HSP 58 inhibition studies.

γδ T cell induction was performed as described above using either RPMI 8226, Daudi, or killed *M.tb.* H37Rv (25) (100μg/ml) (Difco Laboratories, Inc., Detroit, MI) as a stimulus. CD16+ cell induction was set up in a similar manner except RPMI 8866 and Daudi were used as stimuli (19,27). At day 0 either normal rabbit serum (1/40), rabbit anti-HSP 58 (1/40) or mouse anti-HSP 72 (1/250) were added to each culture and the cells were cultured as previously described for γδ cell induction.

RESULTS

RPMI 8226 stimulation of γ/δ T cells.

Upon coculture of PBMC's with irradiated RPMI 8226 and rhIL-2 there was an increase of γ/δ T cells from day 0 (0.9%) to day 10 (57%) which represented a 143 fold increase in γ/δ T cell number (Fig 1a). This enrichment for γ/δ T cells was a result of proliferation rather than a selective loss of α/β T cells as there was a 90±20 SEM fold increase in absolute numbers of γ/δ T cells from day 0 to day 10 (Fig.1b). Other tumour cell lines have previously been tested for their ability to induce γδ T cells and thus far only RPMI 8226 and another B lymphoblastoid cell line, Daudi have been found to be effective inducers (15,19).

γ/δ T cell receptor expression by immunoprecipitation.

The TCRδ1 antibody detects framework elements of the δ chain

which are common to all isotypes of the δ receptors. Therefore the nature of the radioimmunoprecipitable material was examined by SDS-PAGE analysis. Under non-reducing conditions, 40 and 80 Kd species were observed (Fig 1c). Following reduction there was a shift in molecular weights leading to the disappearance of the 80 Kd species and the appearance of multiple bands in the 40 to 50 Kd range. The specificity of the immunoprecipitation reaction was confirmed using the γ/δ negative T cell line, Jurkat and normal PBMC,s. In both cases TCRδ1 reactive material was not detected by SDS-PAGE. These results suggest that the predominant form of γ/δ TCR present on these stimulated cells was disulphide-linked.

Phenotype of γδ T cell clones.

In order to better assess the stimulating mechanism(s) potentially involved in the RPMI 8226 induction of γδ T cells, clonal populations were developed and examined for their marker phenotype, proliferative and cytotoxic specificities.

Following purification the polyclonal population of RPMI-8226 induced γδ T cells were 91% positive for the framework mAb TCRδ1 (Table 1). The majority of these cells (82%) expressed the Vγ9-encoded receptor, all of γδ cells expressed CD2 and CD3, a significant proportion expressed CD8 (14%), or CD56 (42%) while CD4 was expressed on only a few cells. They all expressed the integrin molecules CD18 (ß2) and CD29 (ß1) but lacked ß3 integrins (ß3 not shown). These results were representative of 3 experiments performed.

Thirty-five clones derived from this purified population of γδ T cells were identical in their expression of surface antigens in that they all expressed CD3, CD2, TCRδ1, CD18, CD29 and did not express TCR αß. Four Vγ9 TCR+ clones were chosen and examined in further detail (Table 1). One of these clones (CL3A20) was CD56+/ CD8-, while the other three were CD56-/CD8+. The only other difference was that while three of the clones were highly positive for HLA-DR, CL3R60 was negative.

Proliferative response of γδ T cell clones.

The four clones and the bulk γδ population were assessed for the specificity of their proliferative responses (Fig.2). The bulk population consistently demonstrated a strong response to the original stimulus RPMI 8226 and a moderate response to Daudi but no significant response to mycobacterial antigens. The polyclonal γδ cells did not proliferate to 9 other tumour cell lines tested.

The clones displayed a more variable pattern of responsiveness. Clones 3A16 and 3R60 had a similar pattern of proliferation to that of the bulk population while clone 3A20 proliferated to RPMI 8866 as well as RPMI 8226 and Daudi. The clone 3R9 did not to respond to any of the stimuli other than rhIL-2.

Cytotoxicity of γδ T cell clones.

The polyclonal purified bulk γδ T cells effectively killed both RPMI 8226 and Daudi, with a low kill of K562 and RPMI 8866, with essentially no kill of Colo 205 and Raji (Table 2). Three of

the clones (3A16, 3R60, 3R9) appeared to have a similar pattern of cytotoxicity to the polyclonal bulk $\gamma\delta$ cells while the clone 3A20 killed all of the targets.

To further assess the spectrum of kill by the clones 5 additional tumour cell targets were tested (Table 2). These targets were all susceptible to lysis by clone 3A20. The other three clones once again demonstrated a similar pattern of restriction in cytotoxic activity. Only two of these additional targets JY and Jurkat were susceptible to lysis by these three clones. The difference between 3A20 and these three clones was its expression of the NK cell antigen CD56 and lack of CD8 expression. As well, it was capable of proliferating in response to RPMI 8866.

Cold competition experiments.

Cold competition experiments were performed in an effort to further assess the specificity of kill in an attempt to obtain more information about the possible recognition sites being used for the three targets RPMI 8226, Daudi and RPMI 8866 (Fig.3A-F). It was found that the polyclonal bulk culture and the four clones all demonstrated the same pattern of killing. Each of the cold targets could inhibit the kill of the other three but with a consistent preferential pattern. Generally, RPMI 8226 was four times more efficient than Daudi while RPMI 8866 had little or no inhibitory effect on either RPMI 8226 or Daudi kill except at very high concentrations. However, RPMI 8866 was as effective as RPMI 8226 and Daudi in the inhibition of RPMI 8866 kill.

The effect of anti-HSP 58 in γδ T cell induction.

HSP-58 has been suggested to play a role in the induction of γδ cells by the LBL Daudi (15). Indirect immuno-fluorescence indicated HSP 58 or a homologue to be present on the surface of a number of cell lines including RPMI 8226 and Daudi which induce γδ T cells. However the noninducing cell lines RPMI 8866, BJAB, K562 and Ramos also expressed anti-HSP 58 reactive material (Fig 4). (The results for the latter two cell lines are not shown).

In an attempt to ascertain the role of HSP 58, or a homologue in the induction of γδ T cells anti-HSP 58 was added to the various γδ T cell induction systems (RPMI 8226, Daudi, and mycobacterial) at the beginning of the culturing period. The anti-HSP 58 markedly inhibited the induction of γδ T cells in the Daudi and mycobacterial treated cultures (Fig. 5A). However, the effects of antibody were much lower in the RPMI 8226 system (Fig. 5A).

The specificity of this inhibition was examined in CD16+ cell induction by lymphoblastoid cell lines RPMI 8866 (19,27). When anti-HSP 58 was added it produced a partial inhibition of CD16+ cell induction by RPMI 8866 (Fig. 5B). Interpretation of the inhibitory effects of anti-HSP 58 is further confounded by the general inhibitory effects of this antibody on cellular proliferation (Fig. 6). Anti-HSP 58 was inhibitory to cellular proliferation in 2 of the 3 γδ induction systems, Daudi and mycobacterial, but not in the RPMI 8226 system. It was also anti-proliferative in the RPMI 8866 induction of CD16+ cells as well as in IL-2 stimulation of PBL growth.

DISCUSSION

It has previously been shown that at least two B cell lines, RPMI 8226 and Daudi are capable of inducing γδ T cell activation (15,19). This induction ability did not appear to correlate to tumour cell type beyond cell lineage. However, the inductive capacity is not a general property of B cell lines as 8 other B cell lines tested were not able to induce γδ T cells (19). Nor did it correlate with the presence of Epstein Barr virus (EBV) as Daudi is latently infected and RPMI 8226 is not.

The present experiments further characterize the nature of the RPMI 8226 system of γδ T cell induction. It would appear that the triggering event results in an average 90 fold increase in γδ T cells and that all normal individuals tested apparently do respond. The majority of individuals have a similar response to both Daudi and RPMI 8226. As well, like the Daudi and mycobacterial induced γδ T cells (1,15) the majority of the γδ cells induced by RPMI 8226 were the disulphide-linked form expressing the Vγ9-encoded receptor.

The inducing stimulus, RPMI 8226 was recognized by and was capable of stimulating proliferation in the purified bulk γδ cultures and clones. Daudi another inducer of Vγ9 expressing γδ T cells (15) was also able to stimulate cytotoxic and proliferative responses in the RPMI 8226 induced bulk and clonal populations. In contrast to the Daudi induced γδ cells, the RPMI 8226 induced populations did not proliferate in response to mycobacterial

products (15). It would therefore appear that there may be some response or repertoire differences between γδ cells generated by these two cell lines.

In addition to these generally observed trends in proliferative responsiveness there was some heterogeneity in the clonal responsiveness to these various stimuli. Clone 3A20 responded not only to RPMI 8226 and Daudi but also to RPMI 8866. A γδ clone, which expresses the NK cell marker CD56 and demonstrates an NK pattern of broad spectrum cytotoxicity also proliferates in response to an NK cell inducing lymphoblastoid cell line (RPMI 8866) (19,27). This observation suggests that there may be different subsets of γδ T cells some of which may function like NK cells while some have been shown to be MHC restricted more like αß T cells (1). Another Vγ9 expressing clone, 3R9 did not proliferate in response to any of the stimuli above the level induced by IL-2 alone. Although this clone was functionally active in cytotoxic assays it is possible that the receptor ligand interaction involved in the proliferative response differs from that involved in the cytotoxic event and was not functional. Alternatively the γδ T cells may produce some of their own growth factors upon stimulation and this particular clone was unable to do so.

The RPMI 8226 induced γδ cells displayed a limited spectrum of non-MHC restricted cytotoxic activity. The two effective inducer lines Daudi and RPMI 8226 were the most susceptible targets. However, some non-inducing lines were also killed by the γδ cells. In the case of clone 3A20 the range of kill was much broader

reminiscent of activated NK cells. This was consistent with the expression of CD56, an antigen which is associated with broad spectrum non-MHC restricted cytotoxicity, both by NK cells and αß T cells (28).

Cold competition experiments demonstrated that RPMI 8226 stimulated γδ cells have a preferential pattern of kill. They lyse RPMI 8226 in preference to Daudi, and Daudi in preference to RPMI 8866. These results could be interpreted to suggest that γδ T cells recognize different targets using different cellular interactions. Alternatively, it is possible that these targets all express the same recognition element but in different quantities or different secondary recognition elements such as adhesion molecules maybe involved. These could enhance cellular interactions leading to preferential conjugate formation with one target type.

The exact role of HSPs on the induction of γδ T cells by RPMI 8226 is difficult to interpret. It appears by indirect immunofluorescence studies that antibody to HSP 58 binds to at least 6 tumour cell lines, but this characteristic does not correlate with their ability to induce γδ T cells. This suggests that if HSP 58 or a homologue does play a role in induction, it is not sufficient in itself for γδ T cell induction but requires some other ancillary factor that is present on RPMI 8226 and Daudi. There also remains the question of what forms of HSPs, which have previously been described as proteins restricted to intracellular compartments (29) are expressed on the surface of cells.

Culture with anti-HSP 58 did partially inhibit γδ T cell

growth in the RPMI 8226, Daudi and mycobacterial based induction systems. However, the effect was not restricted to γδ cell induction as CD16+ and IL-2 stimulated cell growth were also inhibited. These results indicate that caution should be exercised in attempting to interpret what the exact role of HSP is in γδ T cell induction. HSPs do appear to play a role in the induction process but not necessarily in the recognition events. HSPs have been postulated to act as antigen presenting molecules in some systems (30). Recently, it has been reported that anti-HSP 58 binds to the surface of γδ T cells (17) raising the possibility that anti-HSP 58 effects may be due to interaction with the responding γδ cell rather than the stimulator population.

There is evidence to suggest that γδ T cells are present in *in vivo* tumours (31,32) and may be involved in the pathogenesis in various disease states such as infections with EBV (33), and measles(34); in autoimmune diseases such as rheumatoid arthritis (35) and polymyositis (36); and diseases of unknown origin such as ulcerative colitis (37). Further characterization and identification of the cellular events involved in this tumour cell induction of γδ T cells could potentially lead to not only a better understanding of their immunological role but to information which may be useful for therapeutic intervention in these and other disease states.

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Figure 1. Phenotypic analysis of PBMC's stimulated with 8226 and rhIL-2. (a) Panel H1 and H3 FITC controls of a day 0 (H1) and day 10 (H3) panels H2 and H4 show TCRδ1 expressing cells day 0, 10 respectively. (b) The absolute number of δ positive cells in 7 individuals present at day 0 (mean, $0.3X10^6 \pm .1X10^6$ SEM) and day 10 ($23X10^6 \pm 6X10^6$) after stimulation (% δ+ cells is expressed in brackets; day 0, 2.2%±0.4; Day 10, 44%±6). Starting cultures contained 10 to $30X10^6$ PBMC's. (c) SDS-PAGE analysis of anti-TCR δ reactive material from 8226 stimulated PBMC's. Lane 1, stimulated PBMC's immunoprecipitated with δ1 mAb (reducing) and 2, control ; lane 3, δ1 mAb (non-reduced) and 4, control. Size markers, relative molecular mass in thousands.

Figure 2. Proliferative response of RPMI 8226 stimulated polyclonal population and four γ/δ T cell clones. Although not shown the cells were also tested against 5 other B cell lines (JY, BJAB, BJAB-P3, Ramos and Raji), 2 T cell lines (Molt4, and Jurkat), the colonic carcinoma cell line Colo 205, the cervical carcinoma line HELA, and the erythroleukemia line K562. The bulk and clonal populations of $\gamma\delta$ cells did not show any proliferative response to these cell lines.

Figure 3. Cold target competition experiments with various labelled targets. Each Cr^{51}-labelled target, RPMI 8226, Daudi and RPMI 8866 was mixed with each of the indicated unlabelled targets prior to incubation with effectors (E:T;1:20). The ability of each cold target to inhibit Cr^{51} release was recorded and expressed as % inhibition of specific lysis. The % specific lysis at E:T ratio 1:20 by clone 3A20 against the three targets RPMI 8226, Daudi and RPMI 8866 without cold target inhibition were 67, 64, and 60 respectively and for clone 3R60 were 57, 53 and 23.

Figure 4. Cell surface expression of HSP 58 on four tumour cell lines by cell surface immunofluorescence. Cells were untreated or reacted with, normal rabbit serum, or rabbit anti-HSP 58 (both used at 1/40 dilution) and stained with a FITC labelled anti-rabbit Ig.

Figure 5 A.) The effect of anti-HSP 58 on induction of $\gamma\delta$ or B.) CD16 bearing cells. The following antibodies were added at day 0 to PBMC's cultured with various stimuli which induce $\gamma\delta$ cells (RPMI 8226, Daudi, mycobacterial extract) or CD16+ cells (RPMI 8866, Daudi): rabbit serum, 1:40; rabbit anti-HSP 58, 1:40. This is a representative experiment of 3 which were performed.

Figure 6. Effect of anti-HSP 58 on overall cellular proliferation in response to various stimuli. This is a representative experiment of 3 which were performed.

Figure 1.

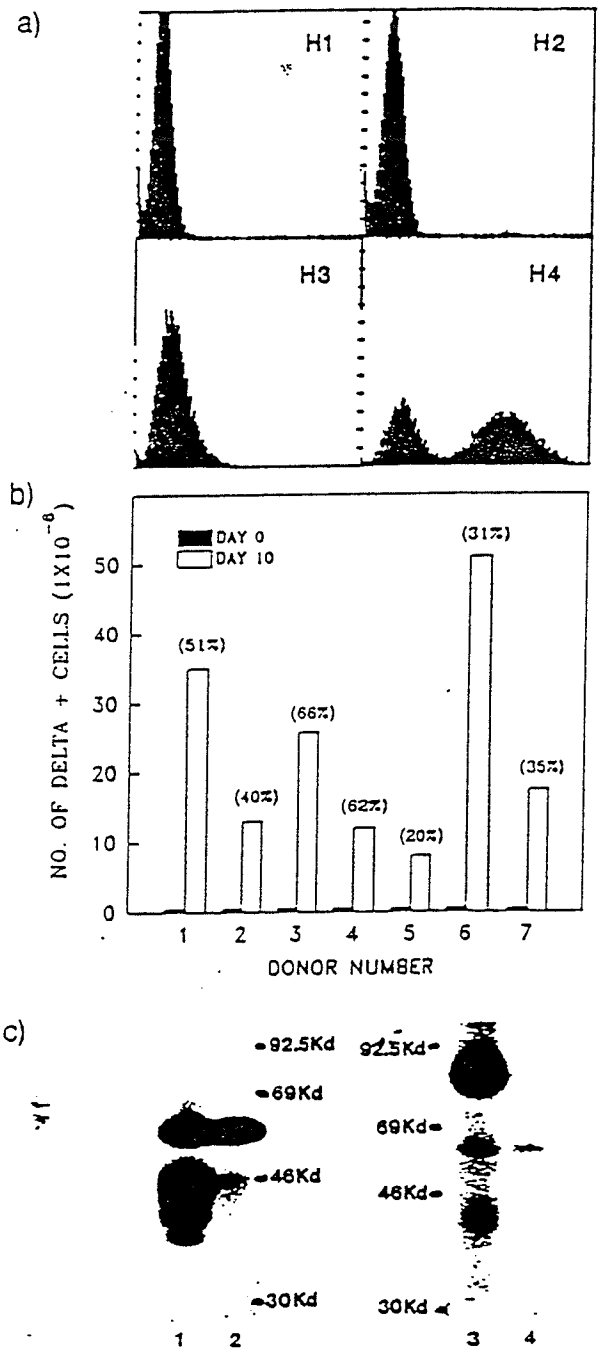
a)
H1
H2
H3
H4
b)
DAY 0
DAY 10
NO. OF DELTA + CELLS (1X10⁻⁶)
(51%)
(40%)
(66%)
(62%)
(20%)
(31%)
(35%)
DONOR NUMBER
c)
92.5Kd
69Kd
46Kd
30Kd
1
2
3
4

Figure 2.

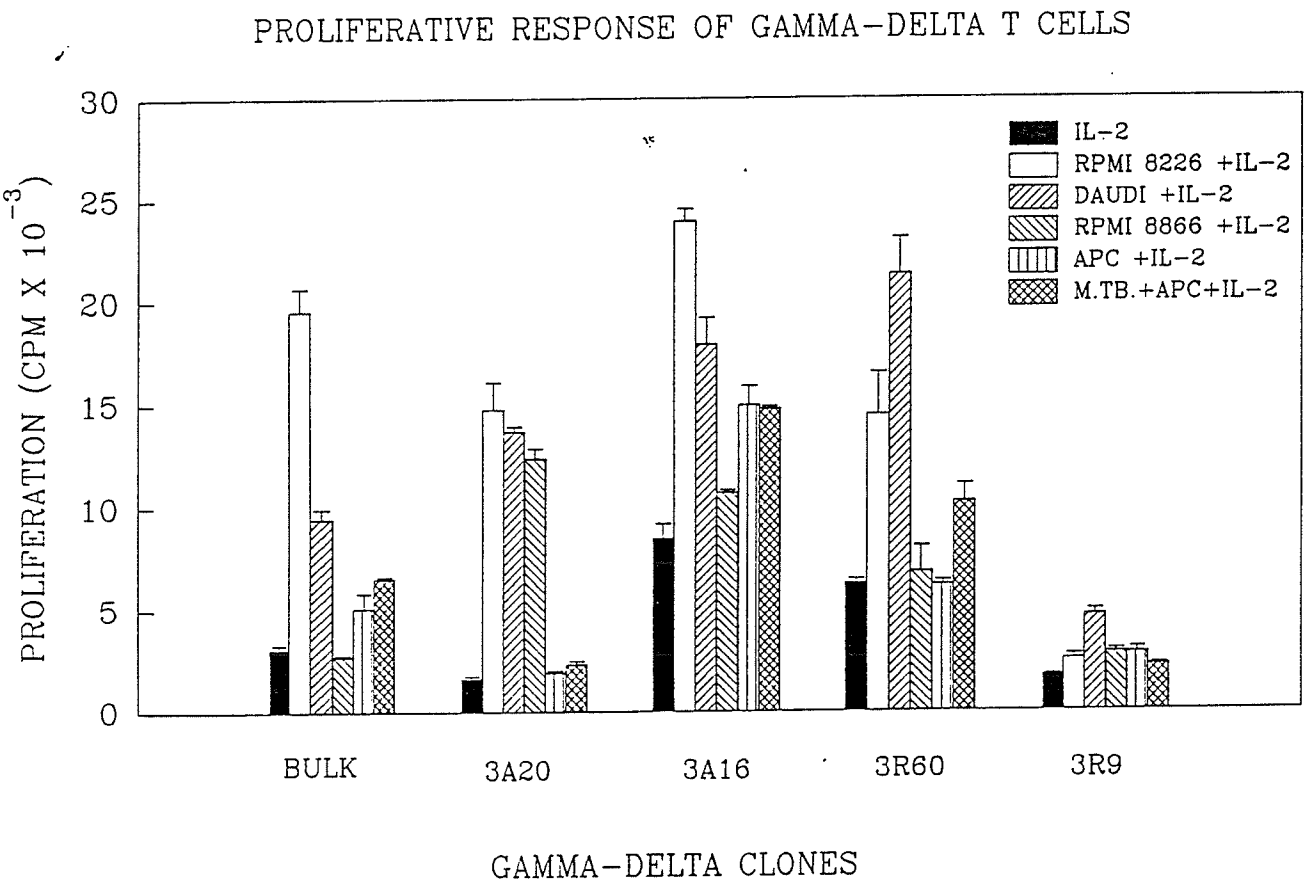
PROLIFERATIVE RESPONSE OF GAMMA-DELTA T CELLS
PROLIFERATION (CPM X 10^{-3})
30
25
20
15
10
5
0
IL-2
RPMI 8226 +IL-2
DAUDI +IL-2
RPMI 8866 +IL-2
APC +IL-2
M.TB.+APC+IL-2
BULK
3A20
3A16
3R60
3R9
GAMMA-DELTA CLONES

Figure 3.

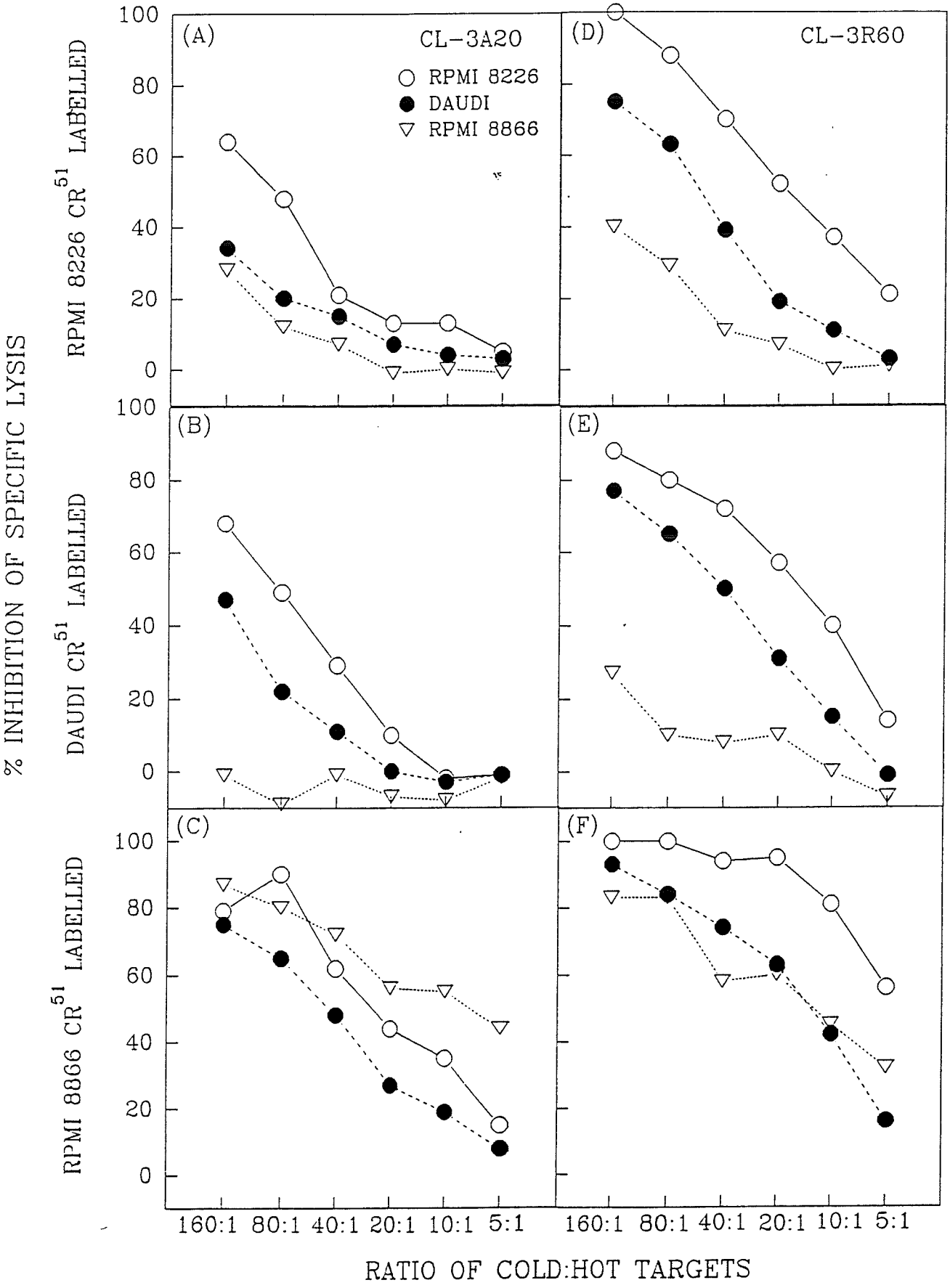
% INHIBITION OF SPECIFIC LYSIS
RPMI 8226 CR51 LABELLED
DAUDI CR51 LABELLED
RPMI 8866 CR51 LABELLED
(A)
CL-3A20
RPMI 8226
DAUDI
RPMI 8866
(D)
CL-3R60
(B)
(E)
(C)
(F)
100
80
60
40
20
0
160:1 80:1 40:1 20:1 10:1 5:1
RATIO OF COLD:HOT TARGETS

Figure 4.

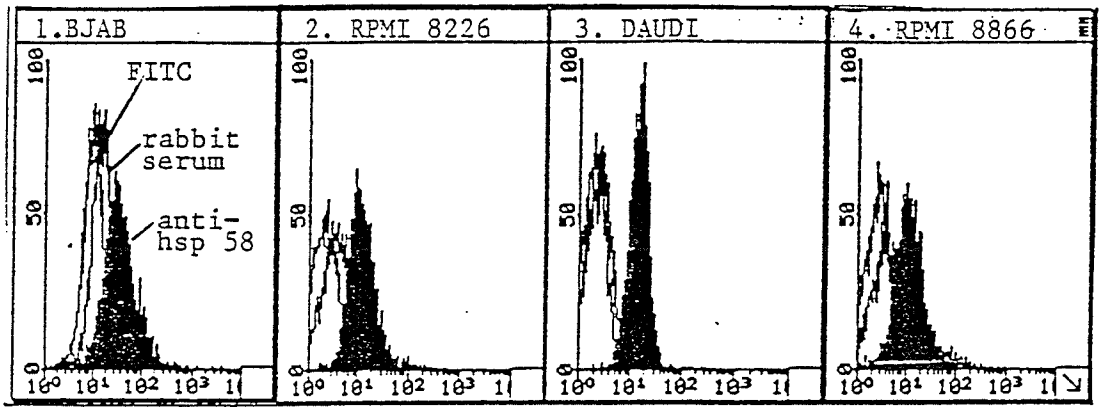
1.BJAB
2. RPMI 8226
3. DAUDI
4. RPMI 8866
FITC
rabbit serum
anti-hsp 58
100
50
0
10⁰ 10¹ 10² 10³ 1

Figure 5.

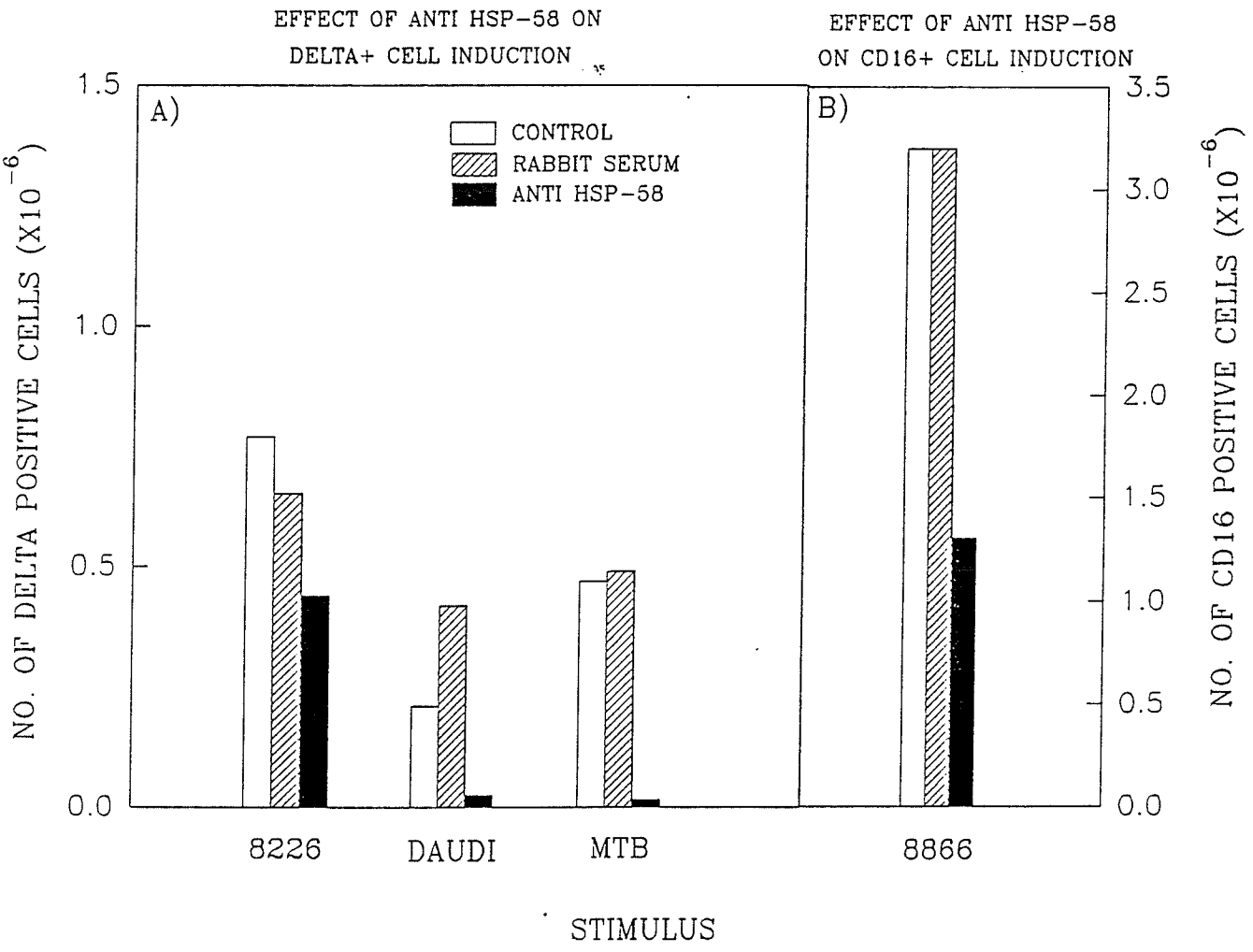
EFFECT OF ANTI HSP-58 ON
DELTA+ CELL INDUCTION
EFFECT OF ANTI HSP-58
ON CD16+ CELL INDUCTION
A)
B)
CONTROL
RABBIT SERUM
ANTI HSP-58
NO. OF DELTA POSITIVE CELLS (X10^{-6})
NO. OF CD16 POSITIVE CELLS (X10^{-6})
1.5
1.0
0.5
0.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
8226
DAUDI
MTB
8866
STIMULUS

Figure 6.

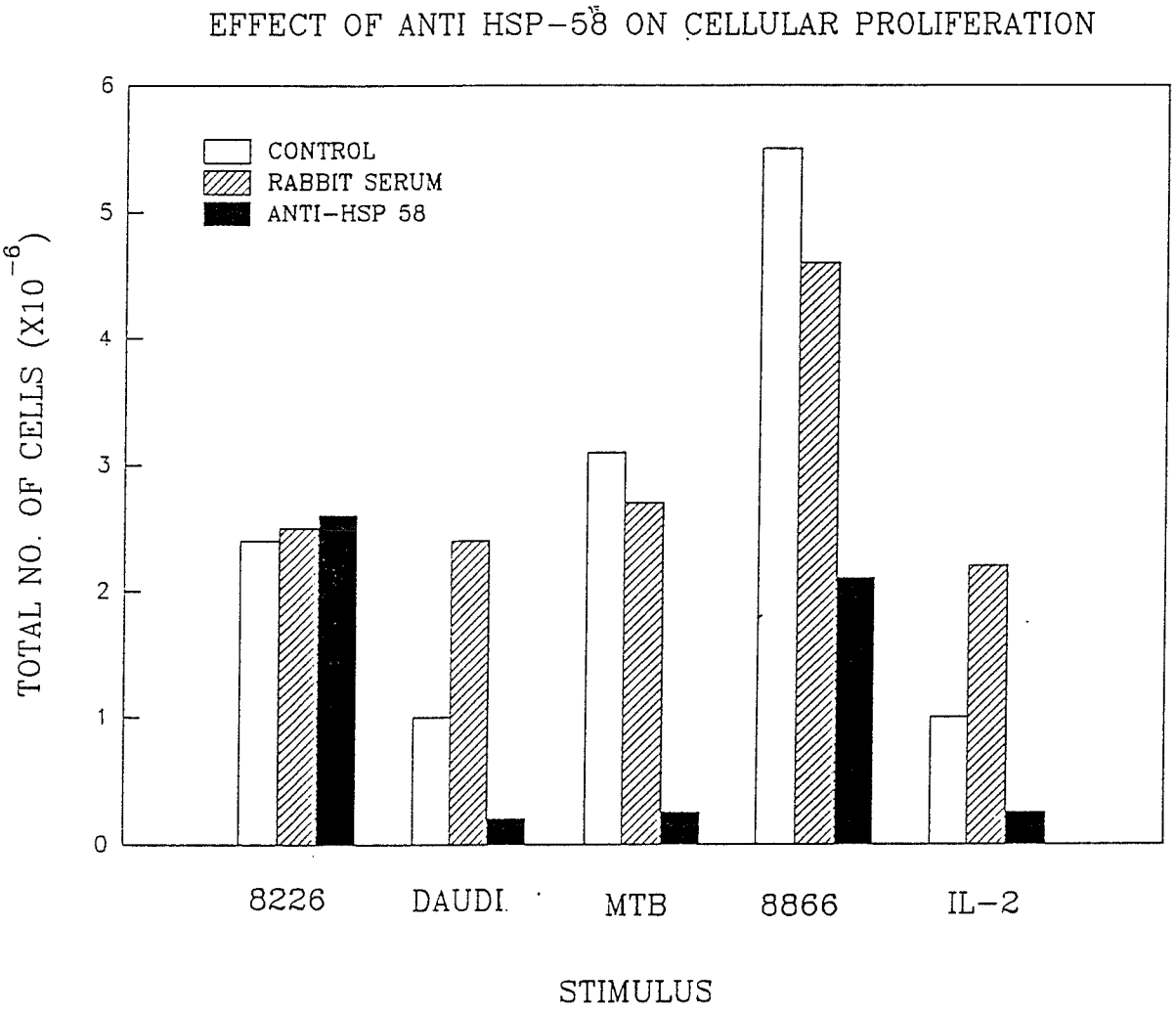
EFFECT OF ANTI HSP-58 ON CELLULAR PROLIFERATION
CONTROL
RABBIT SERUM
ANTI-HSP 58
TOTAL NO. OF CELLS (X10^{-6})
0
1
2
3
4
5
6
8226
DAUDI
MTB
8866
IL-2
STIMULUS

TABLE 1. PHENOTYPIC MARKERS FOR PURIFIED RPMI 8226 STIMULATED γδ POLYCLONAL T CELLS AND FOUR CLONES

	BULK*	CL3A20@	CL3A16@	CL3R60@	CL3R9@
CD3	93	+	+	+	+
CD2	95	+	+	+	+
TCRδ1	91	+	+	+	+
Vγ9	75	+	+	+	+
α/ß	5	-	-	-	-
CD4	2	-	-	-	-
CD8	14	-	+	+	+
CD16	3	-	-	-	-
CD56	42	+	-	-	-
HLA-DR	97	+	+	-	+
CD18 (ß2)	99	+	+	+	+
CD29 (ß1)	97	+	+	+	+

* percentage of cells expressing particular surface marker.
@ clonal data presented as +/- based on whether the marker was present or not.

TABLE 2. % SPECIFIC LYSIS BY BULK CULTURE AND γδ T CELL CLONES (E:T RATIO; 20:1)

TARGETS	BULK	3A20	3A16	3R60	3R9
RPMI 8226	52	67	62	57	58
DAUDI	67	64	56	53	49
RPMI 8866	11	60	28	23	13
K562	20	72	40	33	56
COLO	6	18	1	0	4
RAJI	1	14	2	2	0
JY	NT	80	30	18	8
BJAB	NT	27	5	9	7
BJAB-B95	NT	13	0	5	0
JURKAT	NT	70	33	25	39
MOLT-4	NT	33	0	0	7

DISCUSSION

Non-MHC restricted killer cells, the major effector of the nonadaptive innate early immune response play an essential role in the normal host defense against viral infections and tumour cell growth. Potentially these effectors when inadequately controlled may be involved in the pathogenesis of autoimmune diseases such as rheumatoid arthritis. It is therefore important to understand the mechanisms involved in their activation and expansion. This information would be potentially useful in therapeutic intervention in viral infections and cancer as well as leading to methods of intervention which could break the cycle of inflammation and destruction in chronic autoimmune diseases.

Previous research in this area has demonstrated that non-MHC restricted killer cells may be derived from at least three different lymphocyte phenotypes including NK cells, $\alpha\beta$ and $\gamma\delta$ T cells (Hersey Bolhuis, 1987; Grimm *et al.*, 1983; Ortaldo *et al.*, 1977; Porcelli, Brenner & Band, 1991; Reynolds & Ortaldo, 1987) . The NK cell has classically been considered synonymous with non-MHC restricted killer cells and the early non-adaptive innate immune response. However, both types of T cells have been demonstrated to be capable of this type of activity and investigators are currently examining their potential role in the early nonadaptive response.

Most of the research involving the activation of these nonspecific effectors has focused on NK cells. It has been

demonstrated that resting NK cells can be activated and expanded by the cytokine IL-2 alone as they spontaneously express the intermediate IL-2 receptor (Dukovich *et al.*, 1987; Le Thi Bich-Thuy, Lane & Fauci, 1984). They also can be activated by IFN-α, -ß and -γ (Lucero *et al.*, 1981; Perussia, Santoli & Trinchieri,1980; Weight *et al.*, 1982). However, IFNs do not have the ability to enhance NK cell proliferation (Trinchieri & Santoli, 1978). These results differ from the generally accepted two signal activation process described for the majority of αß T cells which do not express IL-2 receptor spontaneously. The αß T cells are usually activated via the CD3-TCR complex, most often by a foreign antigen being presented to the T cell in association with self-MHC (Allison & Lanier, 1987; Greene & Leonard, 1986). This results in expression of IL-2 receptor, secretion of IL-2 and expansion of the population in response to the IL-2. Some αß T cells do appear to respond directly to IL-2 (London, Perussia & Trinchieri, 1986) and can be activated directly by IFN-α and -ß (Brooks, Holscher & Urdal, 1985).

There has been a very active and so far unsuccessful search for a unique NK cell receptor analogous to the TCR. The majority of the research looking for the NK cell receptor has focused on a receptor specifically involved in the recognition of targets and activation of the lytic machinery. This body of work has identified numerous molecules on the surface of NK cells including CD16 (Anegon *et al.*, 1988; Werfel *et al.*, 1989), CD2 (Schmidt *et al.*, 1985a; 1987; 1988), p80 molecule (Ortaldo *et al.*, 1989), vimentin

(Evans *et al.*, 1988; Harris *et al.*, 1991), and a family of lectin-like receptors (Giorda & Trucco, 1991; Sentman *et al.*, 1989; Yokoyama *et al.*, 1991) which can result in activation of cytotoxic mechanisms and signal transduction in NK cells. However, only in the case of CD16 molecules has activation of NK cells been shown to be induced by their natural ligand, immune-complexed IgG (Hendrich *et al.*, 1990). Since no apparently unique receptor has so far been found one of the prevailing postulates concerning NK cell receptors suggest that all these molecules discussed previously might play some role in either the recognition or postbinding phases, with no single molecule playing a unique and essential role. Different sets of molecules might be involved in each combination of NK-target cells and a heterogeneity in the functional role of the various molecules might exist at both the NK and target cell levels (Timonen, 1990).

The activation events which trigger $\gamma\delta$ T cells are unknown. It would appear that they are unlike the majority of $\alpha\beta$ T cells in that they do not appear to recognize antigen in association with self MHC via the TCR. It has been extremely difficult to define what type of antigens $\gamma\delta$ T cells respond to and how they recognize the antigens. They appear to be activated into non-MHC restricted killer cells and proliferate in response to various structures such as *Mycobacterium tuberculosis,* other bacterial products, classical and nonclassical MHC molecules and some LBL tumour cells (Allison & Havran, 1991; Fisch *et al.*, 1990b; Porcelli, Brenner & Band, 1991). Exactly how these structures are recognized and interact

with the γδ T cell has not been discerned. There is certainly evidence using Mabs to CD3 (Ferrini *et al.*, 1988) and TCR γδ (Bottino *et al.*, 1988; Ciccone *et al.*, 1988) that these receptors are capable of activating the cell. The natural ligands for the γδ TCR are however more difficult to find. As well, γδ T cells do express the intermediate IL-2 receptor spontaneously and are able to be activated and expand in the presence of IL-2 alone (Aparicio *et al.*, 1989).

The current project set out to examine activation of non-MHC restricted killer cells from another approach. Since these cells have a major cellular interaction with tumour targets which results in activation of the lytic machinery we were interested in assessing whether such a NK cell-tumour target cell interaction could also result in activation and triggering of NK cells into cell cycle. In other words what is the relationship between the ultimate targets of NK cells and their potential to act as inducing agents ? Such an observation would be significant as it could provide a new model for activation and expansion of NK cells. Such an induction model could potentially involve soluble mediators, or receptor-ligand interaction(s) between the effector cells and the cellular targets.

The first paper addressed whether tumour cells were able to induce non-MHC restricted killer cell activity and PBMC proliferation. It demonstrated that one type of tumour cell, the LBL was a potent inducer. These results suggest that if the recognition event which occurs during tumour cell lysis is

identical with all tumour cells it is unlikely that this event is sufficient for non-MHC restricted killer cell induction as not all tumour cells had this ability. Only LBL were capable of induction. If lysis of each tumour cell involves different recognition events then it is still possible that for LBL the recognition for lysis of the target and for activation and expansion of non-MHC restricted killer cells involves the same surface structures. These experiments did confirm that IL-2 was able to activate and expand non-MHC restricted killer cells. However, it did not have the same capacity that the potent LBL did suggesting that other factor(s) were involved. In order to further define the nature of the LBL response this paper went on to examine the phenotype of the effectors that were induced to determine if there was a common element. Purified cell populations were used to determine their cytotoxic and proliferative characteristics.

LBL appear to have some unique feature which enables them to induce non-MHC restricted killer cells. In fact, they were able to stimulate all 3 types of non-MHC restricted killer cells including NK cells, $\gamma\delta$ and $\alpha\beta$ T cells. Which phenotype was induced was dependent on the specific LBL used in the induction system as well as the presence or absence of exogenous IL-2. Without the presence of IL-2 the potent inducers were effective at generating populations markedly increased in CD16+ cells, while poor inducers activated predominantly $\alpha\beta$ T cells. When exogenous IL-2 was added to the cultures with poor inducers there was a synergism between these two factors resulting in potent non-MHC restricted killer

activity and enhanced PBMC proliferation. Two of the poor inducers, BJAB and Ramos plus IL-2 resulted in populations with significantly increased CD16+ cells. A third poor inducer, RPMI 8226 induced a significant increase in $\gamma\delta$ T cells. The potent inducer, Daudi was also able to stimulate the growth of both CD16+ and $\gamma\delta$ T cells.

When supernatants from these culture systems were analyzed for the presence of spontaneous IL-2 only the potent inducer cultures contained measurable amounts (Data not shown). It therefore appears that IL-2 is an important ancillary factor required for activation of these different populations of non-MHC restricted effector cells by LBL. The potent inducers were capable of spontaneously producing IL-2 in the culture system and if anti-IL-2 was added their ability to induce non-MHC restricted activity was significantly inhibited (data not shown). Perussia *et al.*, (1979) demonstrated in a similar induction system that this reduction was due to a significant reduction in CD16+ cell numbers. The poor inducers were able to stimulate low levels of non-MHC restricted killer activity which appears to be mediated by $\alpha\beta$ TCR+ cells with very limited proliferation. These LBL may induce only minimal amounts of IL-2 which is consumed during the culture period or some other lymphokine which can contribute but is much less effective in the induction of PBMC proliferation and CD16+ or $\gamma\delta$ TCR+ cells. When large doses of exogenous IL-2 were added to these culture systems they became potent inducers of non-MHC restricted killer activity.

One of the differences between RPMI 8226 and the other LBL

tested is that it secretes only lambda light chain while the other LBL secrete whole immunoglobulin, either IgG, IgA or IgE. This raises the question whether there is a formation of immune complexes in the culture mixture or if the antibodies produced can in some manner bind back on to the LBL surface exposing the Fc portion which then makes the cell a large immune complex. Then CD16 cells could be activated via their CD16 Fc receptor. Although CD16 is an IgG Fc receptor there are reports that Fc receptors for IgA or IgE can be expressed on NK cells when they are cocultured with IgA or IgE secreting B cell lines respectively (Kimata & Saxon, 1988). If immune complexes in the culture were sufficient then a supernatant should be able to induce CD16+ cells. As it was later demonstrated this was not the case. It is also possible that some ancillary molecule that is expressed on the surface with whole immunoglobulin is the activating molecule, and this is not present on RPMI 8226 cells. These postulates would need some further testing examining the ability of other LBL which secrete only light chain or no immunoglobulin at all for their induction potential. Light chain secreting LBL could be obtained from culture of other myeloma patient tumours. Such lines could be available through existing cell cultures banks. The non-immunoglobulin secreting LBL could be obtained by cloning and/or mutating EBV transformed B cell lines and screening for lack of immunoglobulin production.

Purified populations of these activated CD16+ cells and $\alpha\beta$ as well as $\gamma\delta$ T cells all demonstrated non-MHC restricted killer cell activity. The CD16+ cells were much more potent effectors than the

αß T cells on a per cell basis. Both these cell populations were able to lyse all the targets tested. The γδ T cells most likely use a different mode of recognition for targets than the NK cells as they were not able to recognize and lyse as many targets. They also were able to proliferate in response to the original stimulus RPMI 8226 as well as to the other LBL, Daudi which stimulates γδ T cells. This would suggest that a similar inducing element or factor(s) are present on both these LBL. The NK cells were however, not able to respond to the original stimulating LBL, RPMI 8866. This would suggest a number of possibilities. The NK cells cannot be reactivated by this stimulus once they have been activated. NK cells may require other growth factors besides IL-2 that were present in the original PBMC culture. Or it is possible that they do not actually interact with the RPMI 8866 initially but are induced by some ancillary factor that is produced in the original culture. However, our later work demonstrated that culture supernatants cannot induce a growth of NK cells, contact with the LBL is required. It is also possible the purification technique using a positive sort with anti-CD16 can interfere with future responses of the cells by shedding or blocking of the CD16 receptor. However, the cells were rested for at least three to four days before using them in proliferation assays. This lack of response to the original LBL has been observed by others as well (Perussia *et al.*, 1987).

The second paper further addressed the question of the factors responsible for LBL induction of non-MHC restricted killer cells

The role of EBV, soluble mediators and the need for cellular contact were examined. Since EBV infection of B cell lines has been reported to have significant effects on cell functions and their surface antigen expression (Robinson & Miller, 1982) it was postulated that this feature may affect the ability of LBL to induce non-MHC restricted killer activity. The 3 LBL which were poor inducers stimulating predominantly αß T cells were EBV noninfected. The LBL which were potent inducers stimulating predominantly CD16+ cells were all EBV infected. It was also noted that the supernatants from the LBL-EBV$^+$ but not LBL-EBV$^-$ cultures contained measurable amounts of IL-2. These results suggested that the presence of EBV was important for induction of potent CD16+ non-MHC restricted killer cell activity. This observation was further confirmed when responses to an EBV noninfected LBL, BJAB and a derivative BJAB-P3, which had been infected with EBV (Fresen & Hausen, 1976) were compared. BJAB-P3 induced increased levels of cytotoxic and proliferative responses similar to other LBL-EBV$^+$ and unlike BJAB was able to induce a population markedly increased in CD16+ cells.

These results confirm that multiple factors are involved in the LBL induction of non-MHC restricted killer cell activity. The presence of EBV infection in inducer lines is able to induce IL-2 secretion by the PBMCs. This suggests that IL-2 is required for potent non-MHC restricted killer cell induction. However, a second factor is present on LBL, including LBL-EBV$^-$ which results in induction of activation and proliferation of potent cytotoxic

effectors. This activation correlates in most cases with an increase in NK cells. With RPMI 8226 there is an increase in $\gamma\delta$ T cells and with Daudi there is an increase in both cell types. The LBL cell surface molecule(s) responsible for stimulation of IL-2 production was not pursued further. It is possible that EBV antigens expressed on the LBL surface or EBV induced cellular changes led to the induction.

Studies could be designed to assess whether it is actually the presence of the virus proteins on the cell surface which results in this activation. One potential IL-2 stimulating antigen is the Lydma antigen which is expressed on EBV infected B cells and recognized by specific cytotoxic T cells (Robinson & Miller, 1982). Or perhaps it is some combination of the high level of MHC class II antigen expression containing EBV peptide for presentation which makes the MHC class II molecule even more effective at activating a MLR type response and IL-2 production. Experiments could be designed to pursue these concepts further. A simple beginning would be to use LBL which have had different sections of the EBV genome inserted to determine which part of the EBV genome is required.

Another possible molecule involved in the activation of IL-2 secretion by the PBMC when stimulated with LBL is the presence of the costimulatory molecule B7 on LBL. The presence of antigen and the costimulatory molecule B7 which interacts with CD28 on the T cell have been reported to be required for activation of T cells resulting in lymphokine production and T cell proliferation (Koulova *et al.*, 1991). LBL have been reported to express high

levels of B7 on their surface (Freedman *et al.*, 1987). Whether B7 has any role in this activation process could be assessed by using anti-B7 in blocking studies.

The next pertinent question concerning LBL induction of non-MHC restricted killer cells was whether the activation required cellular contact or soluble mediators. When the concentration of RPMI 8866 was titrated a ratio as low as 625:1 (PBMC:RPMI 8866) was found effective in inducing non-MHC restricted killer cell activity. These results suggested that either a soluble mediator was involved or this was a very potent cellular antigen. It was also possible that an amplification cascade could be involved. LBL cocultured with PBMCs could result in the activation of the B cells in the PBMC preparation. These activated B cells as well as the LBL could then act as NK cell inducers. Preliminary experiments did demonstrate that PBMCs that had been cocultured with LBL-EBV$^+$ were able to induce an increase in CD16+ cells in fresh autologous PBMCs (Data not shown).

Supernatants from LBL cultures were not able to induce potent non-MHC restricted killer cells and although supernatants from PBMC and LBL coculture were able to induce non-MHC restricted killer activity this was predominantly CD3+ cells most likely induced by the IL-2 present. Kobayashi *et al.* (1989) were able to isolate a factor produced by stimulated RPMI 8866 which has been identified as Il-12 (D'Andre *et al.*, 1992). This factor did enhance IL-2 induced non-MHC restricted killer cell activity, PBMC proliferation and IFN-γ production but did not increase CD16+ cell numbers.

Experiments using filters to separate the PBMC from the LBL confirmed that cell contact was required for induction CD16+ non-MHC restricted killer cell activity. These results would therefore suggest that a LBL cellular ligand is most likely involved in this activation process. This ligand appears to be either a protein or glycoprotein as it was trypsin sensitive and it was apparently already expressed on the surface of RPMI 8866 as *de novo* protein synthesis was not required. Attempts to use paraformaldehyde fixed cells were unsuccessful at generating CD16+ cells but did generate the less potent CD3+ effectors. This suggests that the NK cell inducing ligand was sensitive to paraformaldehyde fixing or that live cells were required. The CD3+ inducing molecule was not sensitive to fixing nor did it appear to require to be present on live cells. Experiments using LBL cell membrane preparations (vesicles) or disrupted LBL cells could be one way to document if a live cell or a cell surface ligand alone is sufficient for this induction. These types of experiments have not been pursued in the present study.

Since the data seem to indicate that a cellular antigen was involved in the activation process a number of known potential cell surface antigens were examined in antibody blocking studies. MHC class II molecules were examined as they are increased on EBV infected LBL (Robinson & Miller, 1982) and also as they are felt to be involved in the generation of CD3+ non-MHC restricted killer cells in an MLR. Antibodies to MHC class II did not block the LBL-EBV^+ induction but did block the LBL-EBV^- induction of CD3+

effectors. This suggested that the LBL-EBV$^-$ response is perhaps analogous to an MLR while the LBL-EBV$^+$ induction of CD16+ cells involves other cellular molecules.

The second group of cellular antigens which were examined were the adhesion molecules CD2, CD18, and CD29. These 3 classes of molecules have all been reported to have some role as potential recognition structures in the lytic process by NK cells and T cells (Axberg *et al.*, 1987; Mentzer, Krensky & Burakoff, 1986; Santoni *et al.*, 1989; Schmidt *et al.*, 1985a; 1987; 1988). As well, NK and T cells have been reported to be activated via the CD2 surface molecule. It was possible that they could be important recognition or binding structures involved in the activation of non-MHC restricted killer cells by LBL. Antibodies to all 3 classes of molecules demonstrated inhibition of LBL induction non-MHC restricted killer cell activity. CD2 and CD18 also significantly inhibited PBMC proliferation. These results could be interpreted in a number of ways: 1) the adhesion molecules are required to stabilize intercellular adhesive processes allowing a completely separate activating antigen to be effective but in no way reflecting a direct role for adhesion molecules in the inductive process; 2) these antibodies may bind to structures not directly involved in activation of the specific effectors but rather to structures involved in ancillary processes required for PBMC activation; 3) ligands for CD2 or possible CD18 maybe acting as a stimulatory antigen in these systems. NK cells have been shown to be triggered via CD2. LFA-3 is a known ligand for CD2. However, it

is rather ubiquitously expressed suggesting it alone is unlikely to be the activating ligand unless it has been altered in some manner by its presence in LBL making it highly efficient at activating NK or T cells via CD2. Perhaps it is some combination of these cellular antigens which are required for the activation event to occur, as has been postulated for the cytotoxic effector target recognition event.

To pursue further the question of which ligands are involved in the induction of non-MHC restricted killer cells specifically CD16+ cells with the LBL model there are two feasible approaches. One would be to generate Mab's to LBL surface molecules and then screen these for their ability to block the induction. Then identify the ligands for these Mab's. One of these ligands could potentially be the unique NK receptor. A second approach would be to use to a subtractive library of CDNA probes and use the procedure of differential hybridization. In this technique one would develop a CDNA library of a potent LBL and screen these probes against messages expressed in the LBL and another B cell line which is unable to stimulate CD16+ cells. Once the MRNA'S which are specific for the potent LBL have been isolated, their protein products could be identified. The genes could be cloned into expression vectors, the proteins produced and antibodies could be developed to these molecules. The molecule could be tested for its ability to induce CD16+ cells or the gene could be transfected into other cell types to determine if they could be able to induce CD16+ cells.

The third paper went on to examine in more detail some of the features involved in LBL induction of the second phenotype of non-MHC restricted killer cells, the γδ T cell. If the cellular interactions involved could be identified it could lead to a better understanding of the ligands involved in γδ T cell responses and help in determining their immunological role. The nature of the receptor interaction with antigen is unclear but data suggest that these structures are not recognized in a manner similar to αß T cell antigens, ie. antigen presented in context with self MHC. It has been postulated that these cells may play a major role in clearing stressed cells, such as viral infected cells, tumour cells or otherwise damaged cells from the host (Allison & Havran, 1991; Born *et al.*, 1990; Janeway, Jones & Hayday, 1988; Kaufmann, 1990). One of the ligands which can activate γδ T cells is the HSP 65 family or groEL family of HSPs. This potentially may be one of the stress proteins γδ cells are interacting with. In order to address these questions concerning γδ cell activation the γδ T cell response to the LBL, RPMI 8226 was characterized both at the bulk culture and clonal level. The specificity of their proliferative responsiveness to the original stimulator as well as their response to other activators of γδ T cells such as Daudi and mycobacterial products was assessed, and the potential role of HSPs in this response was addressed.

It has previously been noted that at least two B cell lines, RPMI 8226 and Daudi were capable of inducing γδ T cells (Selin, Stewart & Wilkins, submitted; Fisch *et al.*, 1990). This induction

did not appear to relate to tumour type beyond cell lineage as RPMI 8226 is a myeloma and Daudi is a Burkitt's lymphoma. Nor did it correlate with the presence of EBV as Daudi is infected and RPMI 8226 is not. Although both these tumours are B cell in origin this was not a general feature of B cell lines as 8 other lines tested did not have this ability. Since two B cell tumour lines can induce $\gamma\delta$ cells it leads to the possibility that under certain circumstances activated normal B cells could potentially activate $\gamma\delta$ T cells. It should be pointed out that just as for the induction of CD16+ NK cells by LBL it was demonstrated by supernatant and filter experiments that cellular contact was required for this induction to occur although the data are not shown.

All normal individuals tested appeared to respond and there appeared to be a consistency in each individuals magnitude of responsiveness suggesting some form of individual control over this response. It is possible there are suppressive factors being produced in any individual which can control the level of response. It may be an aberration in this control which could lead to chronic stimulation of these cells and potentially to their role in pathogenesis of chronic diseases such as coeliac disease or the autoimmune diseases. It is also possible that the higher responders have a higher precursor frequency of $\gamma\delta$ responsive cells to these LBL antigens. It would be of interest to note if the higher responders are more prone to develop these types of diseases. The majority of individuals have a similar response to both Daudi and RPMI 8226 suggesting a common activating event (Table 2). However,

there does appear to be some individual variation as a few people had divergent responses. The fact that many individuals responded to both these two tumour cell lines equally does not necessarily suggest that the same antigen is being recognized. It just suggests that whatever the activating structures are they are very potent and that most normal humans have the ability to respond.

In an attempt to further define the nature of the RPMI 8226 inducing element the reactivity patterns of these $\gamma\delta$ T cells both purified bulk and clonal populations were assessed in proliferation and cytotoxicity assays. The original stimulus, RPMI 8226 was recognized by and capable of stimulating proliferation in the bulk cultures and clones. Daudi was also capable of inducing these cells to proliferate while mycobacterial products were not effective generally. In addition to these observed trends there was also some heterogeneity in the clonal responsiveness to these various stimuli. One clone responded not only to RPMI 8226 and Daudi but also to RPMI 8866, but not other tumour cell lines tested. Another clone had some marginal responsiveness to mycobacterial products. Another clone did not respond to any of stimuli above the levels induced by IL-2 alone. Although this clone was functionally active in cytotoxic assays it is possible that the receptor ligand interaction involved in the proliferative response differ from those involved in the cytotoxic event and was not functional. Alternatively, the $\gamma\delta$ T cells may produce some of their own growth factors upon stimulation and this particular clone was unable to do so. These proliferative results overall suggest that the $\gamma\delta$ T cells

induced by RPMI 8226 are capable of specifically recognizing a structure(s) present on both RPMI 8226 and Daudi cells but not mycobacterial products, suggesting that a similar structure may be present on these two stimuli. In contrast, however, Daudi stimulated γδ T cells have been reported to also respond to mycobacterial products suggesting that perhaps these two types of γδ cells are different.

The heterogeneity in proliferative response in the clones could suggest that all these stimulating structures express the same antigen and the γδ TCR is triggered as has been previously suggested in a manner analogous to the superantigen induction of αß T cells (Fisch *et al.*, 1990; Porcelli, Brenner & Band, 1991; Rust *et al.*, 1990). Or it is possible that one γδ TCR can respond to a number of different stimuli. At present γδ T cells are felt to have only limited variable chain diversity although there is tremendous diversity in the junctional sequences but its significance in recognition of antigen is still undetermined (Allison & Havran, 1991; Porcelli, Brenner & Band, 1991). If one believes that γδ TCR diversity is limited than it is possible that one γδ T cell clone can recognize more than one antigen. It is also very possible that if the antigens they recognize are very common and limited the human PBMCs used in these experiments have probably already been exposed to these antigens. Perhaps that is why some of these clones are recognizing antigens they were not exposed to at the time of these experiments once again analogous to a superantigen type response.

The RPMI 8226 induced γδ T cells demonstrated a non-MHC restricted cytotoxicity pattern. However, analogous to the LBL induction of CD16+ NK cells all targets were not effective inducers which suggests that the requirements for induction and cytotoxic recognition are not the same. However, it should be noted that the most effective targets were Daudi and RPMI 8226 the targets capable of induction. It is possible that for these two targets the same recognition elements are involved for cytotoxicity and proliferation while for other targets different recognition events are required.

There was also some evidence of clonal heterogeneity in their cytotoxicity patterns. The 3 CD8+ clones had the same distinct restricted pattern while the CD56+ clone had a very broad spectrum of kill, reminiscent of activated NK cells. This further supports the previous observation that CD56 antigen is almost invariably expressed on clones with very broad spectrum non-MHC restricted specificity, both NK cells and αß T cells (Ritz *et al.*, 1988). Although this association exists the presence of CD56 has not been shown to be directly involved in this process.

This clonal heterogeneity in cell surface markers, in proliferative responses and cytotoxic pattern suggest that RPMI 8226 results in a polyclonal stimulation of γδ cells. The majority express the Vγ9 TCR, however some of these clones have markedly different response patterns as one did not even respond to the original stimulus. This variation in responsiveness could relate to clones with tremendous TCR junctional diversity as has been

reported for clones stimulated by mycobacterial products (Porcelli, Brenner & Band, 1991). There have been reports that junctional diversity may correlate with γδ T cell antigen specificity (Rellahan *et al.*, 1991). Alternatively, there may be γδ TCR functional differences during different states of differentiation.

Later experiments examined the the expression pattern of ß1, ß2, and ß3 integrins by these cloned γδ T cells. These results demonstrated very little heterogeneity with a functional utilization pattern of adhesion molecules very similar to αß T cells and B cells (Wilkins *et al.*, 1992) (Appendix A).

The question still remains if the same element is involved in the induction or cytotoxic recognition by RPMI 8226 and Daudi. As noted earlier both are B cells and generally stimulate all individuals to respond at some level. Both cells stimulate the three responsive clones to proliferate and both are effectively lysed by the clones. As well, Daudi stimulated clones are reported to be responsive to mycobacterial products which RPMI 8226 stimulated cells are not (Fisch *et al.*, 1990). However, there was some evidence of differential induction of γδ T cells in donors by these two targets. As well, cold competition experiments demonstrated that RPMI 8226 stimulated γδ cells have a preferential pattern of kill. They lyse RPMI 8226 in preference to Daudi and Daudi in preference to RPMI 8866. These results could be interpreted to suggest that γδ T cells recognize different targets using different cellular interactions or that other structures involved in stabilization are differentially expressed. These

differences will only be resolved once the stimulating ligand on these cells is identified.

One potential ligand, groEL HSPs has been identified in the mycobacterial system and in the Daudi system of induction. It was therefore decided to examine its potential role in the RPMI 8226 system of induction. It was demonstrated that antibody to HSP 58 could bind to the surface of 6 tumour cell lines, but this characteristic did not correlate their ability to induce $\gamma\delta$ T cells. This suggests that if HSP 58 or a homologue does play a role in induction, it is not sufficient in itself for $\gamma\delta$ T cell induction. There also remains the question of how HSP's, which have previously been described as proteins restricted to intracellular compartments (Young & Elliot, 1990) are expressed on the surface of cells.

Anti-HSP 58 did partially inhibit $\gamma\delta$ T cell growth induced by RPMI 8226, Daudi and mycobacterial systems of induction. However, the specificity of the effect was not absolute as this antibody could act as a polyclonal inhibitor of CD16+ and IL-2 stimulated cell growth as well as $\gamma\delta$ T cells. Anti-HSP 58 appeared to have a significant anti-proliferative effect in all induction systems except the RPMI 8226, which suggests that anti-HSP 58 may have an inhibitory effect on $\gamma\delta$ T cell induction which is unrelated to its anti-proliferative effects. These results do suggest that caution should be exercised in attempting to interpret what the exact role of HSP is in $\gamma\delta$ T cell induction. HSP's do appear to play a role in the induction process however they may not be the specific inducing

antigen. HSPs have been postulated to act as antigen presenting molecules in some systems (Vanbuskirk *et al.*, 1989). Recently, it has been reported that anti-HSP-58 binds to the surface of γδ T cells (Jarjour *et al.*,1990) suggesting that anti-HSP 58 effects may be directed at the effector rather than the stimulator.

In order to pursue further the potential ligand present on RPMI 8226 cells capable of inducing γδ T cells similar approaches suggested for identification of the NK cell inducing ligand present on LBL could be taken. These include generation of mAb's to RPMI 8226 surface molecules or using a subtractive library of cDNA probes from RPMI 8226 for differential hybridization. However, these is also another method feasible with the development of techniques for producing specific solubilized γδ and αß TCR (Slanetz & Bothwell, 1991). The genes for the human Vγ9δ2 TCR have been transfected into a thymoma cell line and these transfected cells have been found to rosette around Daudi cells (personal communication with A.L.M. Bothwell). Soluble TCR could then be used to purify the ligand it binds to on the tumour cell line either Daudi or RPMI 8226.

Since the target structures for non-MHC restricted killing by all 3 effector phenotypes have not been clearly identified it remains an open question whether they all use the same or different target recognition structures. Or even whether each effector phenotype is capable of using multiple target structures dependent on the target phenotype. The fact that activated NK cells versus fresh NK cells recognize different targets suggests that there is

some variability in target structure recognition. This is further supported by the evidence that non-MHC restricted γδ+ T cells recognize a different pattern of targets then activated NK cells. Furthermore, various clones of γδ T cells recognize different patterns of targets. There is a suggestion that CD56+ NK cells, γδ and αß T cells are all capable of recognizing an extremely broad range of targets suggesting that this subset of cells may be using a common target recognition structure.

Based on this target recognition pattern by non-MHC restricted killer cells it seems unlikely although possible that one common tumour cell target structure could also act as an inducing element. The present study confirms this observation. Overall it is apparent from these results that only some tumour lines may have inducing structures present on their surface. Specifically, the lymphoblastoid cell lines appear to be not only targets of non-MHC restricted killer cells but are potent inducers of this function. It is still possible if non-MHC restricted killer cells use different target structures for lysis recognition of different target phenotypes that the inducing structure on LBL is also the recognition structure of this subpopulation of tumour cells. However, it is unlikely that there is only one common inducing element for non-MHC restricted killing present on all LBL. Different LBL alone or in conjunction with IL-2 were able to stimulate non-MHC restricted cytotoxic activity in all 3 effector phenotypes, NK cells, γδ and αß T cells. However, the phenotype(s) which were induced was dependent on the specific LBL used in the

induction system as well as the presence or absence of IL-2. This suggests that different surface structures were required at least to induce a specific phenotype of non-MHC restricted effectors.

The adhesion molecules which are rather ubiquitously expressed have been demonstrated to play a role in non-MHC restricted lysis. Whether they are the recognition structures or ancillary molecules contributing to target cell recognition has not been clearly established. From the present investigation they do appear to have some role in the induction process by LBL as well, the exact nature of which was not determined and needs further investigation.

These studies demonstrate that there are certain shared properties between the different stimuli that induce non-MHC restricted killers. First, LBL were the best inducers of effectors. Second, cell contact was required for the stimulation to occur. Third, EBV infection resulted in IL-2 secretion, and potent induction of effectors, EBV uninfected LBL were poor inducers unless cultured with exogenous IL-2. This suggested that the presence of IL-2 was required for potent induction of non-MHC restricted killers by LBL.

Different effectors were however induced by different LBL stimuli. All phenotypes would generally appear with each stimulus but only one phenotype would predominate. This suggests that these effectors can coexist and are not mutually exclusive. It however, also suggests that there is some specific stimulus required to induce a specific effector. Most likely the presence of IL-2 was able to stimulate the lower levels of the other two effector

phenotypes while the specific LBL enhanced the activation of the predominant effector phenotype. All EBV infected LBL tested induced good levels of CD16+ cells. As well, two of three EBV uninfected lines induced predominantly CD16+ cells in the presence of IL-2. This suggests that whatever the activating factor is it is a general feature of LBLs. Far fewer LBL induced $\gamma\delta$+ T cell effectors. One EBV infected line, Daudi was capable of inducing high levels of both CD16+ and $\gamma\delta$+ cells. One EBV uninfected line (RPMI 8226) induced predominantly $\gamma\delta$ T cells. It is most likely that the mechanisms of induction for NK cells and $\gamma\delta$ T cells are quite different although LBL are capable of expressing structures which will stimulate both. As well the $\gamma\delta$ cells are able to respond to the original tumour stimulus which the NK cells do not.

As stated earlier there is no clear common characteristic between RPMI 8226 or Daudi which explains their ability to induce $\gamma\delta$ T cells. They are both B cell lines but not all B cell lines induce $\gamma\delta$ T cells. They are of different tumour origin, one is infected with EBV, the other is not and they differ in their MHC expression. Daudi does not express MHC class I, while RPMI 8226 is a poor expresser of MHC class II. Until the actual stimulating factors are identified on LBL $\gamma\delta$ T cells it will be difficult to determine if it is the same or similar stimulating structure(s) present on RPMI 8226 and Daudi cell lines.

One question which arises from this research into induction of non-MHC restricted killer cells is what the relationship of these nonspecific effectors is to the highly antigen specific T and B

cell responses. For instance, did immunological effector mechanisms evolve after the sophisticated recognition systems that now trigger them, or are the recognition systems recent additions to pre-existing, non-clonal systems of host defense. How does an NK cell that lacks a clonally distributed receptor identify which target cell to attack? It has been suggested that an NK cell may have multiple recognitive mechanisms. Thus, NK cells may function in addition to cytolytic T cells by destroying cells that lack MHC class I molecules; by using their Fc receptor they can kill antibody-coated target cells; they kill some virus infected cells; they appear to regulate haemopoiesis; and they can directly kill some bacteria.

That NK cell and cytolytic T cells share cytolytic effector mechanisms suggests that the NK cell is an evolutionary forerunner of the cytolytic T cell. It seems likely that the host defence systems of primitive organisms consisted of effector cells like the NK cell with multiple monoclonally distributed recognitive mechanisms. Primitive immune systems would be expected to have few cells, so the effector cells must have been multi-specific. The acquisition by NK cells of a clonally distributed receptor capable of discrimination between self and non-self could be sufficient to generate a cytolytic T cell. A clonally distributed receptor would allow recognition of any virus as well as the establishment of immunological memory. The zeta-chains, which are required along with the CD3 receptor for signal transduction in T cells have recently been identified in NK cells associated with larger

structures that may participate in ligand recognition. This observation as well as the finding of cytolytic T cells being able to acquire in certain circumstances NK-like non-MHC restricted cytotoxic activity supports the idea that T cells arose from NK cells. The generation of $\gamma\delta$ T cells, which appear to have TCRs with much less diversity in the variable portions of the γ and δ chains and a less specific pattern of responsiveness, another cell able to recognized possibly more than one ligand or only very common ligands may actually have been an earlier evolutionary step in the development of the antigen specific $\alpha\beta$ T cell.

Another issue which arises from this research into LBL induction of non-MHC restricted killer cells is what could be the potential significance of this nonspecific response. What purpose could this serve? This response was observed to be equally effective whether EBV infected autologous or allogeneic B cell lines were used. This suggests that this may have been an early primitive inductive response to EBV or other viral infection of B cells. The infection results in induction directly by the B cells of the innate response, inducing IL-2 secretion, possibly other cytokines, and potentially direct cellular activation generating nonspecific NK, and $\alpha\beta$ T cell effectors which would lyse the abnormal cells. It would have been highly advantageous to survival during evolution of the immune system to develop effective methods to clear EBV infected B cells as in certain circumstances the genetic events which occur during infection result in B cell transformation and malignancy.

It is also still possible that these B cell tumours are expressing some antigen that can be expressed by activated B cells after invasion of the host by a foreign antigen. Then in the presence of IL-2 or other cytokines these activated B cells can directly activate the non-MHC restricted killer cell involving both NK cell and αß T cell responses. It is possible that under some other stressful circumstances they are able to express antigens such as HSP's which result in the activation of γδ T cells, which would then clear away any of the abnormal stressed cells from the body.

Another potential reason for LBL ability to induce non-MHC restricted killer cells is the possibility that at least some cells in the host, such as B cells when they become tumorigenic do actually have the ability to activate immune mechanisms which clear them from the host. However, only EBV transformed cell lines are able to directly induce the cytokines required for the activation while other LBL require exogenous IL-2 or IL-2 production in the system by some other event. But since EBV infection has such a high rate of B cell transformation it would be very efficient for the body to have these cells direct their own clearance.

The results reviewed here suggest that if one antigen structure is involved in NK cell recognition and lysis of all tumour cells that antigen is not sufficient for induction as all of the targets are not able to induce. However, it is still possible as has been postulated that if different structures are involved in interactions with different types of targets that the same

structure is required for LBL activation and proliferation of both NK cells and γδ T cells as are needed in lysis recognition. It would appear that the adhesion molecules are a crucial part of both the cytotoxic and inductive processes but we still do not have the answer whether these are just secondary or primary structures involved in the event.

Another inference of potential significance which can be drawn from this ability of LBL to induce non-MHC restricted killer cells is that chronically activated or EBV infected B cells may play a role in some of the chronic autoimmune diseases. If such a cell was not cleared from the host it could act as a chronic stimulus for activation of non-MHC restricted killer cells. These cells besides being capable of nonspecific cytotoxicity can release soluble mediators which recruit other inflammatory cells or directly result in perpetuation of chronic inflammation, as in rheumatoid arthritis. It is possible that activated B cells expressing stress antigens are able to induce γδ T cells in some of the autoimmune diseases such as rheumatoid arthritis and polymyositis. Or they may be involved in the pathogenesis of the other diseases of unknown origin where γδ T cells are increased, such as coeliac disease and sarcoidosis.

Among the most valuable knowledge which could be gained by continued investigation of LBL induction of non-MHC restricted killer cells is the chance to identify potential cellular ligands involved in the activation of both NK cells and γδ T cells. Knowing what these ligands are could lead to a better understanding of the

activation events and functional roles of these two categories of non-MHC restricted killer cells. This would be exceedingly useful information as few natural cellular ligands have been identified to activate NK cells and the information available on ligands recognized by $\gamma\delta$ T cells is still very preliminary. It could potentially lead to insight into the receptor interactions involved in identifying a unique NK receptor or helping to define exactly how $\gamma\delta$ T cells interact with antigens.

As well, the molecule involved in IL-2 induction by EBV infected LBL could be invaluable to identify. If it could be isolated it may be helpful in tissue culture propagation or even as a method to enhance spontaneous IL-2 secretion rather than giving exogenous IL-2 in the therapy of cancer. This molecule may well be one of the antigens which could be triggering the continuation or maintenance of an autoimmune process or inflammatory state by induction locally of a continuous supply of IL-2. It is even possible that under certain circumstances such as stress these particular ligands either the ones inducing IL-2 , NK cells or $\gamma\delta$ cells may be expressed on other cells surfaces of the host resulting in activation of these non-MHC restricted killer cells.

This type of information on triggering events in both NK cells and $\gamma\delta$ T cells could lead to better methods for activation of tumour clearing mechanisms, either enhancing present therapeutic modalities for active cancer or enhancing immune tumour surveillance in individuals at higher risk for development of cancers. As well this information could be useful in understanding

the pathogenesis of chronic disease such as rheumatoid arthritis and lead to therapeutic intervention which could inhibit the cycle of on going inflammation.

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Figure 3. Genomic organization of murine and human γ and δ gene families. For simplicity the exons of the C genes are not depicted. Brackets (//) indicate gaps; the murine γ genes have been linked by pulsed field electrophoresis, although the orientation of the Vγ1.3, Jγ3 and Cγ3 genes relative to the other genes is not known. Functional genes are listed above the lines. The human Vγ1.5 sequence is not determined. The maps are drawn roughly to scale. The ticks below the lines of the murine γ and δ genes correspond to the approximate location of EcoRI sites in the BALB/c strain.

Figure 5. Schematic representation of three forms of the human TCR γδ complex. Form 1, represented by PBL C1 cells, displays short (40kDa) TCR γ polypeptide that is disulfide linked to TCR δ. The TCR γ chain is encoded by the Cγ1 gene that contains a single copy of the CII exon (hatched segment), bearing a cysteine in the connector region. Form 2abc, represented by IDP2 or PEER cell lines, displays a long (55 kDa) TCR γ polypeptide that is noncovalently associated with TCR δ. The TCR γ chain is encoded by the Cγ2 gene that contains three copies (copy a, copy b, copy c) of the CII exons (hatched segments), none of which bear a cysteine in the connector region. The TCR γ polypeptide encoded by this form is larger due to the CII exon repeats and the attachment of additional asparagine-linked carbohydrates. Form 2bc, represented by the MOLT-13 cell line, displays a short (40 kDa) TCR γ polypeptide noncovalently associated with TCR δ. The TCR γ chain is encoded by a version of the Cγ2 gene that contains two copies (copy b and copy c) of the CII exons, neither of which encodes a cysteine. In this form, less carbohydrate is attached to the potential asparagine-linked glycan acceptor sites that are located in the connector region, largely accounting for the smaller size of TCR γ.

Figure 4. Expression of TCR chains during fetal thymic development. The values for Vγ3, Vγ2 and pan αß represent actual data points. The values for the Vγ4 and the other γδ curves represent the number of cells remaining after subtraction of the sum of cells expressing Vγ3 and γ2 from the total number of γδ cells obtained with pan γδ. The other γδ cells are primarily Vγ4 expressing from day 14 until day 17, based on PCR analysis of cDNA obtained from timed fetal tissue. The Vγ4 expressing cells decline after birth and V`5+ cells become the other major γδ bearing population in the adult thymus.

Figure 1.

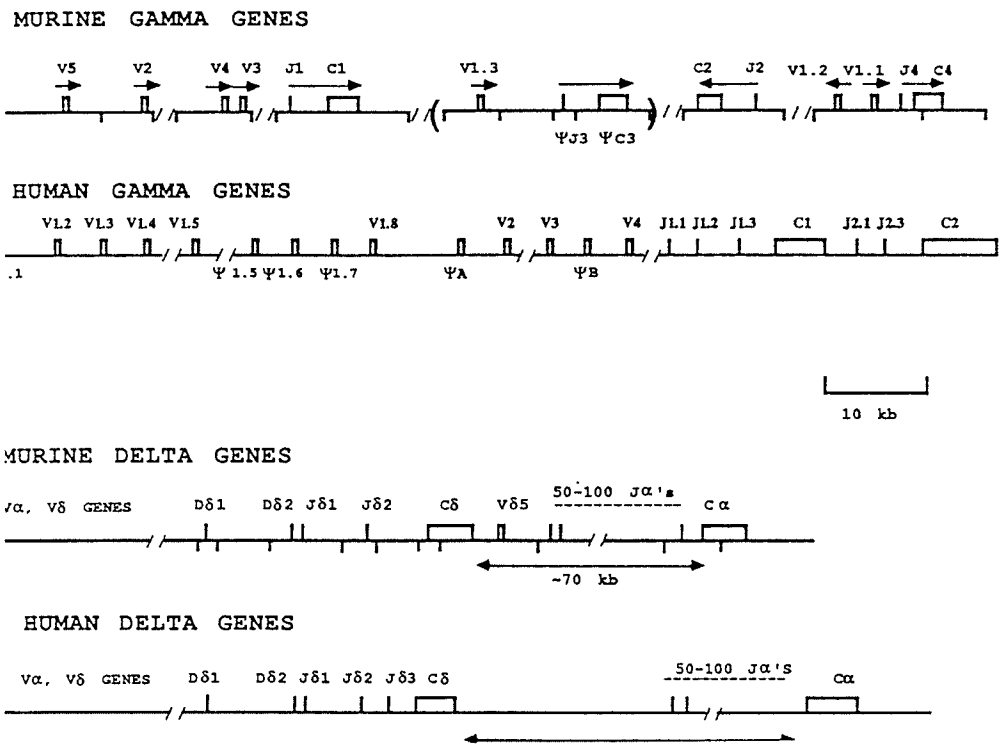
MURINE GAMMA GENES
V5
V2
V4 V3
J1 C1
V1.3
ΨJ3 ΨC3
C2 J2
V1.2 V1.1 J4 C4
HUMAN GAMMA GENES
VL2 VL3 VL4 VL5
V1.8
V2 V3
V4 JL1 JL2 JL3 C1
J2.1 J2.3 C2
.1
Ψ 1.5 Ψ1.6 Ψ1.7
ΨA
ΨB
10 kb
MURINE DELTA GENES
Vα, Vδ GENES
Dδ1 Dδ2 Jδ1 Jδ2
Cδ
Vδ5
50-100 Jα's
Cα
~70 kb
HUMAN DELTA GENES
Vα, Vδ GENES
Dδ1 Dδ2 Jδ1 Jδ2 Jδ3 Cδ
50-100 Jα'S
Cα

Figure 2.

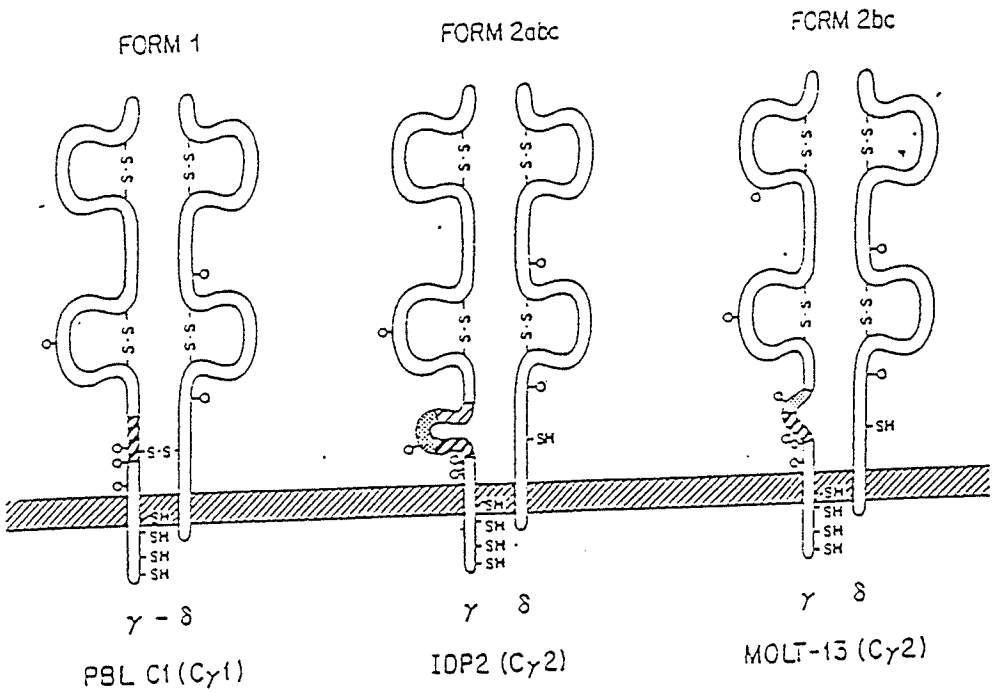
FORM 1
FORM 2abc
FORM 2bc
S-S
SH
γ - δ
γ δ
γ δ
PBL C1 (Cγ1)
IDP2 (Cγ2)
MOLT-13 (Cγ2)

Figure 3.

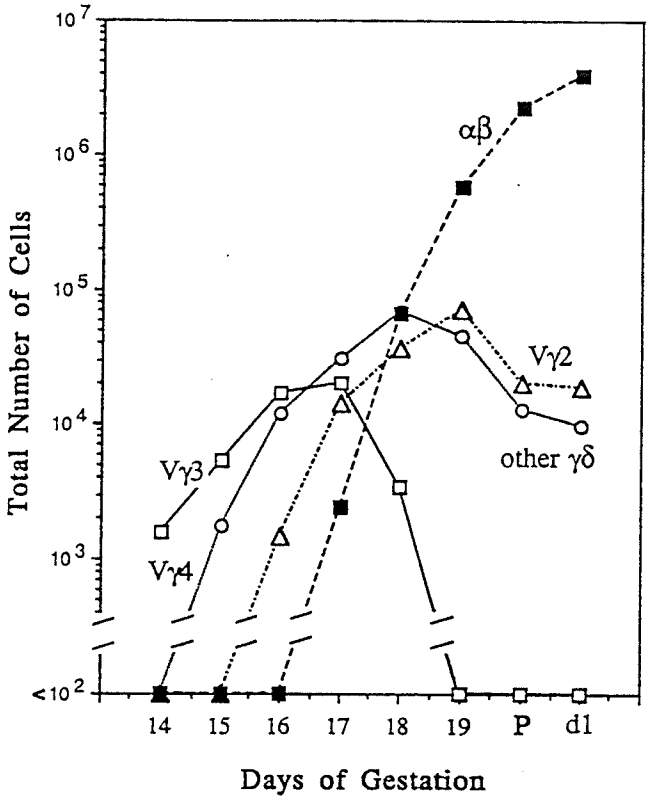
Total Number of Cells
10^7
10^6
10^5
10^4
10^3
$<10^2$
αβ
Vγ2
other γδ
Vγ3
Vγ4
14
15
16
17
18
19
P
d1
Days of Gestation

Table 1 Epithelial $\gamma\delta$ T lymphocytes

Tissue	γ	δ	Diversity	$\alpha\beta$ Cells
Skin	V_3/J_1	$V_1/D_2/J_2$	minimal	absent
Vagina, uterus	V_4/J_1	V_1/D_2J_2	minimal	subepithelial
Tongue	V_4/J_1	$V_1/D_2/J_2$	minimal	?
Intestine	V_5/J_1	V_5, V_4, V_6, V_7	+++	lamina propria
Lung	V_2, V_4	V_6, V_5, V_4, V_7	++	?

Table 2. Percentage of δ-expressing cells in normal individuals after stimulation with LBL.

DONOR NO.	STIMULUS RPMI 8226	DAUDI
1	8	50
2	22	18
3	33	25
4	86	75
5	28	39
6	50	60
7	21	7
8	33	25
9	22	23
10	32	38
11	17	15

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APPENDIX A

Scand. J. Immunol. 36, 213–219, 1992

The Interactions of $\gamma\delta$ T Cells with Extracellular Matrix: Receptor Expression and Utilization Patterns

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Wilkins J, Selin L, Stewart S, Sivananthan K, Stupack D. The Interactions of $\gamma\delta$ T Cells with Extracellular Matrix: Receptor Expression and Utilization Patterns. Scand J Immunol 1992;36:213–19

Purified populations and clones of human $\gamma\delta$ T cells were examined for their ability to interact with extracellular matrix (ECM) components. The stimulation of these cells with phorbol ester induced cellular adhesion for ECM. The adhesion structures for fibronectin and collagen were shown to be members of the CD29 integrin family. The expression patterns of β_1, β_2 and β_3 integrins by these cells were examined. The receptor expression and utilization patterns suggest that $\alpha\beta$, $\gamma\delta$ T cells and B cells have similar repertoires of adhesion structure.

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The predominant T-cell type in the human peripheral circulation expresses an antigen receptor which consists of an $\alpha\beta$ dimer [1]. However, a minor subset of T cells utilize an alternate dimeric antigen receptor containing γ and δ chains [2]. These cells differ from $\alpha\beta$ TcR-bearing cells in terms of anatomical distribution and activation of γ,δ cell subsets with tissues implying that these cells selectively accumulate at specific sites [4]. The basis for this patterning is unknown but it has been suggested that interactions with the extracellular matrix (ECM) of the tissues may contribute to the selective accumulation and retention of cells [5].

Lymphocyte adherence to extracellular matrix appears to be predominantly mediated by members of the β_1 subfamily of integrins (VLA antigens) [6, 7]. These heterodimeric molecules consist of one of at least 8 α chains non-covalently associated with the common β_1 (CD29) chain to generate a functional receptor [7, 8]. There is considerable redundancy in receptor specificities as several species of β_1 integrins recognize a single ECM ligand (e.g. $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are all capable of binding to fibronectin). Individual receptors are also capable of interacting with multiple ligands (e.g. $\alpha_3\beta_1$) [7]. The significance of this receptor degeneracy and redundancy is unknown. However, it may be that the receptors elicit distinct responses to an individual ligand. Thus patterns of receptor expression and utilization may define the responses of a cell to interactions with ECM.

There appear to be some qualitative and quantitative differences in the β_1 integrin expression patterns of lymphocyte subsets such as CD4 'naive' and 'memory' cells [9] and CD4 versus CD8 T cells [10]. Also, in the case of B cells it was suggested that $\alpha_4\beta_1$ was exclusively employed as the fibronectin receptor [11]. This contrasted with the results obtained with CD4 T lymphocytes where both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ were found to be functional receptors for fibronectin [12]. However, an examination of a larger panel of B-cell lines and normal B cells indicated that there is heterogeneity in the B-cell fibronectin receptor use, as both the $\alpha_4\beta_1$ and the $\alpha_5\beta_1$ forms can be expressed and employed by these cells [13]. Furthermore, it has recently been demonstrated that subsets of B cells express and utilize (D.

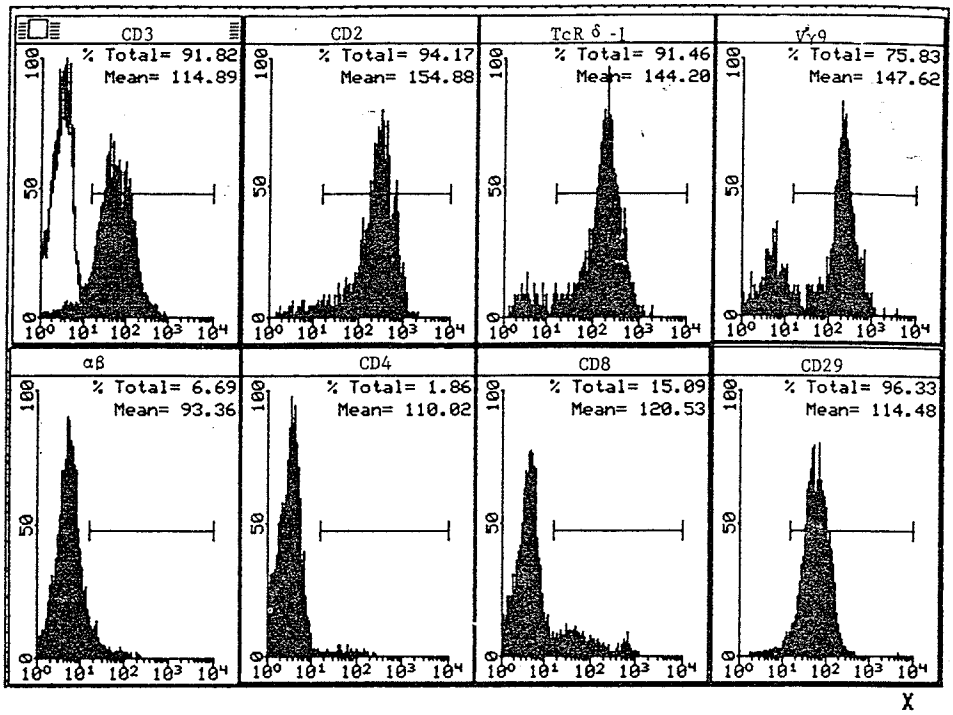

FIG. 1. Phenotypic analysis of purified $\gamma\delta$ T-cell populations on day 15 of culture. The unfilled histogram in the CD3 frame represents the profile obtained with cells stained with control antibodies.

Stupack *et al.*, submitted) integrin $\alpha_v\beta_3$ for adherence to fibronectin and vitronectin. Thus even within a given cell lineage there can be heterogeneity in the adhesion structures employed for binding to extracellular matrix.

The present studies were initiated to examine the interactions of human peripheral blood derived $\gamma\delta$ cell lines and clones with components of the extracellular matrix. The expression of the β_1 integrins and their roles in the adhesive process were also examined. As a recent report indicated that $\alpha_v\beta_3$ was involved in the activation of murine intraepithelial $\gamma\delta$ cells, the clones were also examined for the presence of the $\alpha_v\beta_3$ vitronectin receptor [14, 15].

MATERIALS AND METHODS

Monoclonal antibodies. The specificies and sources of the antibodies used in these studies are listed in Table I. The antibodies were used at saturating concentrations for flow cytometry and at concentrations in excess of those required to inhibit adherence of control cell lines.

The surface phenotype of the clones was determined by flow cytometry as previously described [16]. The detailed analyses were performed at least twice and as many as five times with reproducible patterns of staining being observed in all cases.

Generation and cloning of $\gamma\delta$-expressing T cells. The $\gamma\delta$ cells were induced from normal peripheral blood by stimulation with irradiated (50 Gy) human myeloma cell line RPMI 8226 (L. Selin *et al.*, submitted). The cells

TABLE I. Antibodies and sources

Specificity	Antibody	Source (Ref.)
α_1 (CD49a)		T-Cell Sciences
α_2 (CD49b)		Telios
α_3 (CD49c)		Telios
α_4 (CD49d)	44H4	[17]
α_5 (CD49e)	JBS5	[18]
β_1 (CD29)	JB1A	[13]
$\alpha_v\beta_3$ (VnR)	Rabbit	Telios
CD2	OKT11	ATCC
CD3	OKT3	ATCC
CD4	OKT4	ATCC
CD8	OKT8	ATCC
CD16		Becton-Dickinson
CD18	TS1/18	ATCC
CD56		Beckton-Dickinson
TcR-$\alpha\beta$		T-Cell Sciences
TcR-δ framework	TCR-δ1	[15]
TcR-Vγ9		T-Cell Sciences

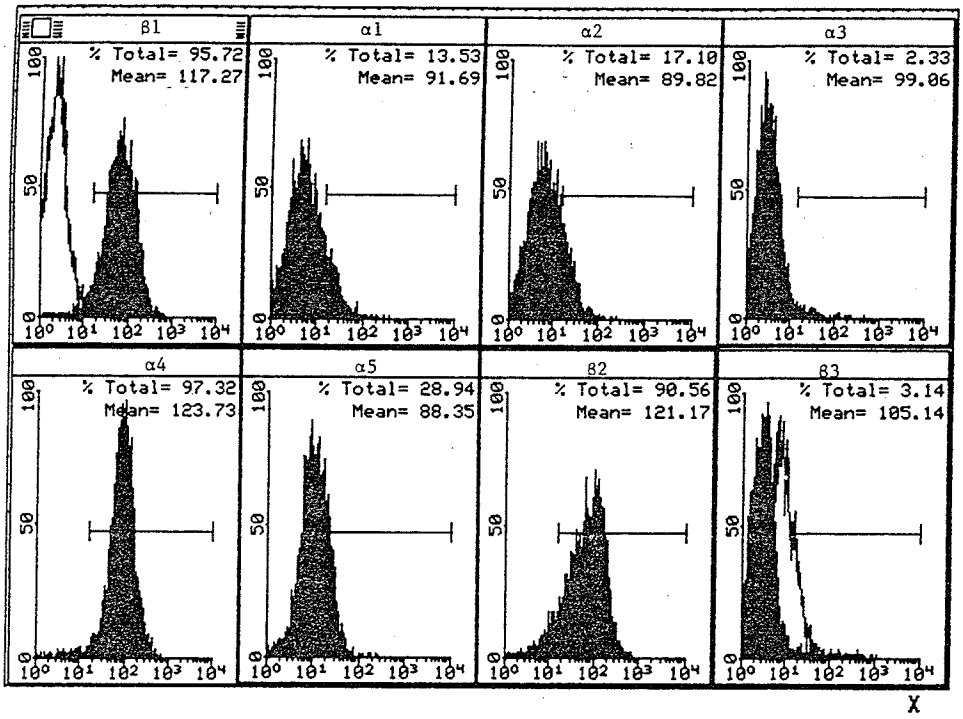

FIG. 2. The integrin expression patterns of purified γδ T cells. The populations were stained with the indicated antibodies to the α chains of the β_1 integrins, or to the β_2 integrin chain. In the case β_3 the cells were stained with a polyclonal anti-β_3. The unfilled histogram in this frame represents the staining obtainined with normal rabbit serum as first antibody.

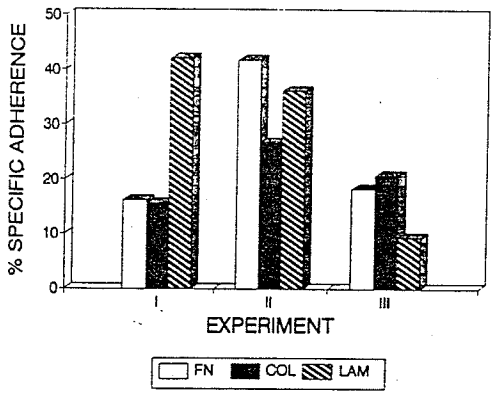

FIG. 3. The adherence of purified γδ T cells to fibronectin, collagen, or laminin following PMA stimulation. The results of three separate experiments are listed. The variation was less than 5% within each of the quadruplicate assays performed.

were cultured at 10^6/ml in AIM-V (Gibco, Paisley, UK) supplemented with 50 U/ml of highly purified recombinant human IL-2 [20]. The cells were diluted on day 6 to 2×10^5/ml in media containing IL-2. The cells were collected on day 10, reacted with anti-δTCS1 and a FITC-labelled $F(ab)_2$ goat anti-mouse immunoglobulin (Tago, Burlingame, CA) and separated using a Coulter Epics IV FACS system. The resulting cells were >95% γδ and <2% αβ as judged by staining with anti-TcR αβ and anti-TCR-δ1.

The γδ cells were cloned either directly at the time of FACS separation or by limiting dilution following a 24-h incubation period. In both cases the cloning was carried out in round-bottom 96-well plates containing 10^5 irradiated peripheral blood mononuclear cells, 10^3 irradiated RPMI 8866 cells, 0.01% (vol/vol) PHA-P (Difco Laboratories, Detroit, MI), 50 U/ml rhIL-2 in RPMI-1640 supplemented with 10% human ab serum. The clones were selected on day 14 and expanded by dilution in the presence of rhIL-2 supplemented RPMI-1640 10% human serum. The clones were restimulated every 7–10 days as described for the original cloning procedure. Prior to further analysis, the γδ expression of the clones was confirmed by flow cytometry.

Adherence assays. The adherence of cells to immobilized collagen (Sigma Chemical Co., St Louis, MO), fibronectin, laminin and vitronectin (Telios Pharmaceuticals, La Jolla, CA) were performed in microtitre trays as previously described [16]. In some cases the cells were labelled with ^{51}Cr and the results expressed as per cent specific adherence: (cpm bound to ECM − cpm bound to control wells)/(total cpm added − cpm bound to control well) × 100. The results are the mean of triplicate values and all clones were examined on 2–5 occasions.

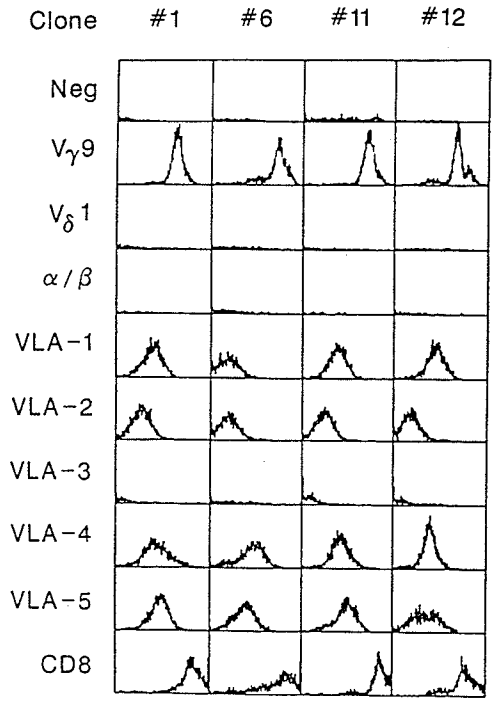

FIG. 4. Phenotypic analysis of $\gamma\delta$ clones. The clones were reacted with antibodies to the indicated antigens and analysed by flow cytometry.

RESULTS

Characteristics of bulk populations

The stimulation of PBL with RPMI 8226 resulted in the generation of populations which were 45% γ/δ positive ($n=10$). The γ/δ cells were purified by FACS to yield populations which were >90% γ/δ positive as assayed by reactivity with TCRδ1 and <5% $\alpha\beta$ positive (Fig. 1). The majority of cells were $CD2^+$, $CD3^+$, $CD4^-$, and $CD29^+$ with a minor subset (15%) of cells expressing low levels of CD8 (Fig. 1). The antigen receptor utilization of this population appeared to be restricted as 75% of the cells expressed Vγ9 (Fig. 1) and only a small proportion <5% displayed Vδ2 (data not shown).

The patterns of integrin expression of this population was examined in detail. The majority of the cells (>90%) expressed CD29, CD18 and CD49d α_4, while 10–30% of the cells reacted with antibodies to α_1, α_2 or α_5 (Fig. 2). There was no detectable α_3 or β_3 on the three bulk populations examined in detail.

Preliminary results had indicated that there was inconsistent basal binding by unstimulated cells (data not shown), therefore cellular adherence was examined following stimulation with PMA. Adherence of the cells to fibronectin, collagen, and laminin (Fig. 3) was observed. However, the relative levels of binding to these ligands varied with the individual cell preparations. Furthermore, as these cells were not clonal it was not possible to ascertain whether the same subpopulations adhered to all substrates. Therefore a panel of clones was generated in order to examine adhesive properties of individual cells.

Characterization of clones

A total of 150 clones were examined for the expression of δ chain, CD3, the α/β T-cell receptor and CD29. All of the clones were negative for α/β T-cell receptor and positive for the other markers. A sampling of 19 representative clones was selected for analysis in greater detail.

All of the clones were $CD3^+$, $V\gamma9^+$, $V\gamma1^-$, $\alpha\beta^-$ and $CD8^+$ (Fig. 4). Analysis of the β_1 expression on the cells indicated that all clones displayed high levels of the CD29 molecule (data not shown). However the distribution of α chains varied within the different clonal populations. Generally $\alpha_1\beta_1$ was low or absent from the cell surface. In contrast $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ were highly expressed on all clones (Fig. 4). Similar patterns were obtained for all 19 clones which were analysed in detail.

The adherence properties of the clones to collagen and fibronectin were examined in detail. Both the bulk cultures and the clones displayed a variable basal level of adherence to fibronectin and collagen which was consistently enhanced by treatment with PMA (Fig. 5). The basis for this variability is unclear but it did appear to correlate with the time after restimulation. In contrast, treatment with anti-CD3 and a cross-linking antibody did not prove to be an effective inducer of adherence.

Preliminary results had indicated that the adherence of the γ/δ cells to collagen and fibronectin was mediated by members of the CD29 integrin family. Therefore the nature of the adhesion structures was examined using α chain-specific monoclonal antibodies capable of inhibiting adherence. Similar patterns of adherence and inhibition were observed with all 19 clones examined and representative data are presented.

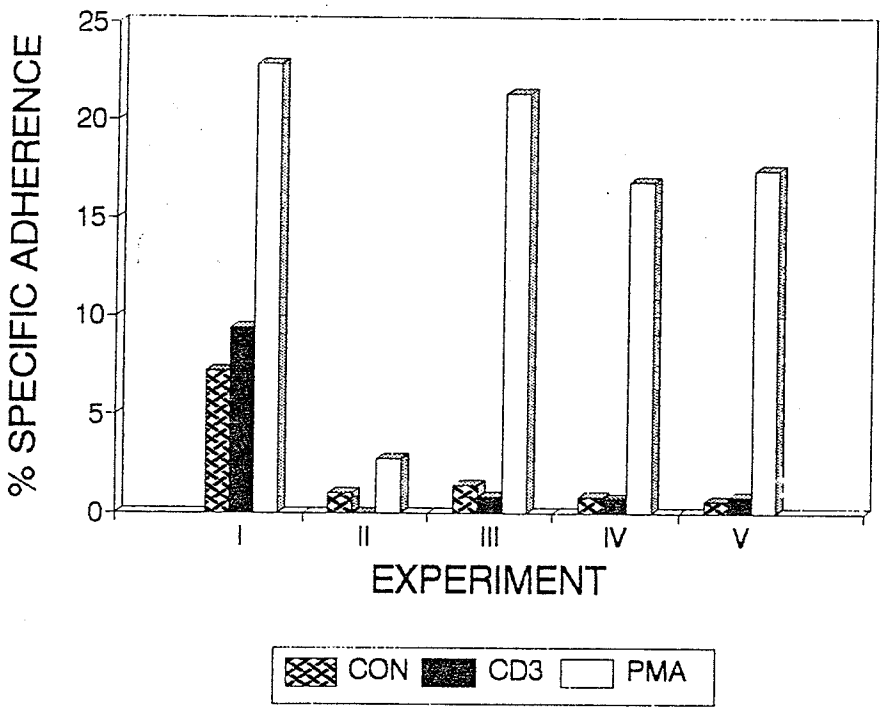

FIG. 5. The adherence of γδ clones to fibronectin requires stimulation with PMA. Clones were assayed directly or following 10-min treatment with PMA (20 ng/ml) or anti-CD3 and a secondary anti-mouse antibody. The adherence to the fibronectin was then determined. The variation from the mean was less than 5% in all groups. The results of five independent experiments are presented.

Antibodies to $\alpha_2\beta_1$ completely inhibited the adherence of γ/δ cells to collagen (Fig. 6a) suggesting that collagen binding was exclusively mediated by this receptor. The adherence to fibronectin was somewhat more complex as it involved both $\alpha_4\beta_1$ and $\alpha_5\beta_1$. Antibodies to either of these species almost completely eliminated the binding to fibronectin and in combination the antibodies did fully inhibit the adherence (Fig. 6b).

It has been reported that murine γ/δ cells express the vitronectin receptor, $\alpha_v\beta_3$, which plays a role in the activation of these cells. FACS analysis of the uncloned populations and of the clones with a polyclonal rabbit anti-VnR or a monoclonal anti-α_v failed to reveal the presence of α_v or β_3 on any of these cells. These cells also failed to adhere to vitronectin suggesting that they lacked adhesion structures for this ligand.

DISCUSSION

The present results indicate that γδ T cells can express β_1 integrins and that these receptors appear to be the predominant adhesion structures for collagen and fibronectin. Qualitatively the β_1 expression patterns of the clones and the bulk populations were similar. However unlike the clones, where it was observed that all clones expressed multiple species of β_1 integrins, the majority of the uncloned cells expressed only $\alpha_4\beta_1$. These differences may reflect the longer duration of the culture period required to generate the clonal populations as it is known that extended culture leads to qualitative and quantitative increases in β_1 expression of T cells [7]. Alternatively it may be that the clones represented a subset of cells which were preferentially selected for by the conditions of the cloning procedure.

Contrary to reports on murine γδ intraepithelial T cells, the cells generated in the present system did not express the vitronectin receptor, $\alpha_v\beta_3$, nor did they adhere to vitronectin [15]. As the $\alpha_v\beta_3$ was reported to be an activation antigen on these cells, both resting and proliferating cells were examined with similar lack of detection of this receptor. The cells generated in the present study were restricted in their V_γ gene utilization as

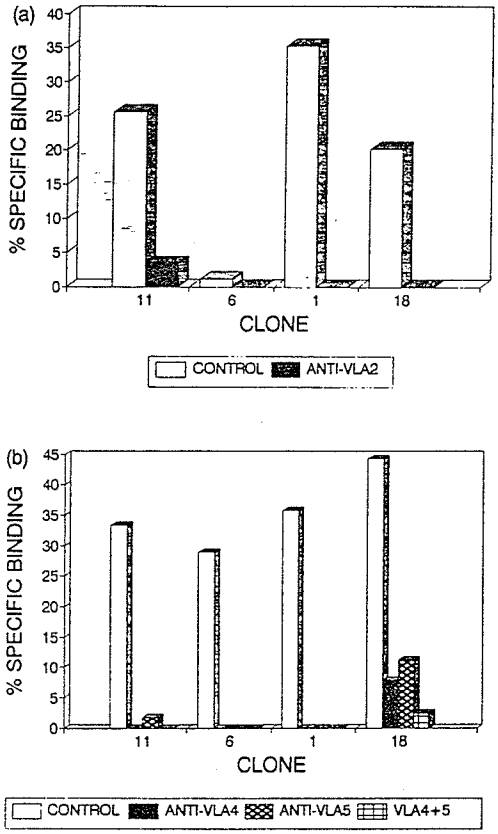

FIG. 6. (a) The adherence to collagen is mediated by α_2-containing complexes. Clones were pretreated with PMA and incubated with anti-VLA-2 or control antibody and assayed for binding to collagen. (b) The adherence to fibronectin is mediated by α_4- and α_5-containing complexes. Cells were pretreated with PMA and treated with anti-α_4, anti-α_5 anti-$\alpha_4+\alpha_5$, or control antibody and cell binding to immobilized fibronectin was assayed.

75–85% of the uncloned $\gamma\delta$ cells and all of the clones were $V_7 9$ positive. Tissue resident $\gamma\delta$ cells, such as in the synovium of rheumatoid arthritis patients, have been reported to use a different isoform of the δ chain to those in the peripheral blood of the same individual [21]. Thus the differences in expression patterns may reflect the fact that the cells used in these two studies were derived from different sources i.e. blood and tissue. Indeed it might be expected that the cellular environment in which a cell resides or the state of differentiation of cells could contribute to the receptor expression and utilization pattern.

The induction of $\gamma\delta$ cell adherence required activation by PMA much like that necessary for the demonstration of $\alpha\beta$ T cells [22]. However, contrary to the case for primary T cells the adhesive potential persisted for several days post stimulation [22]. It is not clear at this time if this represents a property of long-term cell lines in general or specifically of $\gamma\delta$ cells. However, preliminary results with $\alpha\beta$ T-cell receptor-bearing clones suggests that it is not a property unique to $\gamma\delta$ cells (J. A. Wilkins, unpublished results). Although the basis for the induction of the adhesive potential by PMA is unknown, it has been observed in a continuous T-cell line that the acquisition of the adherent phenotype does not require increased expression of integrins or de novo protein synthesis [16]. The kinetics of the induction of $\gamma\delta$ T-cell adherence by PMA is rapid, requiring 10–15 min, suggesting that de novo synthesis of β_1 integrins is not the basis for the developments of the adhesive phenotype. Thus it appears that the stimulation of lymphocyte adherence to ECM involves both the activation of pre-existing integrins on the cell surface as well as the eventual up-regulation of receptor expression following extended stimulation of lymphocytes.

The results of our study relate to the expression patterns of activated $\gamma\delta$ cells and as such do not necessarily address the phenotype of cells in the circulation. The data demonstrate the β_1 expression potential of these cells, not necessarily their in vivo status. Nonetheless these results do indicate that the expression patterns of the T-cell populations in general do not appear to be restricted by the antigen receptor utilization pattern. The differentiation status of the cells may play a more important role in defining pattern than does subset. Indeed B-cell lines can display quite distinctive integrin expression and utilization patterns of β_1 integrins [11, 12]. Recently β_3-mediated adherence of B cells to fibronectin and vitronectin has been observed, indicating that lymphocytes can use multiple integrin subfamily members for adhesion. Furthermore one cell may coexpress and use more than one subfamily of integrins (D. Stupack *et al.*, submitted).

Lymphocyte interaction with ECM can be of functional significance. Immobilized laminin or fibronectin can synergize with suboptimal concentrations of stimuli such as anti-CD2 or anti-CD3 to generate proliferative responses [22, 23]. Adherence to fibronectin has been observed to induce the synthesis of the nuclear binding factor, AP1, which is required for the transcription of a number of genes including those for IL-2 [24].

The binding of T-cell clones to ECM can also lead to the synthesis of cytokines such as IFN-γ or fibroblast activating factor (Ref. 25; J. A. Wilkins *et al.*, unpublished observations). Thus the expression pattern of β_1 integrins can potentially influence the responses of a cell to ECM. Preliminary results indicate that the interaction of γδ cell lines with ECM in the presence of anti-CD3 does not result in the proliferation of these cells. It is unclear if this represents a unique property of γδ cells or if there is a loss in responsiveness to ECM as a result of prior activation. Experiments are in progress to examine these possibilities.

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Dr. S. Stewart